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Molecular phylogeny of *Thraupis* Boie, 1826 (Aves: Passeriformes) and taxonomic review of the *Thraupis episcopus* (Linnaeus, 1766) – *Thraupis sayaca* (Linnaeus, 1766) species complex

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Luís Fábio Silveira

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Con todo mi amor para mi madre María Helena, mi padre Héctor y mis hermanas Luchy y Camila. Quienes, con sus sacrificios y esfuerzos siempre me han apoyado y a quienes debo todo. Que el resultado de mi maestría sea una forma de agradecerles todo.

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“The goal of life is knowledge, everything else is secondary. Without knowledge, there can be no understanding of good and evil, no appreciation of the sacred obligations that human beings have to the earth and the Great Mother. With knowledge comes wisdom and tolerance.”

Wade Davis

One River: Explorations and discoveries in the Amazon Rain Forest. p.45

RESUMO

O gênero *Thraupis* Boie, 1826 é um grupo monofilético composto por sete espécies. Entretanto, as relações entre estas espécies continuam obscuras. *Thraupis abbas* é a espécie irmã do clado *T. ornata–T. palmarum*. Um segundo grupo é composto pelo clado *T. episcopus–T. sayaca*. Por outro lado, *T. glaucocolpa* não foi incluída em nenhum dos trabalhos que utilizou dados moleculares, enquanto que a posição de *T. cyanoptera* continua ainda não é clara. O complexo de espécies *T. episcopus–T. sayaca–T. glaucocolpa* inclui 18 subespécies e uma grande variação morfológica, além de uma ampla distribuição de várias delas, com áreas de sintopia entre *T. episcopus* e *T. sayaca*, onde a identificação destas duas espécies é muito difícil. Estudos moleculares prévios só incluíram amostras de dois indivíduos de *T. episcopus* e uma de *T. sayaca*. Este complexo de espécies ainda apresenta uma grande instabilidade taxonômica. Na revisão deste gênero foram analisados 1.171 espécimes. As análises morfométricas mostraram que a massa é o parâmetro que apresenta a maior variação e que *T. cyanoptera* é a única mais claramente diferençável dentre as unidades taxonômicas. Foi também realizada uma análise filogenética com base em dois marcadores mitocondriais (Cyt-β e ND2), além de três íntrons nucleares (íntron 3 do gen MUSK, íntron 5 do gen TGFB2 e uma parte do íntron 5 do gen BF5). Foi realizada uma análise RAxML e das redes de haplótipos independentemente para cada lócus, e esta informação foi utilizada para agrupar as amostras em unidades taxonômicas genéticas. O RAxML e as redes de haplótipos mostraram uma relação próxima entre *T. episcopus* e *T. sayaca*, além de uma alta probabilidade de um processo de introgressão entre as espécies. Há também uma evidente estruturação genética em *T. episcopus*. As unidades taxonômicas genéticas foram utilizadas em uma análise multilocus de árvore de espécies, com um relógio molecular calibrado. A árvore de espécies sugere que a origem do gênero *Thraupis* se deu entre 5.5 e 7.7 milhões de anos. *Thraupis glaucocolpa* é a linhagem mais antiga do gênero e a estrutura genética dentro de *T. episcopus* possui uma relação com as características morfológicas dessa espécie. Finalmente, são sinonimizadas várias subespécies e *T. episcopus cana* é elevada à espécie com base nos dados morfológicos e moleculares.

