UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Phylogeography of hummingbird by means of DNA analysis and taxonomic classification: Chlorostilbon sp. an unexpected species in the Chota valley

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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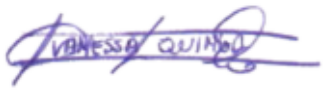

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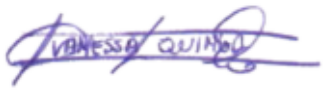

Mayra Vanessa Quinga Nasimba

CI: 172520019-8

Dedication

To God,

To my mother,

To my father,

To my brother,

To my sister,

Thank you for your support and infinite love.

Mayra Vanessa Quinga Nasimba

Acknowledgments

First of all my family God and my family, who supported me during my university career, especially to my father Francisco Quinga and my mother Celinda Nasimba for their trust during my study time at Yachay Tech; thanks to them I was able to finish my university career

Special thanks to Dr. Markus Patricio Tellkamp Tietz, who, as a tutor, has shared as much knowledge and time as possible, and has led me to a successful thesis project.

To Dr. Francisco Javier Álvarez Botas who, as teacher lab, has shared as much knowledge and time, I consider that within help this project is possible.

To my friends from university, Alexandra, Evelyn, Pamela, Patty, Eve, Jonathan, Luis, Stalyn and Leonel for all the shared moments and support throughout my university life.

Mayra Vanessa Quinga Nasimba

Resumen

El *Chlorostilbon* sp. (familia Trochilidae) descrito en este trabajo es una población enigmática que se la encuentra comúnmente en el bosque seco del valle del chota. Para comprender la influencia de los procesos geológicos o ecológicos en la filogeografía de *Chlorostilbon* principalmente al valle de Chota, se analizan las secuencias de los genes de la subunidad 2 de NADH deshidrogenasa (ND2) y el marcador nuclear del intrón 5 de adenilato quinasa (AK1) usando análisis de máxima parsimonia y máxima verosimilitud. Los análisis sugieren que el colibrí enigmático presenta una afinidad molecular con un grupo consistente de *Chlorostilbon melanorhynchus* y *C. assimilis.* Por sus características morfológicas esta especie presenta fenotipo similar al de *Chlorostilbon mellisugus* presente en las provincias de Morona Santiago, Napo, Orellana, Pastaza, Sucumbíos, y Zamora Chinchipe. Postulo que la población enigmática de *Chlorostilbon* posiblemente se originó a través de la hibridación de *C. melanorhynchus* por introgresión de *C. mellisugus*. Se describen y analizan los patrones de variación de *C. melanorhynchus* en el valle del Chota usando especímenes depositados en el Museo Ecuatoriano de Ciencias Naturales.

Palabras clave: Especiación; hibridación; filogeografía; ADN mitocondrial; ADN nuclear; *Chlorostilbon melanorhynchus; Chlorostilbon mellisugus*

Abstract

The *Chlorostilbon* sp. (family Trochilidae) described in this work is an enigmatic population commonly found in the Chota valley's dry forest. To understand the influence of human geological or ecological processes on the phylogeography of these *Chlorostilbon,* sequences of the NADH dehydrogenase subunit 2 (ND2) gene and the Adenylate Kinase (AK1) intron 5 nuclear marker were analyzed using maximum parsimony and maximum likelihood. These analyses suggest that this hummingbird is most closely related to a clade consisting of *Chlorostilbon melanorhynchus* and *C. affinis*, although its morphological characteristics resemble more those of *Chlorostilbon mellisugus* present in the provinces of Morona Santiago, Napo, Orellana, Pastaza, Sucumbíos and Zamora Chinchipe. I hypothesize that the population of the enigmatic species of *Chlorostilbon* possibly originated through the hybridization of *C. melanorhynchus* with introgression of eastern *C. mellisugus*. I analyze and discuss variation patterns of the Chota population in light of museum specimens deposited at the Museo Ecuatoriano de Ciencias Naturales.

Keywords: Speciation; hybridization; phylogeography; mitochondrial DNA; nuclear DNA; *Chlorostilbon melanorhynchus; Chlorostilbon mellisugus.*

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Abbreviations

AK1: Adenylate kinase gene

ND2: NADH dehydrogenase subunit 2 gene

Cyt b: Cytochrome b

MEGAX: Molecular Evolutionary Genetics Analysis across computing platforms

ML: Maximum Likelihood

MP: Maximum Parsimony

NJ: Neibor Joining

AICs: Akaike's Information Criterion

SPR: Subtree-Pruning-Regrafting

MECN: Museo Ecuatoriano de Ciencias Naturales

1. Introduction

Bird phylogeography is the study of historical processes that are responsible for the distributions of bird populations. In the Neotropics, hummingbirds (Apodiformes: Trochilidae) comprise the most diverse avian family, with between 328 and 338 described species depending on classification (Schuchmann, 1999; Fogden et al., 2014; McGuire et al., 2014). Phylogenetic relationships among major hummingbird clades are relatively well resolved, yet due to the lack of morphological synapomorphies and relative phenotypic homogeneity, the systematics of several hummingbird groups remains unresolved (Hernández et al., 2020). Hummingbirds are ecologically diverse, with most communities characterized by sympatric species, and the wealthiest sites being inhabited by 25 or more taxa (Parker et al., 1985; Robbins et al., 1987; Stiles and Levey, 1994; Kromer and Kessler, 2006; Dziedzioch et al., 2003). Because they exhibit substantial species richness, ecological diversity and a unique lifestyle, these small birds approach the upper limits for vertebrate physiological performance and metabolism (Suarez et al., 1991; Chai and Dudley, 1995, 1996; Suarez, 1998),

Studies of phylogeographic patterns provide insight into the processes driving lineage divergence in a particular region and the roles of genetic exchange among populations. Molecular studies have revealed diverse and complex biogeographic processes and patterns of differentiation among populations (García-Moreno et al., 2004; Dingle et al., 2006; Pérez-Emán, 2005). Most bird populations include individuals that perform short and long-distance movements, an adaptive behavior in response to, human habitat alteration, or climate change. Conversely, some species become confined to an area, such as those that survived in “refuges” during Pleistocene glaciation (Hewitt, 2003). Phylogenetic analyses help to identify the factors that caused phylogeographic breaks in time; thus, it is necessary to use a set of appropriate molecular data and historical information to explain current patterns.

The explosion of studies based on polymerase chain reaction (PCR) and DNA sequencing analysis has provided a wealth of new data for hummingbird systematists in recent decades. However, nearly all these sequence data have come from mitochondrial genes inherited as a single unit, typically exhibit strictly matrilineal inheritance, and have other unusual properties (Avise, 1991). The advantages of mitochondrial DNA (mtDNA) to phylogenetic inference have been well established (Moore, 1995).

Mitochondrial DNA (mtDNA) evolves rapidly and provides an abundance of genotypic characters or haplotypes. For these reasons, it has been used in phylogenetic

studies, either by restriction fragment analysis or sequencing of amplified regions (Brown, 1983; Moritz et al., 1987; Dowling et al., 1990). Nevertheless, its utility is limited because it is inherited as a single linkage group (Moore, 1997). In contrast, nuclear loci such as AK1 intron 5 may be more helpful to analyze mutation events at intermediate time depths at which homoplastic mutations may become a severe problem for rapidly evolving mitochondrial genes. In contrast, nuclear protein coding accumulates differences more slowly.

Haldane (1922) observed that the heterogametic sex more often suffers reduced survivorship or fertility than the homogametic sex when hybridization occurs between closely related animal species, an aspect of avian biology that may favor mtDNA's use for resolving phylogenetic studies (Moore, 1995). To hummingbirds, a hybrid zone between species may be an open conduit for introgression of nuclear genes via males but a barrier to mtDNA because females are the heterogametic sex and thus do not readily reproduce. Thus, this results in a decreased likelihood that the mitochondrial genome will introgress between hybridizing hummingbird taxa.

The clade of the emeralds comprises about 108 species belonging to 30 genera. The Western Emerald (*Chlorostilbon melanorhynchus*) prefers scrublands and gardens in dry mountain valleys and the humid forests of the western slopes of the Andes. In northwestern Ecuador, it occupies an area that extends from the Carchi province through the Chota river valley south to the Cumbayá and Quito regions in the Pichincha province. It can also be found in the western lowlands in northeastern Guayas province and southwestern Chimborazo province. In northeastern Ecuador, it lives at altitudes between 1500 and 2700 meters, further south between 600 and 1800 meters (Arzuza, 2019; Lyons, 2002).

This study presents a new phylogeny for the genus *Chlorostilbon* that includes an enigmatic population from the Chota valley. We use the mitochondrial marker NADH dehydrogenase subunit 2 (ND2) and nuclear marker Adenylate Kinase (AK1) intron 5 to reconstruct the phylogeny.

1.1.Problem statement

Hummingbirds have proven an excellent system for comparative biological studies and represent one of the most extensive avian radiations (Altshuler et al., 2004a,b). Recent studies have supplied new insights into hummingbird systematics and generated predictions about the timing of speciation events. Most speciation events likely have occurred during the Pliocene, with a few during the Pleistocene, mainly at higher elevations. Currently, new information has also been shed light on the relationship between genetic and phenotypic divergence; generally, there exists a weak phylogenetic signal in color traits (Parra, Remsen, Alvarez-Rebolledo, & McGuire, 2009).

A population of *Chlorostilbon*, which does not fit with the plumage of the species expected to be found in the Chota valley, *C. melanorhynchus,* has been visiting the hummingbird feeders in the neighborhood of Las Mercedes in Urcuquí. Here we present a multilocus phylogenetic hypothesis for the hummingbird genus *Chlorostilbon* based on one mitochondrial and one nuclear gene to determine its taxonomic status. This study represents a first step in establishing the enigmatic hummingbird's phylogeography and identity.

1.2. Objectives

1.2.1. General objectives

Conduct a phylogenetic analysis using molecular tools and morphological data to determine the evolutionary relationships and taxonomic status of the enigmatic species of *Chlorostilbon* in the Chota Valley, Imbabura, Ecuador. We thus hope to encourage the conservation of this species and highlight the importance of the locality for conservation.

1.2.2. Specific objectives

- Infer a molecular phylogeny using maximum parsimony and maximum likelihood that includes the enigmatic species of the genus *Chlorostilbon.*
- Perform a morphological characterization of the enigmatic species of *Chlorostilbon* for taxonomic purposes.

2. Methods and Materials

2.1 Study Site

The study site is located in the southern portion of the Chota valley in Imbabura province of northern Ecuador (Figures 1 and 2).

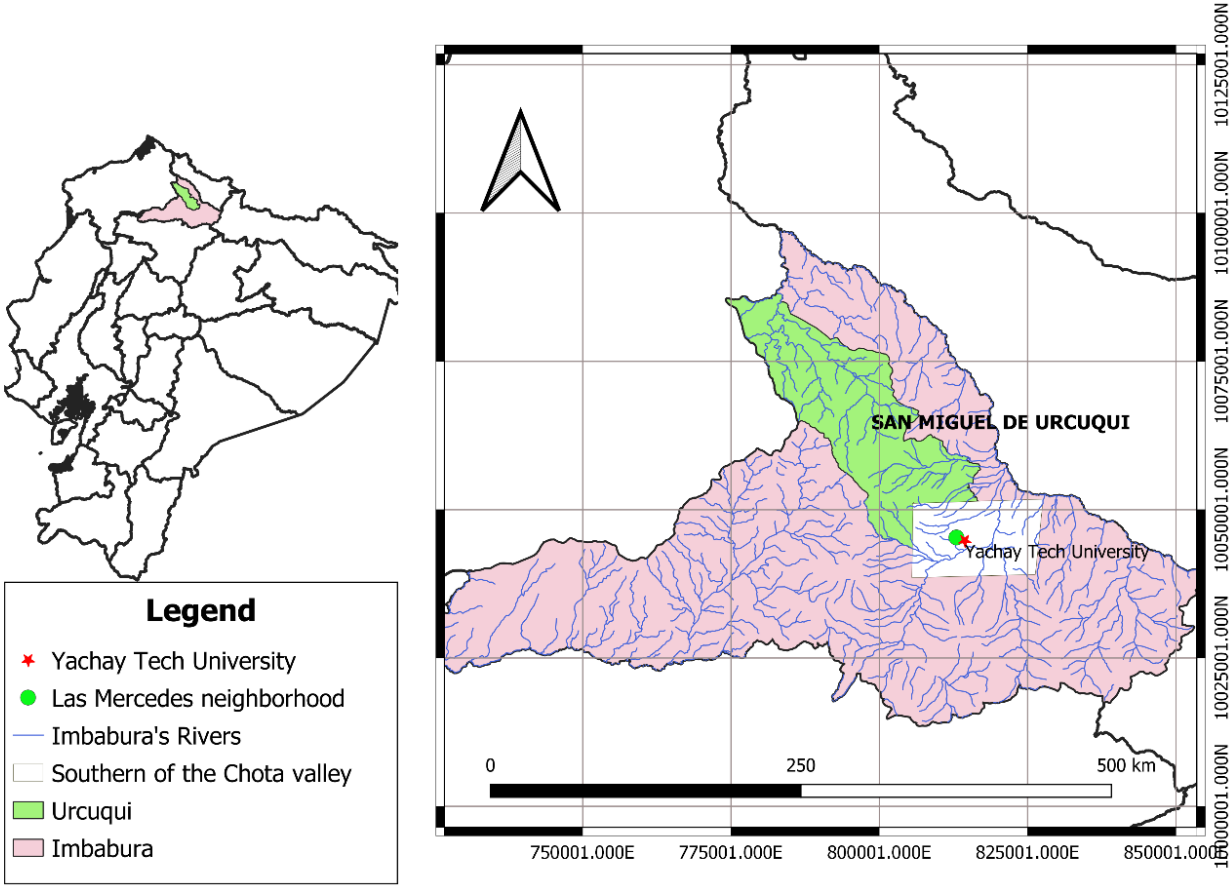

Figure 1. Map showing the location of enigmatic *Chlorostilbon* sp. and the blood collection site in Urcuquí-Imbabura.

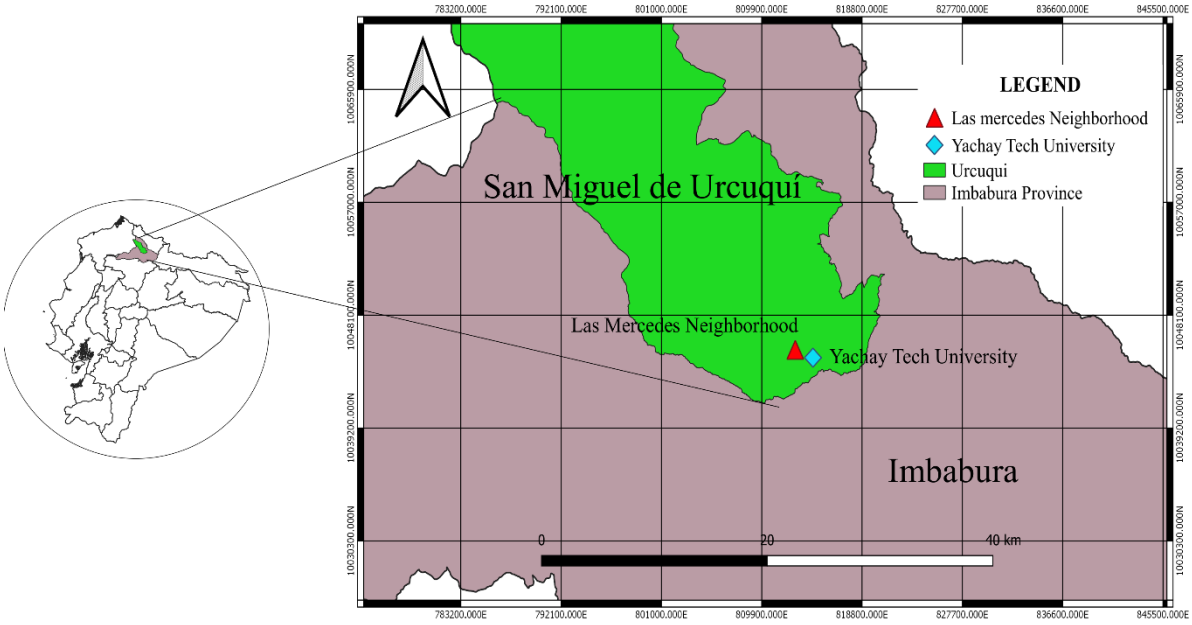

Figure 2. The southern region of the Chota valley where Yachay Tech University is located

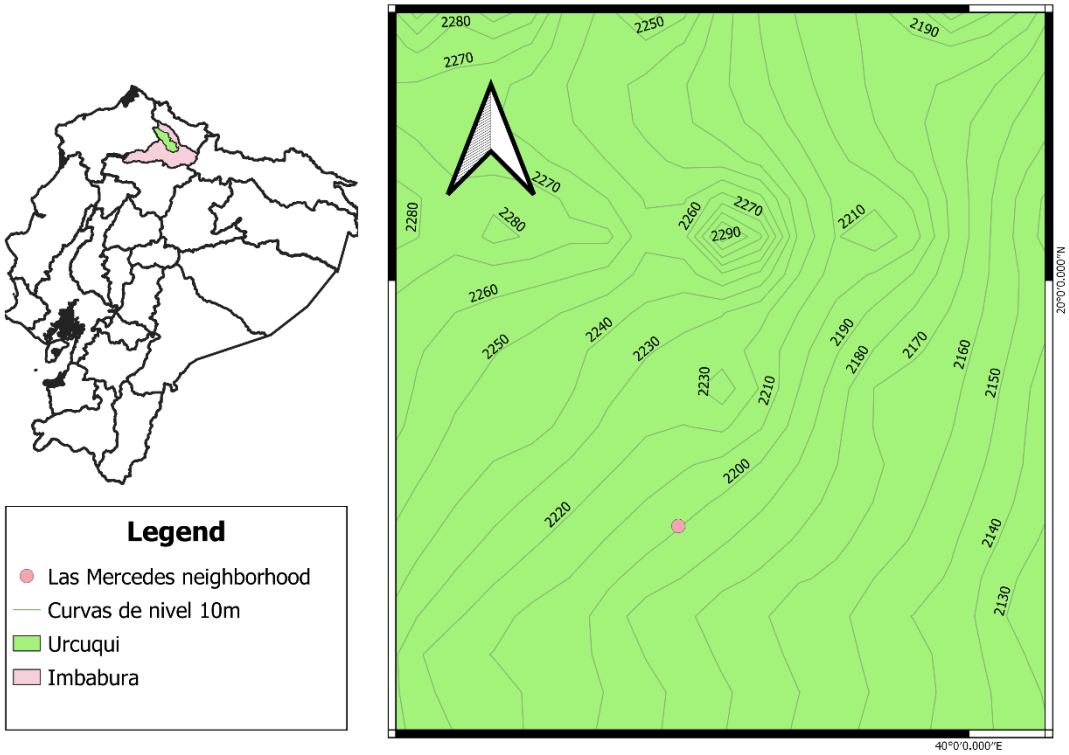

Figure 3. Location of the sampling site in the Las Mercedes neighborhood.

The Chota valley is comprised of heavily degraded inter-Andean dry montane forest and is located in Imbabura and Carchi provinces in northern Ecuador. The vegetation found here includes dry forests covered by lichens and mosses (Baquero, 2004). Various hummingbird species inhabit this area, such as *Colibri coruscans*, *Chlorostilbon*

melanorhynchus, *Hydrocharis grayi*, *Myrtis fanny*, *Amazilia tzacatl*, and *Chaetocercus mulsant*. The sampling site is located in the upper Chota valley near Yachay Tech University in the Las Mercedes neighborhood at a height above sea level of 2200 meters.

2.2. Hummingbird trap

We used two nets to capture hummingbirds without hurting them. Nets were placed above hummingbird feeders, as shown in Figure 3. Net number 1 was built with 2 meters of 16 gauge steel wire to support the trap, 3.5 meters of tule fabric, and weights to facilitate the net to drop. Net number 2 was constructed of 2.5 meters of 16 gauge steel wire as support, 3 meters of Zaran, and weights. The tule fabric and Zaran could be comfortably handled, allowing the trap to be folded and held at the steel wire with a clothespin. This trap has a mechanism that allows the cloth to fall quickly and trap the hummingbirds as they come to drink at the feeders.

Figure 4. Hummingbird traps, A) cloth net N ° 1 extended, B) Zaran net N ° 2 extended, C) cloth net N ° 1 collected, D) Zaran net N ° 2 collected and E) two nets extended with feeders.

2.3. Collection, processing, and storage of blood

The blood was collected from the metatarsal veins of six hummingbirds four *Chlorostilbon* sp. and two *Colibri coruscans* by puncture with a 3 ml syringe needle (Owen, 2011). Blood samples were drawn up with a capillary tube. Alcohol was used to disinfect the puncture site and styptic pencil (aluminum sulfate 56 %) to help stop the

bleeding. Blood samples of *Chorostilbon* sp. and *Colibri coruscans* were collected in a 1.5 ml microcentrifuge tube with 1 ml of lysis buffer, and stored at a temperature of -4°C. The lysis buffer is composed of 10 M Tris-HCl, 2 mM Na- EDTA, and 1% SDS, pH 8.0 and can be kept at room temperature for approximately four weeks (Farhat, 2013). Aliquots of 1.5 milliliters of *Clorostilbon* sp. blood were preserved as follows for six days before DNA extraction. We stored blood at room temperature (20- 24 °C) in 1.0 ml of lysis buffer for approximately 8 hours. In the laboratory, the diluted samples were stored at -80 °C for the long term, and diluted samples at -20 ° C were used for PCR.

2.4. DNA isolation

DNA was isolated from 1ml of whole blood in lysis buffer. Mitochondrial and nuclear DNA were isolated with a QIAamp Tissue and blood Kit extraction (QIAGEN) according to the manufacturer's protocol with additional steps. First, 220 µl of PBS were added for blood samples, centrifuged for 3 minutes at 1300 rpm (18327 g), and 30 µl of proteinase K to were added to the microtube containing the blood sample.

2.5. PCR amplification and mitochondrial DNA and nuclear intron sequencing

The mitochondrial ND2 gene was amplified (fragments of 500 to 700 base pair) and sequenced with the primers listed in Table 1. PCR reactions were mixed in 40 µl total volume with 20 µl of Light Mix Polymerase, 14 µl water, 4µl of each primer, and 2 µl of template DNA. The amplification of ND2 was carried out in Veriti™ 96-Well Thermal Cycler, Thermo Fisher Scientific, using the following program: initial denaturation for 4 minutes at 94°C, 35 cycles of 30 seconds denaturation at 94 °C, 30 seconds primer annealing at 60 °C and 45 seconds extension at 72 °C, final extension for 5 minutes at 72°C, and termination of the reaction was achieved by chilling the mixture to 4 °C

We used the primer pair AK6c- and AK5b+ to amplify and sequence approximately 500 to 650 base pairs of intron 5 of AK1 fragments and sequenced amplicons with the primers listed in Table 1 (Shapiro and Dumbacher, 2001). This amplification product ranged in length from about 350 to 500 bp among the individuals we sampled.

The PCR reaction mix contained 11.3 µl water, 5 µl each 5X Buffer, 2 µl each magnesium chloride, 2.5 µl each dNTPs, 2 µl each primer, 2 µl each template, and 0.2

µl Fusion polymerase. PCR amplifications were in 25 µl total volume. DNA amplification of Adenile Kinase intron 5 was carried out in a Veriti™ 96-Well Thermal Cycler -Thermo Fisher Scientific using the following program: initial denaturation for 1 minutes at 94°C, 35 cycles of 30 seconds denature at 94°C, 45 seconds primers annealing at 58°C, 45 seconds extension at 72°C, final extension for 5 minutes at 72°C, and termination of the reaction was achieved by chilling the mixture to 4 °C

Table 1
Primers used for amplification of a fragment of NADH dehydrogenase subunit 2 (ND2) gene (Hackett, 1996; Johnson, 1998(Hackett, 1996) and the Adenylate Kinase gene intron 5 (Shapiro and Dumbacher, 2001).

Primer name	**Primer sequence (5'to 3')**	Nucleotide position
Pair L5215 and H5766		
L5215	TATCGGGCCCATACCCCGAAAAT	
H5766	GGATGAGAAGGCTAGGATTTTKCG	
Pair AK5b+ and AK6c-		
AK5b+	ATTGACGGCTACCCTCGCGAGGTG	4820 to 4843
AK6c-	CACCCGCCCGCTGGTCTCTCC	5454 to 5474

We visualized PCR products on a 2% agarose gel with a 100bp ladder. PCR products were purified using the ExoASP-IT enzyme to degrade the remaining primers, and nucleotides were incubated at 37°C for 15 min. Finally, to inactivate the ExoSAP-IT, samples were incubated at 80°C for 15 min. DNA sequencing was performed by BioSin Biosciences (Quito, Ecuador). Electropherograms were edited and aligned using FinchTV v1.4.0, used to determine DNA sequence genotypes, and bioinformatically analyzed to obtain consensus sequences in Geneious v11.1.5.

2.6. Phylogenetic analyses

For the phylogenetic analysis, I drew from 151 DNA accessions found in NCBI's GenBank belonging to *Chlorostilbon.* Sequences used are summarized in Table 2 and 3. We used informative characters for maximum likelihood (ML) and maximum parsimony (MP) searches on the following species: *Cynanthus sordidus, Hylocharis*

xantusii, H. leucotis, Campylopterus villavicencio, C. rufus as outgroups. By this means, we avoided assumptions of monophyly of *Chlorostilbon* sp. (McGuire et al, 2014). Our selection of outgroup genera was based on the growing consensus that Emeralds form a monophyletic group.

We used Akaike's Information Criterion (AICs) (Akaike, 1973; see Posada and Buckley, 2004), as implemented in MEGA X to determine the best DNA substitution models. Phylogeny was obtained using the (TN93+G+I) model in ML analyses for NADH dehydrogenase subunit 2 (ND2) gene. The (HKY+I) model was used for Adenylate kinase AK1 intron 5, and a (TN93+G) model for the combination of ND2-AK1. The evolutionary analyses were performed in MEGA X (Kumar et al., 2018).