ABSTRACT

Currently, the genus *Thraupis* Boie, 1826 is a monophyletic group with seven species, all of which have high molecular and morphological support. Nevertheless, the phylogenetic relationship of the species still unclear: *T. abbas* is the sister species of *T. ornata–T. palmarum* clade, and a second group within the genus is composed by the *T. episcopus–T. sayaca* clade. Furthermore, in the remaining species group, one of the species, *T. glaucocolpa,* has not been included in any of the previous molecular studies, even it was believed to be close related with *T. sayaca*. Moreover, the last species within the genus, *T. cyanoptera,* has an uncertain position in the genus phylogenetic tree. The *T. episcopus–T. sayaca–T. galucocolpa* species complex includes 18 subspecies and a high morphological variation and a wide distribution which includes overlapping zones of *T. episcopus* and *T. sayaca,* makes taxa identification almost impossible. Nonetheless, previous molecular studies had only used samples from two individuals of *T. episcopus* and one of *T. sayaca*. Furthermore, the group does not have taxonomic stability, as shown by the multiple changes, which occur at different levels: moving from one genus to another or from species to subspecies level etc. To check the genus, I analyze 1171 specimens. The morphometric analysis outcomes show the weight as the most variable and important measure and *T. cyanoptera* as the only clearly different species within taxonomic units. Finally, I did a phylogenetic analysis based on two mitochondrial genes (Cyt-β and ND2), in addition to three nuclear introns (intron 3 of MUSK gen, intron 5 of TGFB2 gen and a piece of the intron 5 of the BF5 gen). I performed the extractions from tissues collected at different localities around the natural distribution of the species, with emphasis on *T. episcopus* and *T. sayaca.* I ran independent locus RAxML analysis and haplotypes networks and used to group the samples on genetic taxonomic units. RAxML and haplotypes analysis shows a close relationship between *T. episcopus* and *T. sayaca* with high probably introgression process within. Furthermore, exposed a genetic structure within *T. episcopus*. I used this genetic taxonomic units to ran a multilocus species tree with a calibrated molecular clock. The species tree suggests that the origin of the genus *Thraupis* was between 5.5 and 7.5 million years before present, in the Messinian age. Also recovers *T. glaucocolpa* is the oldest linage in the genus and shows a relation between the morphological traits with the genetic structure within *T. episcopus*. Finally, I suggest synonymizing several subspecies and elevating to species level the subspecies *T. episcopus cana,* based on the morphological and molecular data.

FIGURE LIST

TABLES LIST

SUMMARY

1. INTRODUCTION

1.1 Taxonomic diversity within Thraupidae

The Thraupidae is a family of nine-primary oscine Passeriformes. Currently 377 species (3.6% of all birds) are included in the family, making it the second largest family of birds (Clements *et al.*, 2017). It is a family restricted to the Americas (Hilty, 2011), and its origins, diversification and highest richness are concentrated in the Neotropics (Sedano and Burns, 2010; Hilty, 2011). Birds belonging to this family are small-medium sized, usually with colorful and contrasting plumages (Hilty, 2011).

This family represents one of the most diverse radiations of birds, not only in the number of taxa but also in plumage types, colors, ecological traits, morphology, and behavior (Hilty, 2011; Burns, Unitt and Mason, 2016). Genera as *Diglossa*, *Coereba* and *Cyanerpes* are good examples of it. Representatives of these genera have specialized bill morphology adapted for foraging from nectar flowers, although they are not closest relatives (Burns, Unitt and Mason, 2016). *Coereba* and *Cyanerpes* function as pollinizers, showing similar ecological strategies. On the other hand, *Diglossa* exhibits a parasitic behavior by piercing the corolla and sucking the nectar without pollinating the flower (Hilty and Brown, 1986; Rojas-Nossa, 2007). Besides this nectar-eaters, the Thraupidae also includes seed-eater birds such as the ones in the genus *Sporophila*, insectivores as those species in *Conirostrum*, and fruit-eaters as the genera *Thraupis* and *Tangara* (Hilty, 2011; Burns, Unitt and Mason, 2016). Each group has particular adaptations in bill morphology for their specific diets. Due to this enormous variation, relationships within the family have remained unclear and debated for a long time. Some of its current members were previously placed in other families, such as Coerebidae and Emberizidae. This taxonomic assumptions were based on morphology and feeding behavior of the birds (Hilty, 2011). Only recently (1990 to present) the advent of molecular data allowed attaining a better understanding of the relationships within the family (Hilty, 2011; Burns, Unitt and Mason, 2016).