Table 2
GenBank accession number and vouchers of DNA sequences for NADH dehydrogenase subunit 2 (ND2) genes used in phylogenetic analysis, * Note NGBY = No GenBank accession number), *NV= no voucher number

Species	Voucher	GenBank accession number
C?.1	NV	NGBY
C?.2	NV	NGBY
C?.3	NV	NGBY
C?.4	NV	NGBY
Campylopterus rufus	FMNH:434026	KJ602203.1
Campylopterus villaviscensio	LSUMNS B-5588	AY830468.1
Chlorostilbon assimilis	LSUMZ B-46655	KJ602210.1
Chlorostilbon assimilis	LSUMZ B-46483	KJ602209.1
Chlorostilbon canivetii	NV	KC858433.1
Chlorostilbon canivetii	UWBM 69030	KJ602212.1
Chlorostilbon lucidus pucherani	FMNH 396025	KJ602208.1
Chlorostilbon maugaeus	LSUMZ B-11520	KJ602213.1
Chlorostilbon melanorhynchus	JVLP 36	KJ602214.1
Chlorostilbon melanorhynchus	LSUMZ B-31514	KJ602211.1
Chlorostilbon melanorhynchus	LSUMNS B-6327	AY830470.1
Chlorostilbon mellisugus	LSUMNS B-9450	AY830471.1

Chlorostilbon notatus	MNH 392810	EU042539.1
Chlorostilbon notatus	FMNH 392347	KJ602207.1
Chlorostilbon notatus	LSUMZ B-35953	KJ602206.1
Chlorostilbon notatus	LSUMZ B-4558	KJ602205.1
Chlorostilbon ricordii	ANSP 5570	KJ602215.1
Chlorostilbon salvini	MBM_7829	KJ602216.1
Chlorostilbon swainsonii	AMNH_NKK_1017	KJ602217.1
Cynanthus sordidus	ALTBAL081	MN807578.1
Cynanthus sordidus	ALTBAL04	MN807577.1
Cynanthus sordidus	UAG013	MN807561.1
Hylocharis leucotis	LSUMZ_B-22003	KJ602252.1
Hylocharis xantusii	LSUMZ_B-3794	KJ602253.1

Table 3
GenBank accessions number and vouchers od DNA sequences to Adenylate kinase AK1 intron 5 gene used in phylogenetic analysis, * Note NGBY = No GenBank accession number), *NV= no voucher number

Species	Voucher	GenBank accession Number
C?.1	NV	NGBY
C?.2	NV	NGBY
C?.3	NV	NGBY
C?.4	NV	NGBY
Campylopterus rufus	FMNH:434026	KJ601826.1
Campylopterus villaviscensio	LSUMNS B-5588	AY830544.1
Chlorostilbon assimilis	LSUMZ B-46655	KJ601832.1
Chlorostilbon aureoventris	LSUMNS_B-18899	GU167160.1
Chlorostilbon aureoventris	LSUMZ_B-31514	KJ601833.1
Chlorostilbon lucidus pucherani	FMNH_396025	KJ601831.1
Chlorostilbon melanorhynchus	LSUMNS B-6327	AY830546.1
Chlorostilbon mellisugus	LSUMZB-46655	AY830547.1
Chlorostilbon notatus	FMNH_392810	EU042457.1

Chlorostilbon poortmani	JVLP_i4198	GU167161.1
Chlorostilbon ricordii	ANSP_5570	KJ601837.1
Chlorostilbon salvini	MBM_7829	KJ601838.1
Chlorostilbon swainsonii	AMNH_NKK_1017	KJ601839.1
Cynanthus sordidus	ALTBAL081	MN746593.1
Cynanthus sordidus	ALTBAL04	MN746590.1
Cynanthus sordidus	UAG013	MN746595.1
Hylocharis leucotis	LSUMZ_B-22003	KJ601875.1
Hylocharis xantusii	LSUMZ_B-3794	KJ601876.1

2.7. Morphological analysis

For the morphological analysis the following measurements were taken: body mass, exposed culmen, total culmen, wing chord, tail length, rectrice 1 length, rectrice 5 length, total tail, and metatarsus (Styles 1999). All captured individuals were male. We used the following material for the measurements: CEN-TEC 93543 portable balance with a 0.1 g precision, a 10-300 mm bird wing ruler, and a compass. Picture were taken with a NIKON model D5600 camera with a 18-55 mm lens, and for observation we used 10x42 Swarovski binoculars.

3. Results

A total of 6 hummingbirds of 2 species were captured at the Chota valley locality. Our analyses recovered a multilocus phylogenetic estimate based on samples of *Chlorostilbon* and outgroups. Our phylogenetic analysis using maximum parsimony and maximum likelihood of ND2 and AK1 loci approximates the topology McGuire et al. (2014) of the Emerald's clade.

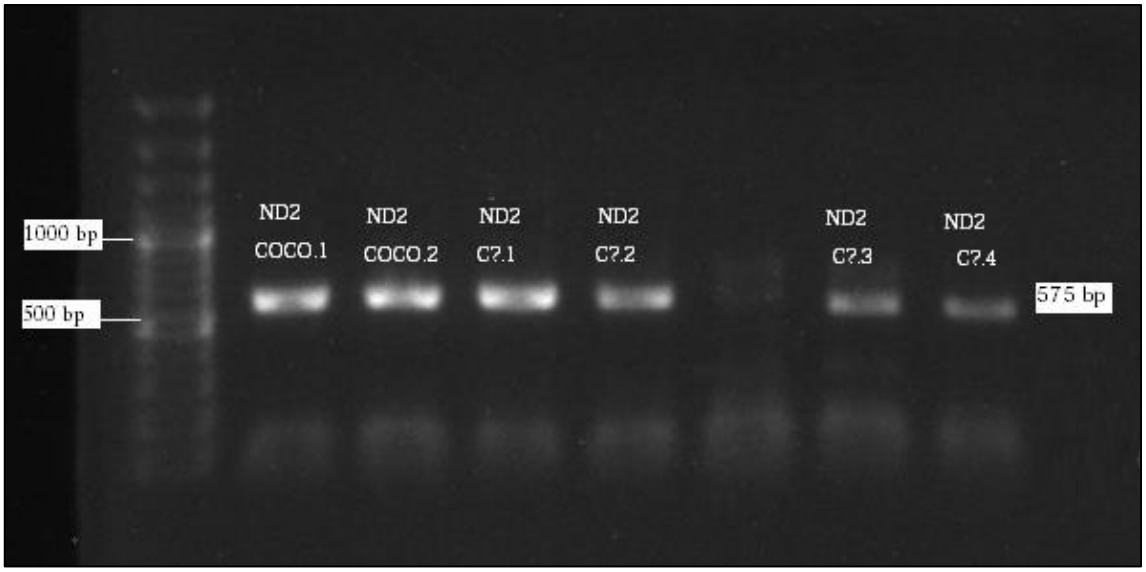

Figure 5. Electrophoresis for detection of the ND2 gene by PCR from blood samples. In lane 1 molecular weight marker, lane 2-8 hummingbird samples.

3.1. Phylogenetic analyses

We use a super matrix made up of concatenated gene alignments in our phylogenetic analysis. We generated partition schemes in which substitution models were specified for each gene or character set.

Our maximum parsimony analysis using ND2 and AK1 produced three slightly different tree topologies. There are several disagreements related to deeper nodes. Phylogenetic analysis, pairwise distances for ND2 gene versus AK1 intron 5, suggests that for relatively recently diverged taxa. AK1 intron 5 exhibits a substitution rate several times slower than ND2 gene, but this difference is effectively reduced at deeper lineage divergences as cytochrome-b saturates relatively to AK1 (Shapiro, 2011).

Maximum parsimony analysis of ND2 gene resulted in a bootstrap consensus tree inferred from 2500 replicates. The MP tree was obtained by the random addition of sequences (10 replicates). This analysis involved 28 nucleotide sequences. There were

a total of 1636 positions in the final dataset. For the clade comprised of individuals C.1, C.2, C.3, C.4, *C. melanorhynchus* and *C. assimilis* a bootstrap value of 98% (90-100% strongly supported) was obtained, and a bootstrap of 91% for the clade only consisting of C.1 and C.2 with C.3 and C.4 is strongly suggested. (Figure 6).

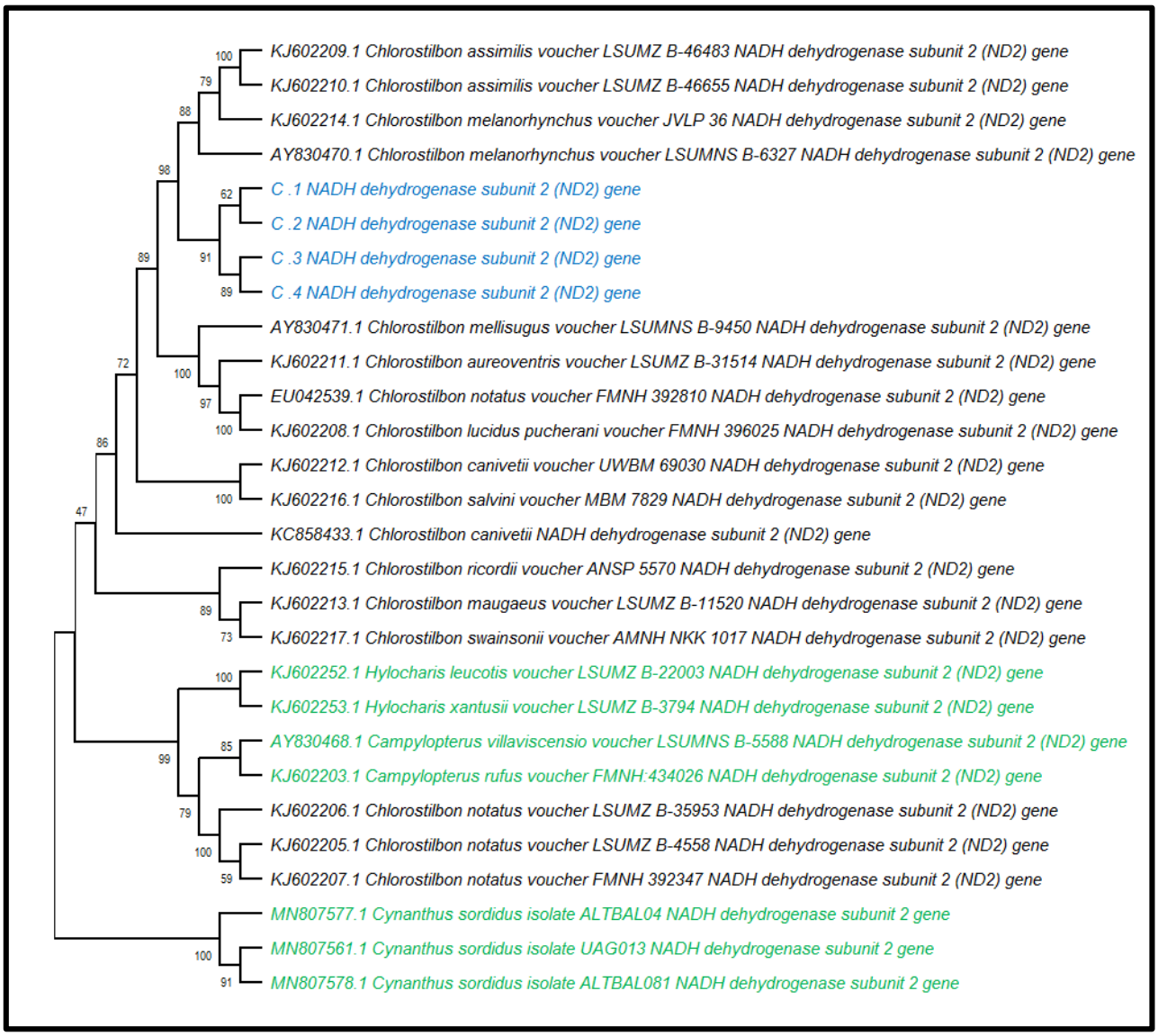

Figure 6. Phylogenetic tree of various species of *Chlorostilbon* with outgroups. Maximum parsimony analysis using the NADH dehydrogenase subunit 2 (ND2) gene. Bootstrap values are shown for each of the corresponding branches. GenBank accession numbers, species names and museum collection numbers are also shown.

For maximum likelihood analyses, the corrected Akaike Information Criterion value (AICc) was used in this study to choose the best-fitting nucleotide substitution model from 24 different models.

We inferred the evolutionary history using the Tamura-Nei model (TN93+G+I) on the ND2 gene. The tree with the highest log likelihood (-5907.20) is shown. The first tree(s) for the heuristic search were obtained by applying Neighbor-Join and BioNJ algorithms. Besides, gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2650)). This analysis involved 28 nucleotide sequences. There were a total of 1636 positions in the final dataset.

Individuals C.1, C.2, C.3, C.4, *C. affinis* and *C. melanorhynchus* form a clade with a bootstrap support of 96% (> 90-100% strongly supported). The clade including only the four individuals of the enigmatic *Chlorostilbon* has a bootstrap support of 68% (50> 70% weakly support). Again, a monophyletic relationship between *C. assimilis*, *C. melanorhynchus*, C.1, C.2, C.3, and C.4 is suggested.