1.2 A brief summary on taxonomic history of the genus *Thraupis*

The genus *Tanagra* was first described by Linnaeus in 1766 (Linné, 1766) with *Tanagra episcopus* as the type species. Thus, the genus *Tanagra* became the type genus of the family Tanagridae. Nevertheless, in 1908 it was pointed out that Linnaeus used the same name for a different group of birds (currently *Euphonia*, Fringillidae) in 1764 (CON, 1963; Hilty, 2011). Since then, a group of ornithologists (mainly from North America) started using *Thraupis* Boie (1826) instead of *Tanagra*, but old-world ornithologists kept using *Tanagra*. At the end of the 1950's, both names became widely used for the same taxon. In 1968, the International Committee of Zoological Nomenclature (ICNZ) decided to suppress the name *Tanagra* based on two proposals from Dr. Dean Amadon and Dr. Ernst Mayr. As the name of the type genus was a not valid name, the family name Tanagridae was not either. The next available name for the genus was *Thraupis* Boie (1826) and based on the "principle of coordination" (Ride, 1999) the family became Thraupidae as required by this principle (ICZN, 1968; Hilty, 2011).

As defined by recent studies, *Thraupis* is a monophyletic genus comprised by seven species: *Thraupis episcopus* (Linnaeus, 1766), *T. sayaca* (Linnaeus, 1766), *T. ornata* (Sparrman, 1789), *T. cyanoptera* (Vieillot, 1817), *T. palmarum* (Wied-Neuwied, 1821), *T. abbas* (Deppe, 1830), and *T. glaucocolpa* Cabanis 1850 (Sedano and Burns, 2010; Hilty, 2011; Burns *et al.*, 2014; Burns, Unitt and Mason, 2016; Remsen *et al.*, 2017). Previous phylogenetic hypotheses of the Thraupidae partially resolved the relationships within *Thraupis* by using DNA markers (Sedano and Burns, 2010; Burns *et al.*, 2014). However, there are taxonomic and phylogenetic uncertainties that have not been solved yet. For instance, the phylogenetic position of *T. cyanoptera* remains uncertain due to low maximum-likelihood and posterior probability support values (Burns *et al.*, 2014); Fig. 1). Additionally, the phylogenetic position of *T. glacucocolpa* remains unknown because no samples have been assessed in a molecular framework (Burns *et al.*, 2014; Burns, Unitt and Mason, 2016) and its placement in *Thraupi*s is based solely on morphology. Thus, T. *glaucocolpa* is assumed to be the sister taxon of *Thraupis sayaca* due to similarities in plumage coloration (Hellmayr, 1936; Burns *et al.*, 2014). Moreover, *T. glaucocolpa*'s taxonomic category is debated. After its description as a species, *T. glaucocolpa* was treated as a subspecies of *Thraupis sayaca*, mainly because of their similarity on plumage coloration (Hellmayr, 1936; Remsen *et al.*, 2017). More recently, it was suggested to be part of a superspecies with *T. episcopus* and *T.*

sayaca (Hilty, 2011). Currently *T. glaucocolpa* is considered as a full species by the South American Classification Committee (SACC) based on the continued use in several classifications, but not as the result of a taxonomic revision (Hilty, 2011; Remsen *et al.*, 2017).