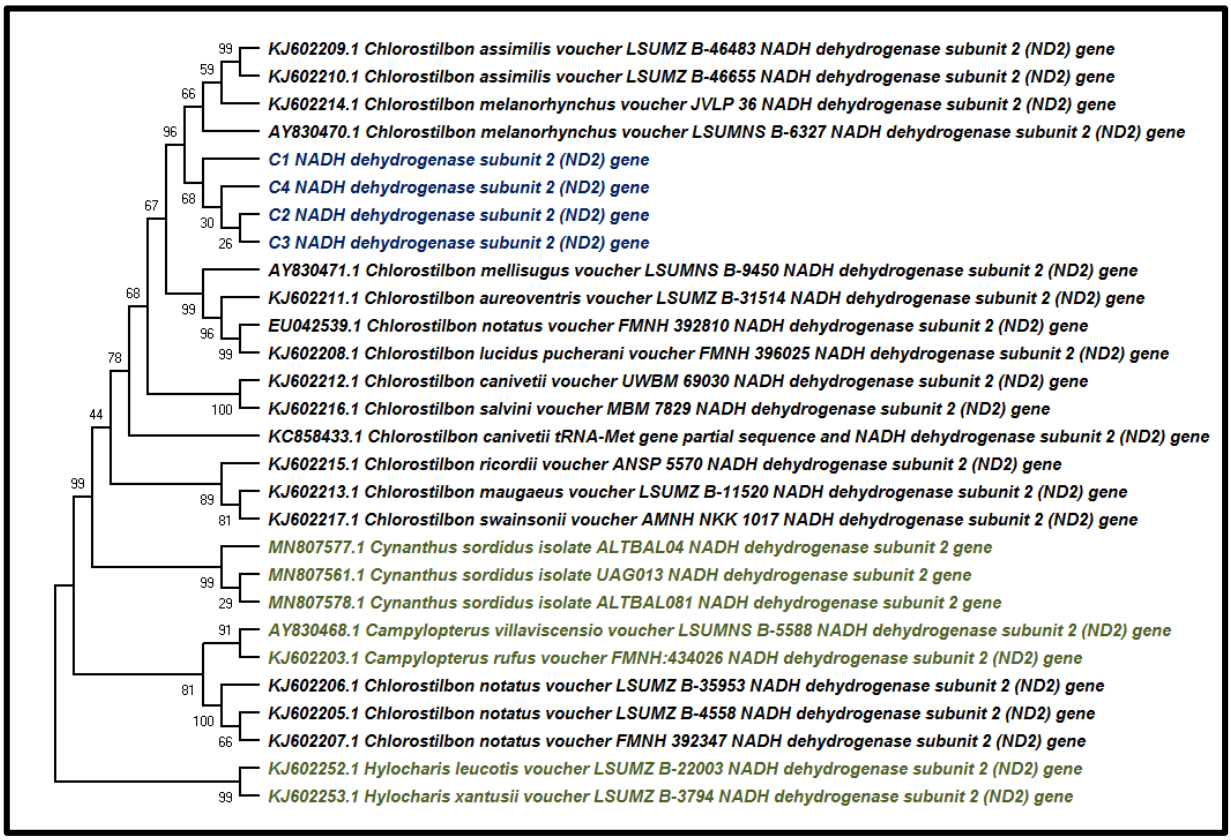

Figure 7. Phylogenetic estimate for hummingbirds based on maximum likelihood analysis. The topology is the Bootstrap consensus tree of the ND2 gene. Bootstrap values are shown for each of the corresponding branches. GenBank accession numbers, species names and museum collection numbers are also shown.

Maximum parsimony analysis of taxa method to AK1 gene resulting in a bootstrap consensus tree inferred from 2500 replicates. The MP tree was obtained by random

addition of sequences (10 replicates). This analysis involved 22 nucleotide sequences. There were a total of 574 positions in the final dataset.

As shown in Figure 8, , a bootstrap value of 68% was obtained for individuals C.1, C.2, C.3, C.4 with *C. melanorhynchus*, and a bootstrap of 62-64% (50> 70% weakly support) for the clade consisting of C.1, C.2, C.3 and C.4. A monophyletic relationship between C.1, C.2, C.3, C.4, *C. assimilis*, *C. melanorhynchu*s, *C. pootmani*, *C. salvini*, *C. mellisugus*, *C. aureoventris*, *C. notatus*, and *C. lucidus pucherani* was confirmed.

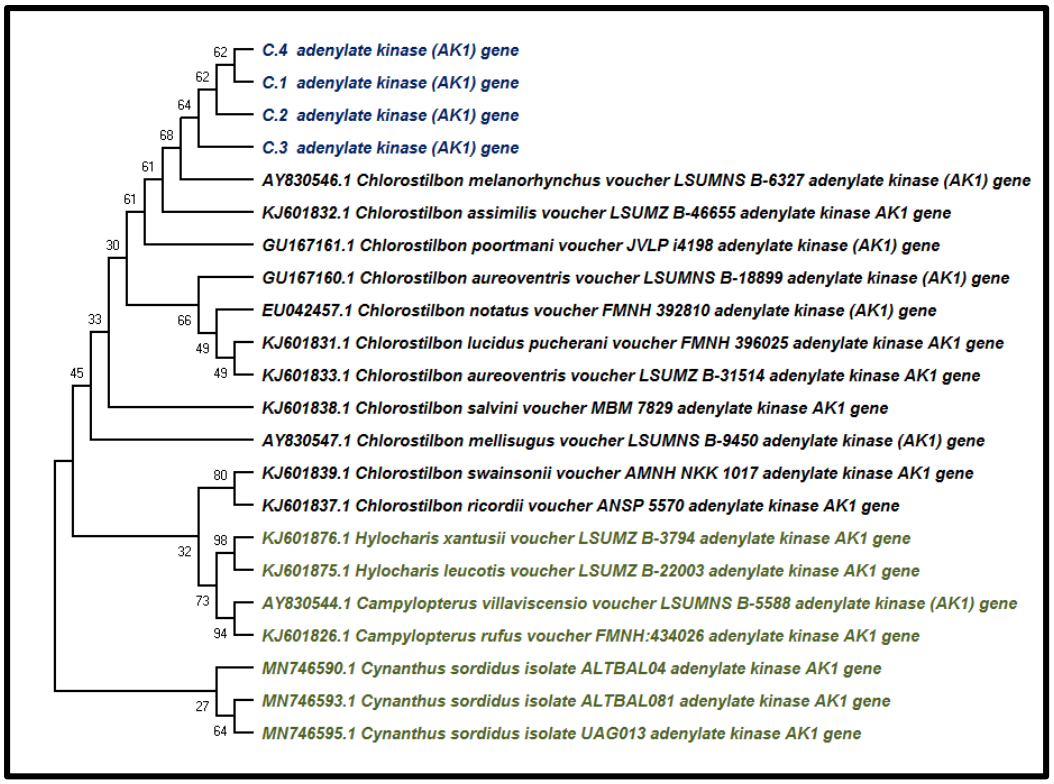

Figure 8. Phylogenetic tree to *Chlorostilbon* with outgroups. Maximum parsimony analysis of taxa to Adenylate kinase (AK1) gene. Bootstrap method (%) values are shown to each of the corresponding branches. Also, include GeneBank accession code of NCBI and voucher location.

Models with the lowest AICc value (Akaike Information Criterion, corrected) are considered to describe the observed substitution pattern the best. The maximum likelihood was inferred applying the Hasegawa-Kishino-Yano model (HKY+I). The bootstrap consensus tree is inferred from 2500 replicates. The heuristic search initial trees were obtained automatically by applying Neighbor-Join and BioNJ algorithms. This analysis involved 22 nucleotide sequences. There were a total of 574 positions in the final dataset.

Chlorostilbon melanorhynchus is observed on the same branch as C.3 for the Ak1 gene. Bootstrap values are relatively low among *Chlorostilbon* species.

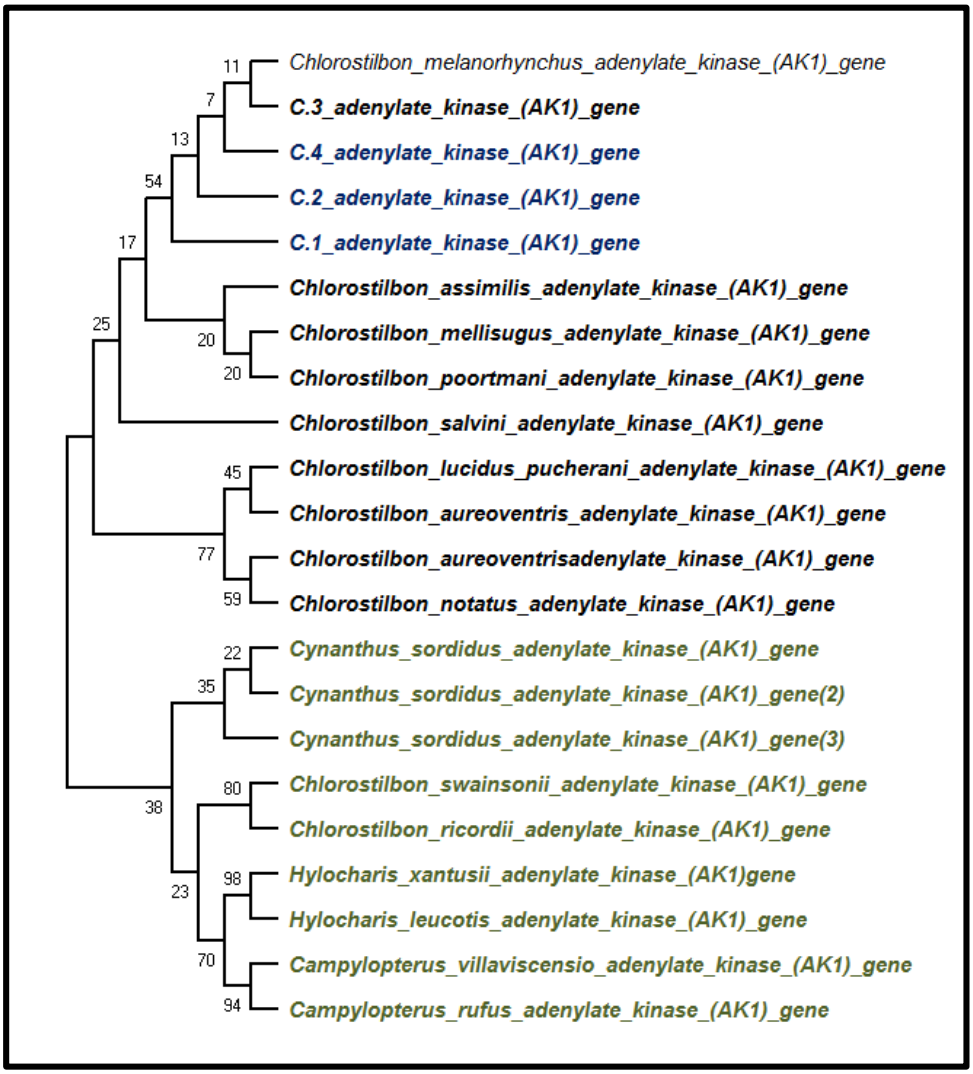

Figure 9. Phylogenetic estimate for hummingbirds based on maximum likelihood analysis. The topology is the Bootstrap consensus tree to AK1 gene. Bootstrap method (%) values are shown to each of the corresponding branches. Furthermore, the code GenBank accession of NCBI is showed in table 4.

Maximum parsimony analysis of taxa method to combination ND2-AK1 genes resulted in the percentage of replicate trees in which the taxa clustered together in 2500 replicates. This analysis involved 19 nucleotide sequences.

Figure 10 shows a bootstrap value of 96% among C.1, C.2 C.3 and C.4. It is evidenced a monophyletic relationship among C.1, C.2, C.3, C.4, *C. assimilis*, *C. melanorhynchus*, C. mellisugus, *C. aureoventris*, *C. notatus*, and *C. lucidus pucherani* is evidenced.

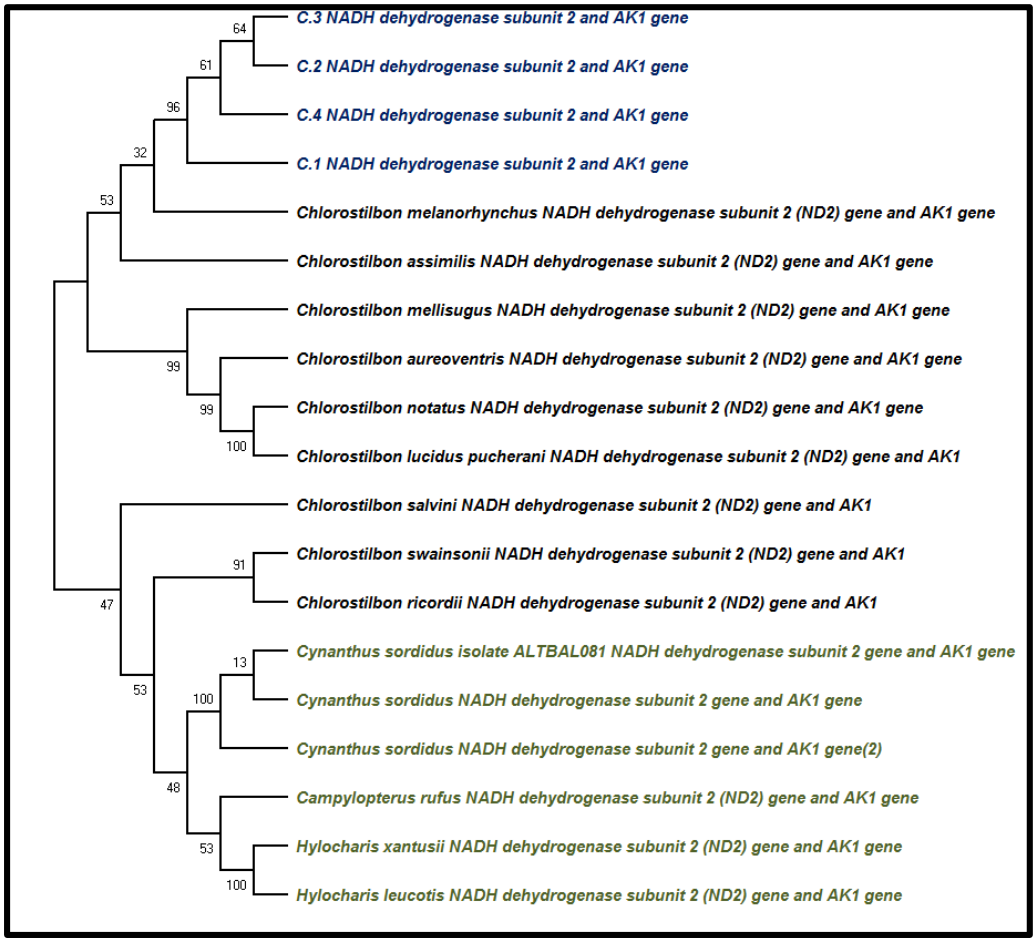

Figure 10. Phylogenetic tree to *Chlorostilbon* with outgroups and samples. Maximum parsimony analysis of taxa to ND2-AK1 genes. Bootstrap method (%) values are shown next to the corresponding branches. Also, include GeneBank accession code of NCBI is showed in table 4 and voucher location.

Models with the lowest AICc value (Akaike Information Criterion, corrected) are considered to describe the substitution pattern the best. For each model, the AICc value, maximum likelihood value (lnL) is -5700, 38565. This analysis involved 19 nucleotide sequences.

Maximum likelihood method to ND2-AK1 genes used Tamura-Nei model (TN93+I). The bootstrap consensus tree was inferred from 2500 replicates to represent the evolutionary history of the taxa analyzed. The first tree(s) for the heuristic search were obtained by applying Neighbor-Join and BioNJ algorithms. This analysis involved 19 nucleotide sequences.

It is observed that in figure 11 for individuals C.1, C.2, C.3, C.4, there is a bootstrap value of 70% among C.1, C.2, C.3 and C.4. *C. melanorhynchus* and specimens of interest bootstrap of 78%. It is evidenced of monophyletic between C.1, C.2, C.3, C.4,

C. melanorhynchus, C. assimilis, C. salvini, C. mellisugus, C. aureoventris, C. notatus, and C. lucidus pucherani.

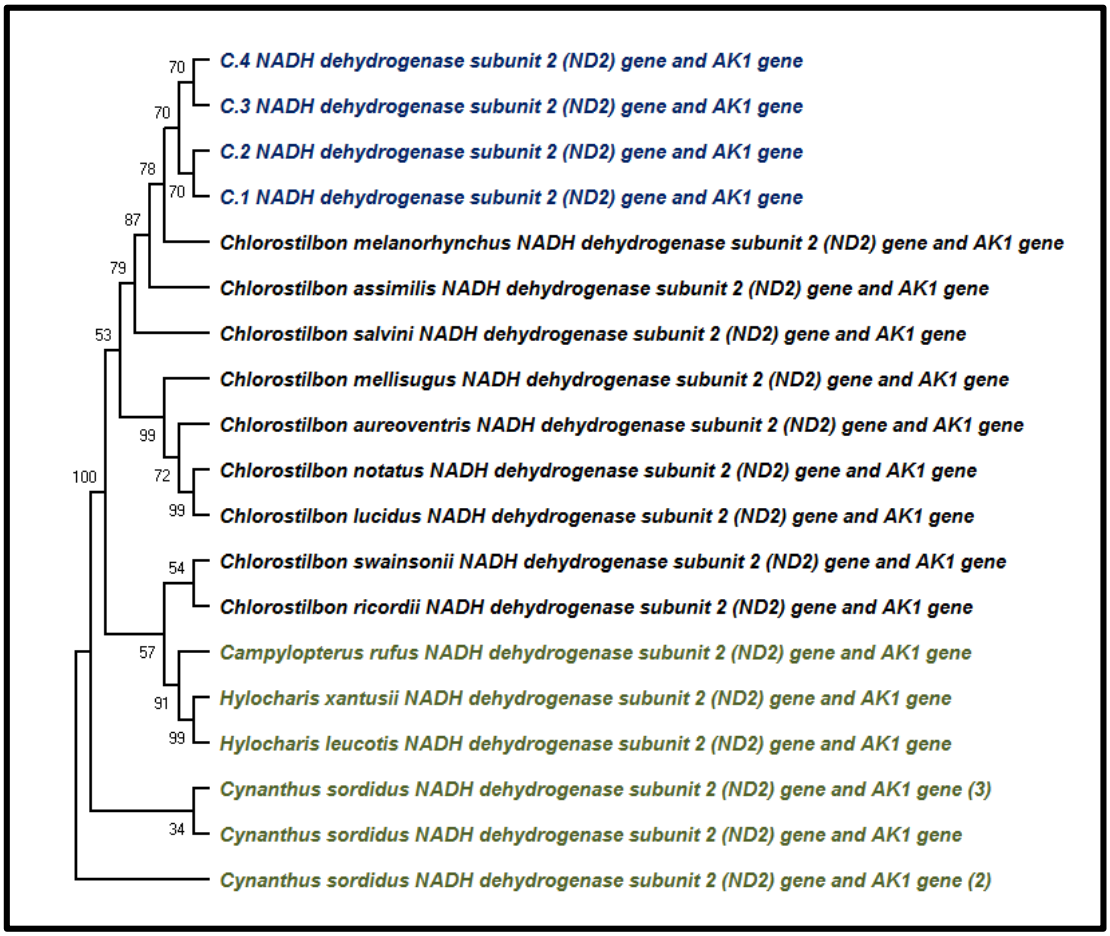

Figure 11. Phylogenetic estimate for hummingbirds based on maximum likelihood analysis. The topology is the Bootstrap consensus tree to ND2-AK1 gene. Bootstrap method (%) values are shown next to the corresponding branches. Also, the code GeneBank accession of NCBI is showed in table 4.

3.2. Morphological analyses

For the taxonomic analysis, we have photographic evidence of specimens C.2, C.3, and C.4. Individual C.1 escaped before picture could be taken.

Specimen C.2 is male and has a yellowish crown; when viewed from another angle, the crown has a green color, the chest is bluish, and seen from different angles an emerald green color (Figure 12).

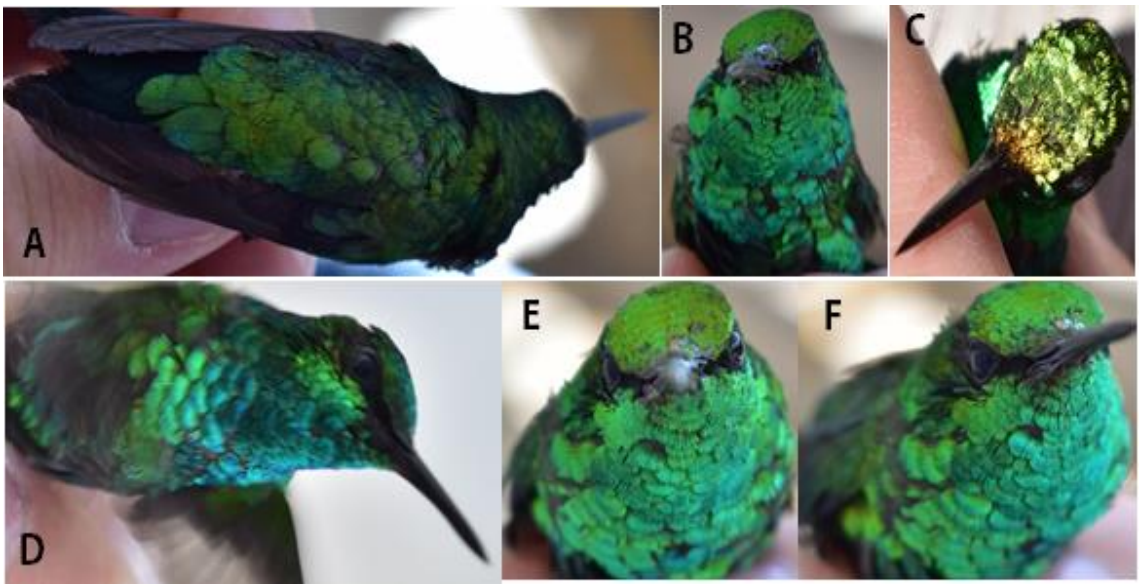

Figure 12. Coloration in the life of enigmatic *Chlorostilbon* sp. individual C.?2. **A**: back, crissum and wing; **B**: crown and chin; **C**: crown and bill; **D**: auriculars, throat and chest; **E, F**: flank, and chest. Adult male.

Specimen C.3 is a juvenile or eclipse plumage male as the plumage was molting ahead of the reproductive season. It has an emerald-colored crown, a bluish chest, seen from different angles. Wingtips are level with the tips of the tail. The tail feathers have a white tip, and it did no longer have the left rectrice #5 (Figure 13).

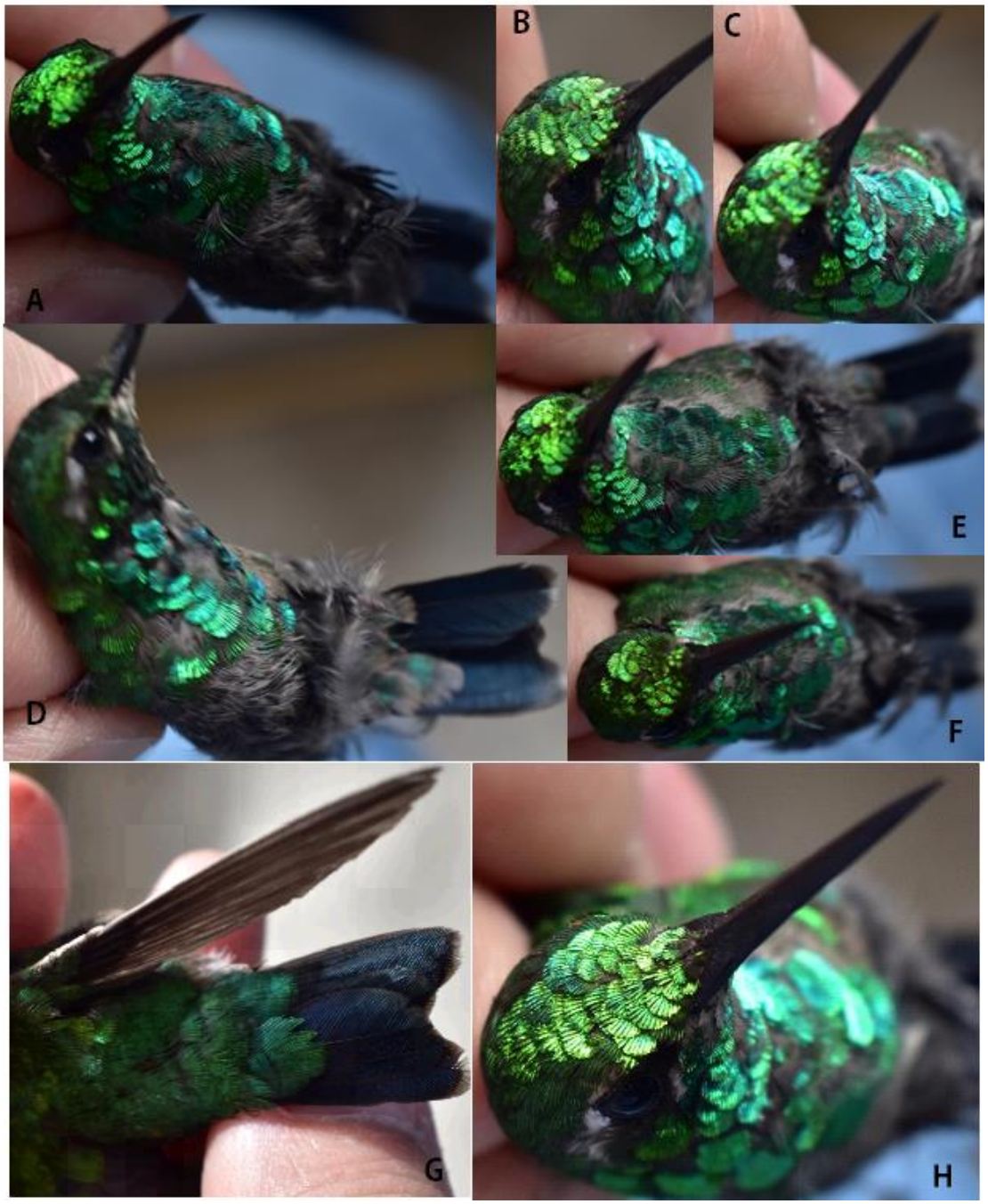

Figure 13. Coloration in life of the enigmatic *Chlorostilbon* sp. individual C?3. **A**: throat, chest, flank and foot; **B**: crown; **C**: bill; **D**: auricular and undertail covers, **E**: chin; **F**: crown; **G**: wing and tail; **H**: crown and bill.

Specimen C.4 is male, has a green crown and, viewed from another angle, is slightly yellow. The feathers are similar in size, the chest is bluish and green observed from another angle (Figure 13).