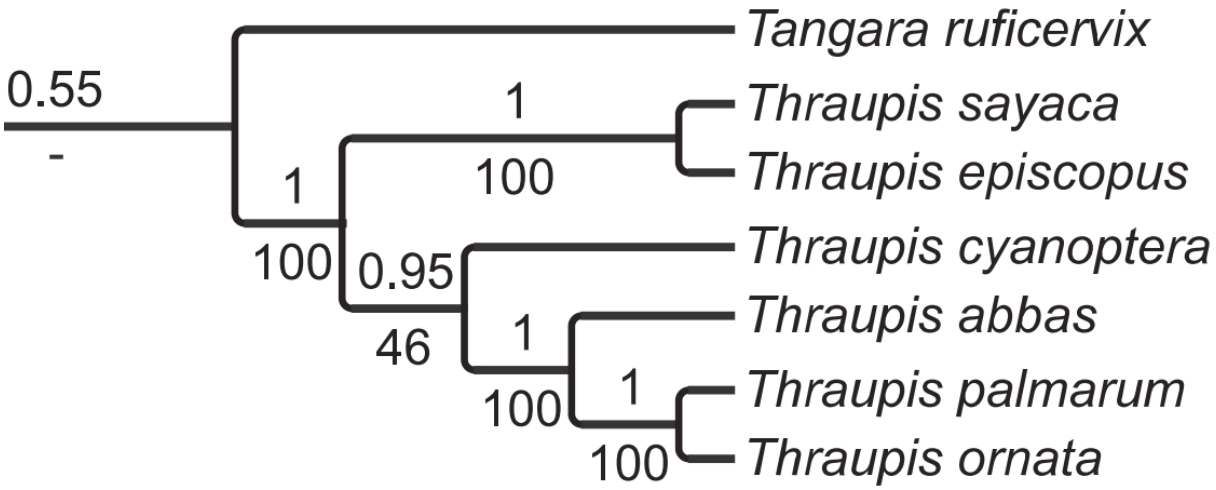

Figure 1. Phylogenetic tree of *Thraupis* based on two mitochondrial markers ND2 and Cyt-β. Posterior probability from Bayesian framework analysis above the branch, maximum likelihood value below the branch. Modified from Burns *et al.* (2014).

Another taxonomic problem pertains the widespread species *T. episcopus.* It exhibits high morphological variation (described as 14 subspecies) that in some cases is structured geographically (Hilty, 2011). Within *T. episcopus*, there are individuals with white lesser-coverts and others with blue lesser-coverts. The first group is distributed east of the Andes and the second group in the west Andes and inter-andean valleys within the northern (Hilty, 2011). Other individuals that show light purple lesser-coverts are located in the Llanos of eastern Colombia and western Venezuela (Fig. 2). This area is where the two aforementioned morphs get into contact. Also, the subspecies *T. episcopus ehrenreichi* seems to be embedded within the distribution of *T. episcopus mediana* (Hilty, 2011). Moreover, the species limits between *T. episcopus* and *T. sayaca* are not clear. Some individuals ascribed to *T. episcopus* show similar plumage to *T. sayaca* and in some regions of southeastern Peru and northwestern Bolivia it is difficult to differentiate them even when hand-held (Schulenberg *et al.*, 2007). Finally, the subspecies *T. sayaca boliviana* (from the borders among Peru, Brazil and Bolivia) shows intermediate traits and some authors suggest those individuals are of intergrades between *T. episcopus* and *T. sayaca* (Zimmer, 1944; Hilty, 2011; Remsen *et al.*, 2017) and others already categorize them as a hybrids (McCarthy, 2006).

Figure 2. Color variation of lesser-coverts within *Thraupis episcopus*: a) *Thraupis episcopus cana* (ICN-AO3361), Colombia, west of the Andes; b) *Thraupis episcopus* cf. *nesophila* (ICN-38869), Llanos of eastern Colombia; c) *Thraupis episcopus leucoptera* (ICN-38786), Colombian Amazon, east of the Andes.

The use of DNA sequence data has generated a reappraisal of traditional morphology-based taxonomy and has greatly improved our understanding of phylogenetic relationships at different taxonomic levels (Reddy, 2011; Isler, Bravo and Brumfield, 2013). Also, the use of DNA has been an important instrument to promoting the development of phylogeography studies (Avise, 2000), which, in turn, have proven useful to define species limits (e.g., Cadena *et al.* 2007, Isler *et al.* 2012) and to identify cryptic lineages i.e., established evolutionary linages with little (or none) external morphological differences (e.g., Carneiro *et al.* 2012). Furthermore, phylogeographic studies bring us closer to a better understanding of mechanisms and patterns behind speciation and diversification (e.g., Moritz *et al.* 2000, Aleixo 2004, Ribas & Miyaki 2004, Cadena & Cuervo 2010). Consequently, using the outcomes of phylogeographic analyses in concert with phenotypic, behavioral, and ecological data is advised toward robust and stable taxonomic classifications (e.g., Isler *et al.* 2012, Cadena *et al.* 2007, Cadena & Cuervo 2010, Carneiro *et al.* 2012). Ultimately, adequate species delimitation procedures will have important implications on species conservation (Dénes *et al.*, 2011).

Here, I integrate information from mitochondrial and nuclear DNA markers with a morphological analysis to define the species limits within the *T. episcopus–T. sayaca–T. glaucocolpa* species complex and to suggest a new phylogenetic hypothesis for the genus *Thraupis* based on DNA sequence data.

2. METHODS

2.1 Morphological data

I analyzed a total of 1.170 specimens, classified as *T. episcopus, T. sayaca, T. glaucocolpa,* and *T. cyanoptera* housed at different museums in Colombia, Brazil, and the United States (Table 1). The revised specimens cover most of the distribution of the respective species, with a few gaps as in Bolivia and northern Argentina (Fig. 3), and included the holotype of *Thraupis episcopus quaesita*. Also, I received high-quality photographs of 13 other type specimens sent by curators from other institutions (Table 1). A total of five types specimens were not analyzed directly. The description of *T. episcopus* and *T. sayaca* were based on descriptions/paintings from antique authors (Hellmayr, 1936) but I had access to these plates by using the Biodiversity Heritage Biodiversity. The type of *Thraupis episcopus nesophilus* (described as *Tanagra sclateri*, Berlepsch, 1880) was destroyed during World War II (Mayr G, pers. comm.) and, despite our efforts, curators of the Varsovia Museum and the Swainson Collection were not able to send the photos of the type specimens of *T. episcopus major* and *T. episcopus cana*.

For phylogenetic analyses, I used a total of 118 tissue samples from vouchered specimens in the Thraupidae housed at different collections (Table 1). These samples include the outgroup species (*Paroaria baeri, Tangara chilensis, Tangara mexicana, Tangara punctata* and *Tangara cayana*) and all species within the genus *Thraupis* (*T. glaucocolpa, T. episcopus, T. sayaca, T. cyanoptera, T. palmarum, T. ornata* and *T. abbas*), with emphasis on the species complex mentioned above (Fig. 4). The outgroup was selected base on previous molecular phylogenies (Burns *et al.*, 2014). I obtained locality information from specimen tags and georeferenced them using the free software Google Earth Pro 7.3, complemented by Gazetteers (Paynter and Traylor, 1991; Paynter, 1992, 1997; Vanzolini, 1992).

Table 1. Museums that allowed access to study skins, tissues samples, and type specimens. Type of sample: S: Specimen, P: Picture, TP: Type picture, T: Tissue for DNA extraction.

Acronym	Museum	Country	Sample
ANSP	Academy of Natural Sciences of Drexel University – Philadelphia	USA	T/TP
AMNH	American Museum of Natural History	USA	S/P/T/TP
COP	Colección Ornitológica Phelps	Venezuela	T
FMNH	Field Museum of Natural History	USA	TP
MCZ	Harvard University Museum of Comparative Zoology	USA	S/P/TP
IAvH	Instituto Alexander von Humboldt	Colombia	T
ICN	Instituto de Ciencias Naturales de la Universidad Nacional de Colombia	Colombia	S/P/T
KU	Kansas University Natural History Museum	USA	S/P/T
LSU	Louisiana State University Museum of Natural Sciences	USA	S/P/T
MZUSP	Museu de Zoologia da Universidade de São Paulo	Brazil	S/P/T
MPEG	Museu Paraense Emílio Goeldi	Brazil	T
MFN	Museum für Naturkunde	Germany	TP
MH	Museum Heineanum	Germany	TP
MSB	Museum of Southwestern Biology – The University of New Mexico	USA	S/P/T
BMNH	Natural History Museum	England	TP
NMNH	Smithsonian Institution National Museum of Natural History	USA	TP
UWBM	University of Washington Burke Museum	USA	T