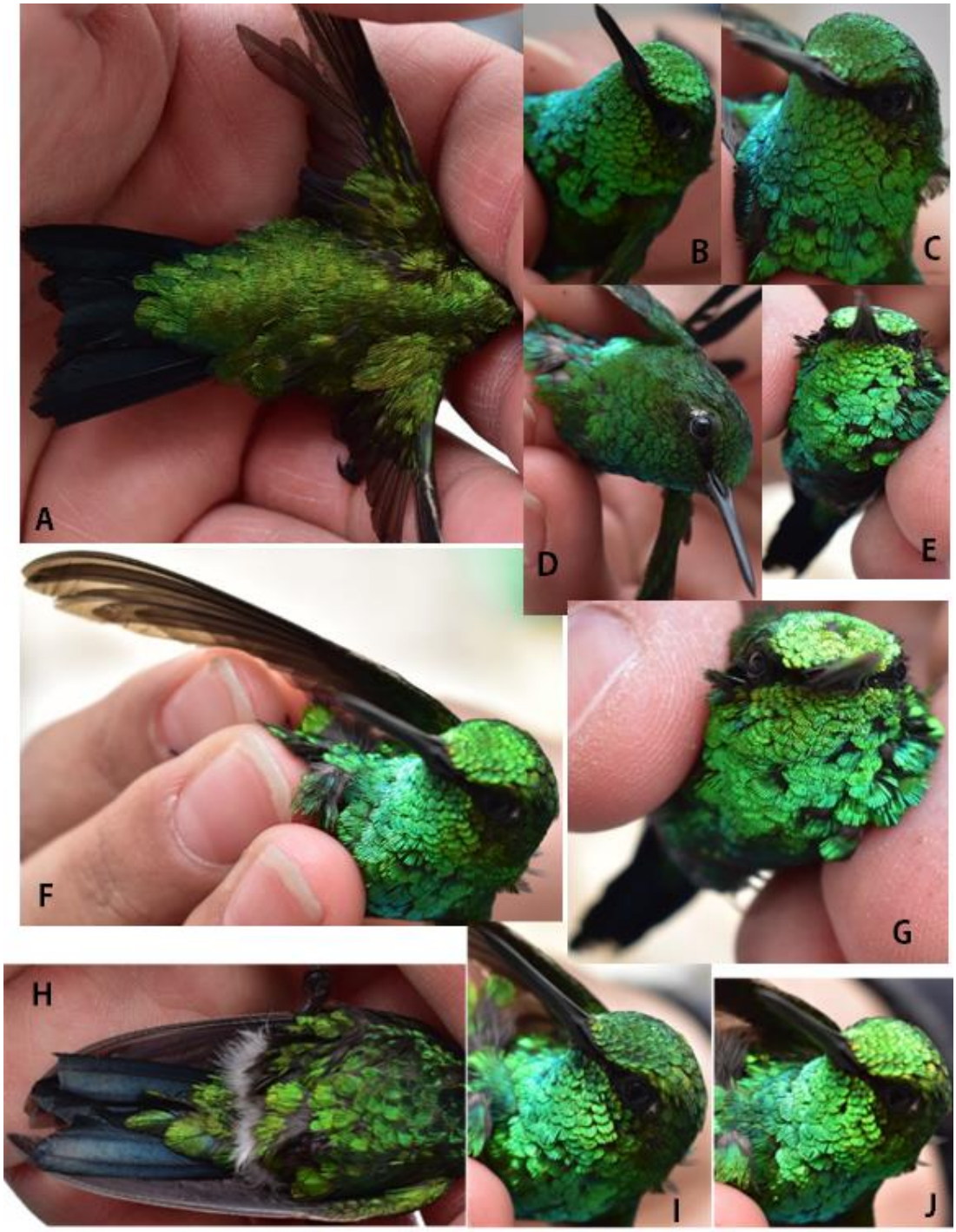

Figure 14. Coloration in life of enigmatic *Chlorostilbon* sp. individual C.?4. A: Back and tail. **B**: Crown, bill, auriculars, **F**: wing, crown and throat; **G**: chest; **H**: flank, undertail coverts, and tail; **I,J**: bill, throat and chin. Adult male.

The four male individuals captured had weights ranging from 2.7 g to 3.2 g. It can be seen that the individual C? .1, C? .2 and C? .3 the measurement of the metatarsal is similar, unlike the individual C? .4, which has a length greater than approximately 0.5 mm.

Figure 15. Coloration in life of *C. (melanorhynchus) mellisugus* individual 3385 **A**: chest **B**: back and tail. **C:** crown, chest, flank, undertail coverts. Adult male.

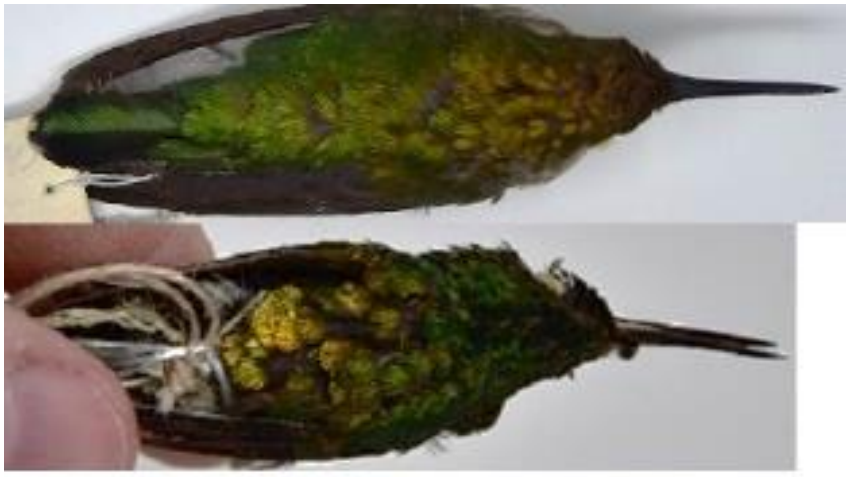

Figure 16. Coloration in life of *C. (melanorhynchus) mellisugus* individual 348. Back and tail, chest, flank, undertail coverts, tail and chin. Adult male.

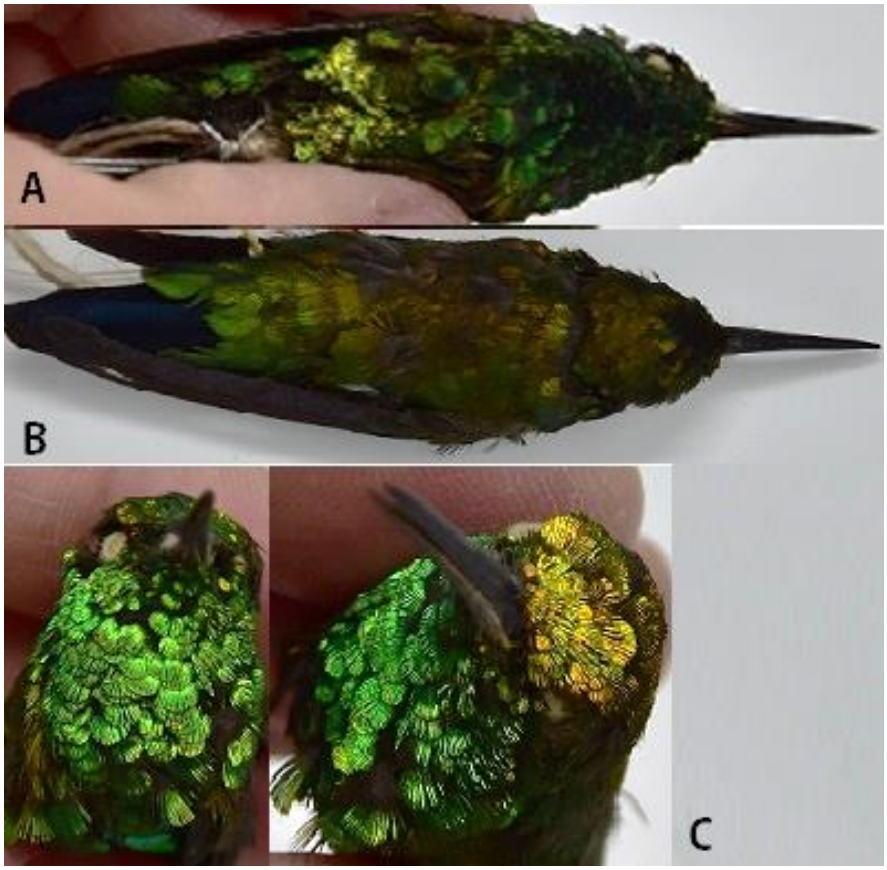

Figure 17. Coloration in life of *C. (melanorhynchus) mellisugus* individual 1910. **A**: flank, undertail coverts, and tail **B**: back and tail. **C**: crown, bill, chest. Adult male.

Figure 18. Coloration in life of *C.* cf. *mellisugus* individual 8660. Crown, chest, flank and undertail coverts. Male.

Figure 19. Coloration in life of *C.* cf.*mellisugus* individual 8657. Bill, throat and chest. Adult male.

Morphological measurements were made for 4 captured individual hummingbirds and 18 hummingbird skins for two species and enigmatic hummingbird (Table 4).

TABLE 4

MEASUREMENTS OF VARIOUS FORMS OF THE *CHLOROSTILBON. CF.MELLISUGUS* COMPLEX FROM CHOTA VALLEY AND COLLECTION OF HUMMINGBIRD SKINS FROM THE ECUADORIAN MUSEUM OF NATURAL SCIENCES

	N°	Sex	Code	Body Mass (g)	Exposed culmen (mm)	Total culmen (mm)	Wing chord (mm)	Tail length				metatarsus (mm)
								Rectrice 1 (mm)	Rectrice 5 (mm)	Relative tail fork	Total tail	
***Chlorostilbon* sp.**	1	M	C?.1	3.20	11.10	20.20	48.00	20.50	26.00	5.50	28.00	5.00
***Chlorostilbon* sp.**	2	M	C?.2	3.20	10.20	19.00	50.00	22.60	31.70	9.10	27.00	5.00
***Chlorostilbon* sp.**	3	M	C?.3	2.70	16.00	21.00	50.00	21.30	26.80	5.50	29.50	5.00
***Chlorostilbon* sp.**	4	M	C?.4	3.20	16.90	20.20	49.00	21.90	28.80	6.90	28.00	5.50
C. (melanorhynchus) mellusigus	7	M	3385	N/A	15.50	21.49	57.00	24.33	28.30	3.97	29.00	N/A
C.* cf. *mellisugus	8	M	8660	2.81	14.10	18.54	49.00	22.12	27.12	5.00	27.00	N/A
C. (melanorhynchus) mellusigus	9	M	1910	N/A	17.14	20.33	53.00	22.66	29.20	6.54	28.00	N/A
C. (melanorhynchus) mellusigus	10	M	1917	N/A	16.20	19.56	47.00	22.11	28.65	6.54	26.00	N/A
C. (melanorhynchus) mellusigus	11	M	1913	N/A	N/A	N/A	47.00	19.97	26.86	6.89	27.00	N/A
C. (melanorhynchus) mellusigus	12	M	348	N/A	13.88	19.31	47.00	21.82	26.82	5.00	26.00	N/A
C. (melanorhynchus) mellusigus	13	M	4490	2.80	15.41	22.28	49.00	23.53	29.24	5.71	29.00	N/A
C. (melanorhynchus) mellusigus	14	M	4242	N/A	14.83	18.03	46.00	22.91	29.83	6.92	27.00	N/A
C.* cf. *mellisugus	15	M	8657	2.90	15.50	19.09	50.00	23.11	28.11	5.00	28.00	N/A

C. (melanorhynchus) mellusigus	16	M	4475	2.85	15.62	18.09	45.00	19.44	26.48	7.04	26.00	N/A
C. (melanorhynchus) mellusigus	17	M	4488	3.05	16.88	19.83	45.00	22.11	26.25	4.14	27.00	N/A
C. (melanorhynchus) mellusigus	18	M	4476	3.10	15.58	19.08	45.00	23.08	26.48	3.40	27.00	N/A
C. melanorhynchus	19	F	337	N/A	15.64	20.65	48.00	25.97	28.20	2.23	28.00	N/A
C. (melanorhynchus) mellusigus	20	F	1911	N/A	17.05	20.67	47.00	26.33	27.68	1.35	29.00	N/A
C. (melanorhynchus) mellusigus	21	F	1915	N/A	15.38	17.87	45.00	22.75	24.75	2.00	27.00	N/A
C. (melanorhynchus) mellusigus	22	F	1914	N/A	15.57	19.25	45.00	17.28	22.06	4.78	23.00	N/A
C. melanorhynchus	23	F	9769	3.90	17.17	19.84	50.00	23.55	26.08	2.53	30.00	N/A
C. melanorhynchus	24	F	8663	2.87	15.47	19.49	45.00	28.40	29.20	0.80	29.00	N/A

4. Discussion

Based on maximum parsimony and maximum likelihood analyses of molecular datasets to determine the phylogenetic placement of the enigmatic *Chlorostilbon* and qualitative morphological examination, we suggest that the enigmatic hummingbird might represent a hybrid population or a species in its own right.

The phylogenetic analysis resulted is very similar to ND2, AK1 and ND2-AK1 trees topologies. Ideally, differences in evolutionary rates within and between gene regions of ND2 and AK1 would be reflected in phylogenetic tree by giving more weight to relatively conserved sites and substitution types that occur less frequently, thus emphasizing changes and sites with a lower probability of homoplasies (Johnson & Sorenson, 1998). The mitochondrial DNA has a high rate at which nucleotide substitutions accrue at silent positions (Thomas et al., 1989; Edwards et al. 1991; Irwin et al. 1991). The ND2 gene is used in studies of recently evolved groups, such as many birds (Kessler and Avise 1984; Avise and Zink 1988).

Our analysis of the mitochondrial gene for 28 sequences resulted in a well-supported estimate of the hummingbird phylogenetic relationship with nodes of maximum likelihood of ND2 gene bootstrap values >95% for our clade of interest. To nuclear gene for 22 sequences of DNA poorly supported our estimate of emerald phylogenetic relationship. Only one node in the maximum likelihood analysis of the AK1 gene yielded a bootstrap value >50%.

The present phylogeny shows the monophyly of the emerald clade, confirming the species groups as proposed by McGuire (2014). Although genetically more related to *C. melanorhynchus*, we suggest that specimens of our enigmatic *Chlorostilbon*, C.?1, C.?2, C.?3 and C.? 4, from southern Chota valley might not be *C. melanorhynchus.* This conclusion is based on their highly similar morphological traits compared to the *C. mellisugus* holotype from the Sierra de Chiribiquete, Depto. Caqueta, Colombia (Stiles, 1996). One C. *melanorhynchus* (AY830470.1) specimen clusters within our samples. It was collected from an unspecified site in Pichincha province. The topology of the tree generated using AK1 gene based on maximum likelihood analysis grouped C. *melanorhynchus* with C?.3.

This study supports previous suggestions of a recent speciation event and recent hybridization between this species. Bleiweiss et al., (1997) proposed a DNA hybridization tree for the Emerald clade that is congruent with the tree determined by our study.

Hybridization may generate an increase in the strength of any barriers to gene exchange and protect larger areas of the genome from introgression. As a result the hybridization may initiate speciation (Wu, 2001; Via, 2009).

Chlorostilbon melanorhynchus and *C. mellisugus* are easily distinguished by body size, length, and plumage coloration (Stiles, 1996). These species occupy opposite slopes of the Andes, *C. melanorhynchus* being found in the Western Andes and *C. mellisugus* in the easterns Andes of Ecuador (Freile, 2019; Stiles et al., 2000). Phylogenetic study of the *C. melanorhynchus* group based on DNA sequences supports enigmatic *Chlorostilbon* sp. as a new species, closely related to *C. melanorhynchus*. However, morphologically we can suggest that the phenotype in more similar to *C. mellisugus*. In addition, the phylogenetic tree shows us that it is a population that is diverging from *C. melanorynchus sensu stricto* (in a strict sense), which appears to move seasonally into the valley, something that needs further studies.

Our results support the morphological validity of the enigmatic *Chlorostilbon* sp. as a distinct species, because the coloration of the plumage and measurement are different from those reported by Stiles (1996) for *C. melanorhynchus* and *C. mellisugus*. Most of the hummingbird skins kept at the Ecuadorean Museum of Natural Sciences differ in their coloration patterns of the head, chest, neck, and feathers that cover the tail from our Chota *Chlorostilbon*. Most specimens of MECN are erroneously identified as *C. mellisugus* but we conclude that they are indeed *C. melanorhynchus.* The skins were collected between 1962 – 2012. Since then, McGuire et al. (2014) proposed a phylogeny of the Trochilidae, based on DNA sequence data that has greatly clarified relationships within the family. For this reason, comparing the skins found in the collection for *C. melanorhynchus* and *C. mellisugus* of the Ecuadorian science museum is insufficient for or study.

Specimens from the eastern slope and Amazon need to be added to this analysis to clarify patterns in morphological variation within *C. melanorhynchus* and *C. mellisugus*. Likewise, DNA analysis for the ND2 and AK1 genes from individuals belonging to the eastern population is suggested for data more complete appreciation of the genetic variation found in these species. In fact, we suggest the inclusion of other nuclear (adenylate kinase (AK1) gene, exons 5, 6 and partial cds) and mitochondrial genes (NADH dehydrogenase subunit 4 gene, partial cds; and tRNA-His, tRNA-Ser, and tRNA-Leu genes) for a greater resolution in phylogenetic relationships.

5. Conclusions

The *Chlorostilbon* sp. described in this work is an enigmatic population belonging to *C. mellisugus* species complex. It is commonly found in the Chota valley's dry forest. Due to its morphological characteristics, this species has a greater affinity with *C. mellisugus,* although genetic data suggest a closer affinity to *C. melanorhychus*.

We conclude that the mitochondrial ND2 gene tree is more likely to be congruent with the species tree than is the nuclear AK1 gene tree. The enigmatic hummingbird appears to be more similar to *C. melanorhynchus*. The population of the enigmatic species *Chlorostilbon* possibly originated through hybridization with the local form of *C. mellisugus*.

6. Recommendations

Based on the results of this study, we propose the following improvements and/or venues for continued research. These recommendations can be summarize as follows.

For sampling in the field:

- Keep the hummingbirds well hydrated during blood sampling; this will help increase the blood volume that can be sampled from the birds. It will also allow the hummingbird not to fatigue.

For lab procedures:

- Let the spin column membrane incubate for 2 minutes at room temperature in Buffer AE and repeat this step two more times; this will help avoid losing the DNA sample during the extraction.

For a better resolution of the phylogenetic tree:

- Sequencing of more genes is required to get a better phylogenetic resolution.
- More museum specimens need to be examined regarding variations in plumage coloration to obtain a better idea of plumage variability in the enigmatic species of *Chlorostilbon*, *C. melanorhynchus* and *C. mellisugus*; this will serve to corroborate the results of this study.

7. References

Altshuler, D.L., Dudley, R., McGuire, J.A., 2004a. Resolution of a paradox: hummingbird flight at high elevation does not come without a cost. Proc. Natl. Acad. Sci. USA 101, 17731–17736.

Altshuler, D.L., Stiles, F.G., Dudley, R., 2004b. Of hummingbirds and helicopters: hovering costs, competitive ability, and foraging strategies. Am. Nat. 163, 16–25.

Akaike, H. 1973. *Information theory and an extension of the maximum likelihood principle.* Pages 267-281 in Second international symposium on information theory (P. N. Petrov and F. Csaki, eds.). Akad. Kiado, Budapest

Arzuza, D 2019. *Chlorostilbon melanorhynchus* En: Freile, J. F., Poveda, C. 2019. *Birds of Ecuador.* Version 2019.0. Museo de Zoología, Pontificia Universidad Católica del Ecuador. https://bioweb.bio/faunaweb/avesweb/FichaEspecie/Chlorostilbon%20melanorhynchus, acceso Jueves, 8 de Abril de 2021.

Avise, J. C. (1991). *Ten unorthodox perspectives on evolution prompted by comparative population genetic findings on mitochondrial DNA.* Annual Review of Genetics 25:45-69.

Avise, J. C., and R. M. Zink. 1988. *Molecular genetic divergence between avian sibling species: king and clapper rails, long-billed and short-billed dowitchers, boat-tailed and great-tailed grackles, and tufted and black-crested titmice*. Auk 105:516-528

Baquero, E, Sierra, R., L. Ordóñez, M. T¡pán, L. Espinosa, M. B. Rivera and P. Soria. (2004) *The Vegetation of the Andes of Ecuador. Explanatory memory of the vegetation maps: potential and remnant at a scale of 1: 250,000 and of the predictive modeling with indicator species.* EcoCiencia / CESLA / EcoPar Corporation / MAG SIGAGRO / CDC- Jatun SachaID Geographical Division - lGM. Quito.

Bleiweiss, R., Kirsch, J. A., & Matheus, J. C. (1997). *DNA hybridization evidence for the principal lineages of hummingbirds (Aves:Trochilidae). Molecular Biology and Evolution, 14(3), 325–343.* doi:10.1093/oxford journals. molbev.a025767

Brown, W. M. (1983). *Evolution of animal mitochondrial DNA.* Pp. 62-88 in M. Nei and R. K. Koehn, eds. Evolution of genes and proteins. Sinauer, Sunderland, Mass.

Chai, P., and R. Dudley. (1996). *Limits to flight energetics of hummingbirds hovering in hypodense and hypoxic gas mixtures*. J. Exp. Biol. 199:2285-2295.

Dingle, C., Lovette, I.J., Canaday, C., Smith, T.B., (2006). Elevational speciation and the phylogenetic relationships of the *Henicorhina* wood-wrens. Auk 123, 119–134.

Dowling, T. E., C. Moritz, and J. D. Palmer. (1990). *Nucleic acids. II. Restriction site analysis.* Pp. 250-317 in D. M. Hillis and C. Moritz, eds. 1990. Molecular systematics. Sinauer, Sunderland, Mass.

Dziedzioch, C, A.-D. Stevens, and G. Gottsberger. (2003). *The hummingbird plant community of a tropical montane rain forest in southern Ecuador*. Plant Biol. 5:331-337.

Edwards, S. V., P. Arctander, and A. C. Wilson. 1991. *Mitochondrial resolution of a deep branch in the genealogical tree for perching birds*. Proceedings of the Royal Society of London B 243:99-107

Fogden, M., Taylor, M. & Williamson, S.L. (2014) *Hummingbirds: A Life Size Guide to Every Species*. Harper Design, East Sussex, 400 pp

Freile, J. F., Poveda, C. 2019. *Chlorostilbon mellisugus* En: Freile, J. F., Poveda, C. (2019). *Birds of Ecuador*. Version 2019.0. Museo de Zoología, Pontificia Universidad Católica del Ecuador. https://bioweb.bio/faunaweb/avesweb/FichaEspecie/Chlorostilbon%20mellisugus, acceso Miércoles, 10 de Marzo de 2021.

Farhat, Y. "Reagent Preparation: Tris-HCl (1.0 M, pH 7.4." Protocol Place. May 2013. Accessed on 02/04/2020. <http://protocol-place.com>

Felsenstein J. (1985). *Confidence limits on phylogenies: An approach using the bootstrap*. Evolution 39:783-791

García-Moreno, J., Navarro-Sigüenza, A. G., Peterson, A. T., & Sánchez-González, L. A. (2004). Genetic variation coincides with geographic structure in the common bush-tanager (*Chlorospingus ophthalmicus*) complex from Mexico. *Molecular Phylogenetics and Evolution*, 33(1), 186–196. doi:10.1016/j.ympev.2004.05.007

Hackett, S. J. (1996). Molecular phylogenetics and biogeography of Neotropical tanagers in the genus Tangara. *Molecular Phylogenetics and Evolution*, *32*(3), 838–854. https://doi.org/10.1016/j.ympev.2004.02.013.

Hernández-Baños, B. E., Zamudio-Beltrán, L. E., & Milá, B. (2020). *Phylogenetic relationships and systematics of a subclade of Mesoamerican emerald hummingbirds* (Aves: Trochilidae: Trochilini). *Zootaxa*, *4748*(3), 581–591. https://doi.org/10.11646/zootaxa.4748.3.11.

Irwin, D. M., T. D. Kocher, and A. C. Wilson. 1991. *Evolution of the cytochrome b gene of mammals*. Journal of Molecular Evolution 32: 128-144.

Johnson, K. P., & Sorenson, M. D. (1998). *Comparing Molecular Evolution in Two Mitochondrial Protein Coding Genes (Cytochrome b and ND2) in the Dabbling Ducks (Tribe : Anatini)*. *10*(1), 82–94.

Kessler, L. G., and J. C. Avise. 1984. *Systematic relationships among waterfowl (Anatidae) inferred from restriction endonuclease analysis of mitochondrial DNA*. Systematic Zoology 33(4):370-380.

McGuire, J. A., Witt, C. C., Remsen, J. V, & Dudley, R. (2014). *Molecular Phylogenetics and the Diversification of Hummingbirds Report Molecular Phylogenetics and the Diversification of Hummingbirds*. (April). https://doi.org/10.1016/j.cub.2014.03.016

Moore. (1997). Inferring Phylogenies From mtDNA Variation: Mitochondrial-Gene Trees Versus Nuclear-Gene Trees Revisited. *Evolution*, *51*(2), 622. https://doi.org/10.2307/2411136

Parra, J. L., Remsen, J. V., Alvarez-Rebolledo, M., & McGuire, J. A. (2009). Molecular phylogenetics of the hummingbird genus Coeligena. *Molecular Phylogenetics and Evolution*, *53*(2), 425–434. https://doi.org/10.1016/j.ympev.2009.07.006

Pérez-emán, J. L. (2005). *Molecular phylogenetics and biogeography of the Neotropical redstarts (Myioborus ; Aves , Parulinae)*. *37*, 511–528. https://doi.org/10.1016/j.ympev.2005.04.013

Shapiro and Dumbacher. (2001). *Adenylate Kinase Intron 5 : A New Nuclear Locus for Avian Systematics*. *118*, 556–562.

Thomas, W. K., and A. T. Beckenbach. 1989. *Variation in salmonid mitochondrial DNA*: evolutionary constraints and mechanisms of substitution. Journal of Molecular Evolution 29:233-245.

Via, S. 2009. *Natural selection in action during speciation*. Proc. Natl. Acad. Sci. USA

106: 9939– 9946.

Wu, C.I. 2001. *The genic view of the process of speciation*. *J. Evol. Biol.* 14: 851– 865.

Appendix 1.