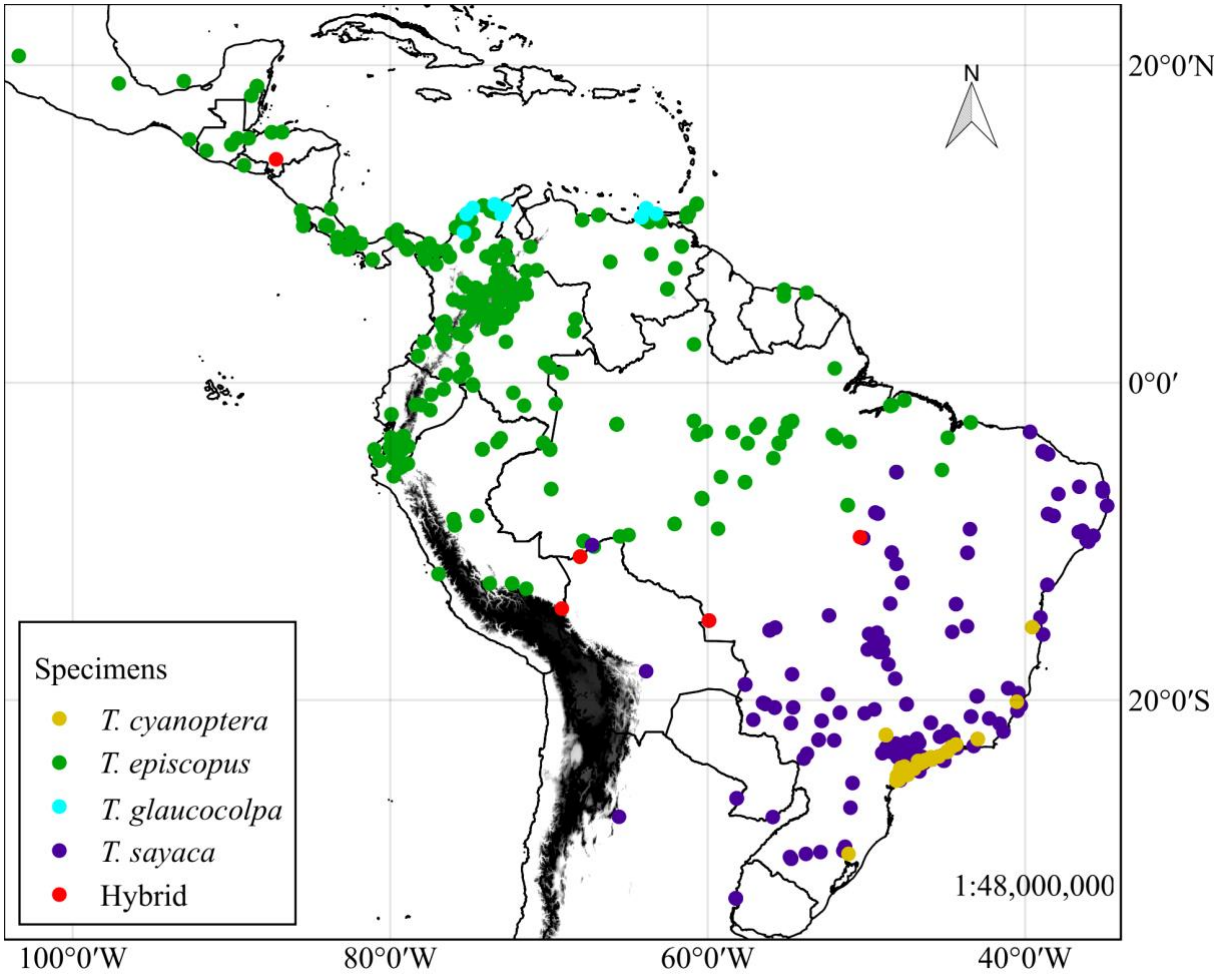

Figure 3. Localities of the 1.170 revised specimens. Specimens with intermediate traits and no clear identification were marked as hybrid (red). The hybrids in South America are *T. episcopus* x *T. sayaca* and the hybrid in Central America is *T. episcopus* x *T. abbas*.