Maximum Likelihood fits of 24 different nucleotide substitution models to ND2 gene

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
TN93+G+I	60	12396,59842	11904,13673	-5891,933667	0,563810057	2,378993294	10,2633291	0,300223993	0,238644292	0,340836485	0,119707708
TN93+G	59	12403,25789	11918,99954	-5900,369496	n/a	0,265085463	10,2928256	0,300223993	0,238644292	0,340836485	0,119707708
HKY+G	58	12424,45755	11948,40269	-5916,075417	n/a	0,262071946	10,8173721	0,300223993	0,238644292	0,340836485	0,119707708
HKY+G+I	59	12424,67268	11940,41433	-5911,07689	0,523428914	1,377723419	10,9001808	0,300223993	0,238644292	0,340836485	0,119707708
GTR+G+I	63	12428,08531	11911,01448	-5892,358837	0,564179413	2,334232902	10,1403952	0,300223993	0,238644292	0,340836485	0,119707708
GTR+G	62	12432,47021	11923,60228	-5899,657377	n/a	0,249563565	10,2263364	0,300223993	0,238644292	0,340836485	0,119707708
HKY+I	58	12446,59855	11970,5437	-5927,145919	0,609019088	n/a	9,56427365	0,300223993	0,238644292	0,340836485	0,119707708
K2+G	55	12506,28103	12054,83753	-5972,305434	n/a	0,302867193	8,20828619	0,25	0,25	0,25	0,25
K2+G+I	56	12509,7131	12050,06566	-5968,915375	0,497165815	1,581346621	8,20935766	0,25	0,25	0,25	0,25
T92+G	56	12525,53022	12065,88279	-5976,823936	n/a	0,303998733	8,23235325	0,269727904	0,269727904	0,230272096	0,230272096
T92+G+I	57	12528,85217	12061,00095	-5973,378821	0,485757772	1,439913252	8,25013902	0,269727904	0,269727904	0,230272096	0,230272096
GTR+I	62	12578,8849	12070,01697	-5972,864721	0,381418093	n/a	8,69711623	0,300223993	0,238644292	0,340836485	0,119707708
K2+I	55	12633,25713	12181,81363	-6035,793483	0,381418093	n/a	7,41203616	0,25	0,25	0,25	0,25
T92+I	56	12653,31921	12193,67177	-6040,71843	0,381418093	n/a	7,42718876	0,269727904	0,269727904	0,230272096	0,230272096
K2	54	12981,66916	12538,42975	-6215,105594	n/a	n/a	6,89226949	0,25	0,25	0,25	0,25
T92	55	13001,15438	12549,71088	-6219,742108	n/a	n/a	6,90342931	0,269727904	0,269727904	0,230272096	0,230272096
TN93	58	13015,13598	12539,08112	-6211,414633	n/a	n/a	7,24255321	0,300223993	0,238644292	0,340836485	0,119707708
GTR	61	13029,29763	12528,63275	-6203,177182	n/a	n/a	7,14450302	0,300223993	0,238644292	0,340836485	0,119707708
HKY	57	13047,62447	12579,77325	-6232,764969	n/a	n/a	7,19791096	0,300223993	0,238644292	0,340836485	0,119707708
JC+G	54	13558,62825	13115,38883	-6503,585135	n/a	0,342011462	0,5	0,25	0,25	0,25	0,25
JC+G+I	55	13559,11052	13107,66702	-6498,720178	0,53965048	3,631829544	0,5	0,25	0,25	0,25	0,25
JC+I	54	13653,00632	13209,7669	-6550,774171	0,381418093	n/a	0,5	0,25	0,25	0,25	0,25
JC	53	13975,68346	13540,64828	-6717,218836	n/a	n/a	0,5	0,25	0,25	0,25	0,25
TN93+I	59	35311,26768	34827,00933	-17354,37439	0,381418093	n/a	8,79744488	0,300223993	0,238644292	0,340836485	0,119707708

Appendix 2.

Maximum Likelihood fits of 24 different nucleotide substitution models to AK1 gene

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
T92	43	2744,15163	2427,539985	-1170,6082	n/a	n/a	1,56478916	0,205358664	0,205358664	0,294641336	0,294641336
HKY+I	46	2744,664038	2405,986906	-1156,808524	0,00001	n/a	2,55606989	0,211279605	0,195774408	0,303970012	0,28531266
T92+I	44	2751,899254	2427,932102	-1169,796719	0,487589158	n/a	1,58842888	0,205358664	0,205358664	0,294641336	0,294641336
TN93+I	47	2752,412162	2406,380557	-1155,997293	0,00001	n/a	2,53829744	0,211279605	0,195774408	0,303970012	0,28531266
K2	42	2755,357086	2446,101294	-1180,896222	n/a	n/a	1,56316931	0,25	0,25	0,25	0,25
T92+G	44	2756,082037	2432,114886	-1171,888111	n/a	0,681490377	2,79547022	0,205358664	0,205358664	0,294641336	0,294641336
T92+G+I	45	2761,409454	2430,08714	-1169,866526	1,01778E-05	0,769724968	1,5874811	0,205358664	0,205358664	0,294641336	0,294641336
K2+I	43	2763,32166	2446,710015	-1180,193215	0,462349761	n/a	1,57828601	0,25	0,25	0,25	0,25
JC	41	2763,697908	2461,798312	-1189,751926	n/a	n/a	0,5	0,25	0,25	0,25	0,25
K2+G	43	2769,770815	2453,15917	-1183,417793	n/a	0,782564959	2,88754115	0,25	0,25	0,25	0,25
JC+I	42	2771,821918	2462,566126	-1189,128638	0,441880682	n/a	0,5	0,25	0,25	0,25	0,25
JC+G	42	2771,932758	2462,676965	-1189,184058	n/a	0,957930935	0,5	0,25	0,25	0,25	0,25
K2+G+I	44	2778,3004	2454,333249	-1182,997292	1,04031E-05	0,773748334	3,07011165	0,25	0,25	0,25	0,25
JC+G+I	43	2781,300432	2464,688788	-1189,182601	1,03131E-05	0,957054233	0,5	0,25	0,25	0,25	0,25
GTR	49	2801,399592	2440,660076	-1171,120421	n/a	n/a	1,56391959	0,211279605	0,195774408	0,303970012	0,28531266
GTR+I	50	2809,215137	2441,122184	-1170,342901	0,480388239	n/a	1,58447481	0,211279605	0,195774408	0,303970012	0,28531266
GTR+G	50	2809,701192	2441,608238	-1170,585928	n/a	0,805155966	1,58278818	0,211279605	0,195774408	0,303970012	0,28531266
GTR+G+I	51	2818,735342	2443,289295	-1170,417709	1,05017E-05	0,801033349	1,58327749	0,211279605	0,195774408	0,303970012	0,28531266
TN93	46	25523,33799	25184,66086	-12546,1455	n/a	n/a	2,44931608	0,211279605	0,195774408	0,303970012	0,28531266
HKY+G+I	47	25560,85665	25214,82504	-12560,21954	0,00001	0,533512022	2,292552	0,211279605	0,195774408	0,303970012	0,28531266
TN93+G+I	48	48328,73381	47975,34807	-23939,47282	0,00001	0,885289628	2,36969221	0,211279605	0,195774408	0,303970012	0,28531266
HKY	45	71074,60161	70743,2793	-35326,46261	n/a	n/a	2,26457994	0,211279605	0,195774408	0,303970012	0,28531266
TN93+G	47	93890,68944	93544,65783	-46725,13593	n/a	0,464546099	1,90300564	0,211279605	0,195774408	0,303970012	0,28531266
HKY+G	46	162194,5199	161855,8428	-80881,73648	n/a	0,534285326	1,97524401	0,211279605	0,195774408	0,303970012	0,28531266

Appendix 3.

Maximum Likelihood fits of 24 different nucleotide substitution models to ND2-AK1 gene.

Model	#Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G
HKY+G	40	11808,11897	11480,89542	-5700,38565	n/a	0,25968029	6,03013272	0,270401995	0,224308599	0,330247846	0,173492519
TN93+G	41	11808,92933	11473,5283	-5695,698985	n/a	0,259103025	5,97691573	0,270401995	0,224308599	0,330247846	0,173492519
HKY+G+I	41	11815,71847	11480,31744	-5699,093555	0,299637991	0,452753469	6,04814997	0,270401995	0,224308599	0,330247846	0,173492519
TN93+G+I	42	11817,52931	11473,95094	-5694,907127	0,232537547	0,389115782	5,97552889	0,270401995	0,224308599	0,330247846	0,173492519
GTR+G	44	11834,13002	11474,19745	-5693,023791	n/a	0,221068851	5,73441694	0,270401995	0,224308599	0,330247846	0,173492519
HKY+I	40	11836,13161	11508,90806	-5714,391972	0,665597022	n/a	5,67849085	0,270401995	0,224308599	0,330247846	0,173492519
TN93+I	41	11837,82759	11502,42656	-5710,148117	0,665202829	n/a	5,62881779	0,270401995	0,224308599	0,330247846	0,173492519
GTR+G+I	45	11842,22483	11474,11539	-5691,97935	0,266370082	0,377822712	5,80036176	0,270401995	0,224308599	0,330247846	0,173492519
GTR+I	44	11863,2338	11503,30123	-5707,57568	0,664973116	n/a	5,52592489	0,270401995	0,224308599	0,330247846	0,173492519
K2+G	37	11872,24984	11569,55964	-5747,726622	n/a	0,244298837	5,25682371	0,25	0,25	0,25	0,25
K2+G+I	38	11880,2357	11569,36757	-5746,627708	0,268796114	0,424425752	5,30498591	0,25	0,25	0,25	0,25
T92+G	38	11882,01372	11571,14559	-5747,516718	n/a	0,244338645	5,2559208	0,248129817	0,248129817	0,251870183	0,251870183
T92+G+I	39	11890,04265	11570,99672	-5746,439334	0,267477945	0,423272949	5,30347598	0,248129817	0,248129817	0,251870183	0,251870183
K2+I	37	11901,06234	11598,37214	-5762,132875	0,655771969	n/a	5,11223255	0,25	0,25	0,25	0,25
T92+I	38	11910,61867	11599,75054	-5761,819193	0,655217922	n/a	5,11187108	0,248129817	0,248129817	0,251870183	0,251870183
TN93	40	12261,2214	11933,99784	-5926,936864	n/a	n/a	4,78258194	0,270401995	0,224308599	0,330247846	0,173492519
HKY	39	12263,77755	11944,73163	-5933,306785	n/a	n/a	4,78459627	0,270401995	0,224308599	0,330247846	0,173492519
K2	36	12269,63245	11975,12033	-5951,509771	n/a	n/a	4,51345453	0,25	0,25	0,25	0,25
T92	37	12279,20469	11976,51449	-5951,20405	n/a	n/a	4,51347588	0,248129817	0,248129817	0,251870183	0,251870183
GTR	43	12281,96757	11930,21203	-5922,034412	n/a	n/a	4,55893829	0,270401995	0,224308599	0,330247846	0,173492519
JC+G	36	12592,68965	12298,17754	-6113,038373	n/a	0,271633017	0,5	0,25	0,25	0,25	0,25
JC+G+I	37	12602,20231	12299,51211	-6112,70286	0,18158009	0,391812566	0,5	0,25	0,25	0,25	0,25
JC+I	36	12613,27825	12318,76614	-6123,332673	0,64351597	n/a	0,5	0,25	0,25	0,25	0,25
JC	35	12946,55755	12660,22368	-6295,064169	n/a	n/a	0,5	0,25	0,25	0,25	0,25

Appendix 4. Nanodrop measurements of Nucleic Acid of Hummingbirds

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
1	C?4b	Admin	19/12/2020 13:31	29,6	ng/µl	0,592	0,363	1,63	1,16	DNA
2	Coco.2	Admin	19/12/2020 13:25	65,4	ng/µl	1,308	0,748	1,75	1,49	DNA
3	C?4	Admin	19/12/2020 13:27	43,4	ng/µl	0,868	0,541	1,60	1,22	DNA
4	Coco.2b	Admin	19/12/2020 13:33	40,6	ng/µl	0,813	0,485	1,67	1,21	DNA
5	Coco.2b	Admin	19/12/2020 13:32	2,2	ng/µl	0,044	0,046	0,96	-0,17	DNA

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
6	Coco.1	Admin	19/12/2020 16:45	57,0	ng/µl	1,139	0,602	1,89	1,13	DNA
7	C?3	Admin	19/12/2020 16:46	69,2	ng/µl	1,384	0,751	1,84	1,26	DNA
8	Coco.1 B	Admin	19/12/2020 17:04	34,7	ng/µl	0,693	0,348	1,99	1,26	DNA
9	C?3 B	Admin	19/12/2020 17:06	32,7	ng/µl	0,654	0,328	1,99	1,16	DNA
10	C?2	Admin	20/12/2020 11:33	19,2	ng/µl	0,384	0,201	1,91	0,81	DNA

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
10	C?2	Admin	20/12/2020 11:33	19,2	ng/µl	0,384	0,201	1,91	0,81	DNA
11		Admin	20/12/2020 11:32	18,8	ng/µl	0,376	0,196	1,92	0,77	DNA
12	C?1B	Admin	20/12/2020 11:37	33,8	ng/µl	0,676	0,363	1,86	1,25	DNA
13	C?1	Admin	20/12/2020 11:34	30,8	ng/µl	0,617	0,328	1,88	1,00	DNA
14	C?2B	Admin	20/12/2020 11:38	21,2	ng/µl	0,425	0,229	1,86	0,96	DNA

#	Sample ID	User name	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type
13	C?1	Admin	20/12/2020 11:34	30,8	ng/µl	0,617	0,328	1,88	1,00	DNA
14	C?2B	Admin	20/12/2020 11:38	21,2	ng/µl	0,425	0,229	1,86	0,96	DNA
15	C?1c	Admin	20/12/2020 12:09	30,5	ng/µl	0,611	0,311	1,97	1,16	DNA
16	C?1c	Admin	20/12/2020 12:10	19,9	ng/µl	0,397	0,191	2,08	0,87	DNA
17	C?2c	Admin	20/12/2020 12:11	20,7	ng/µl	0,414	0,211	1,97	0,92	DNA