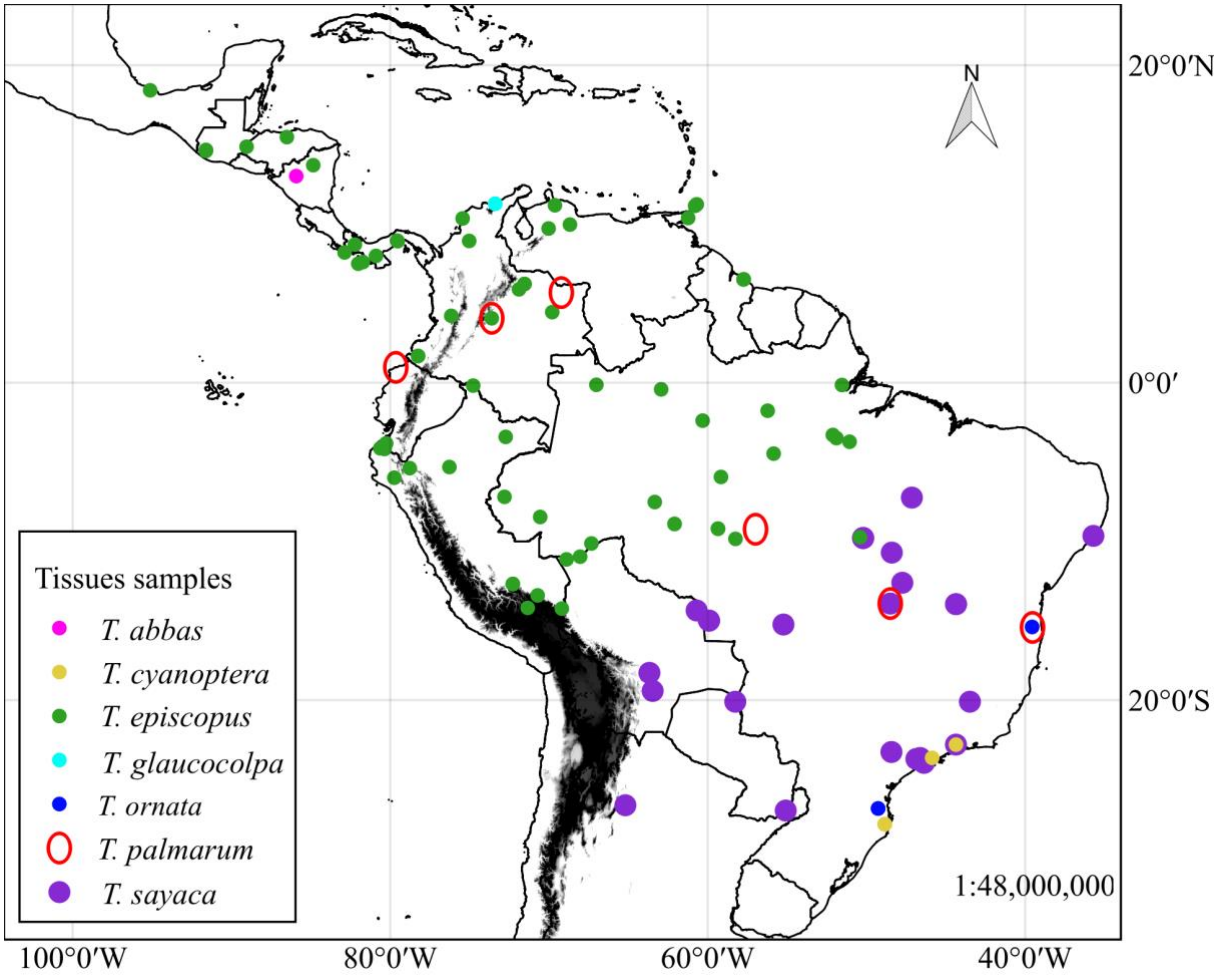

Figure 4. Localities of the 118 tissue samples used in this study. Identification made by curators at each collection. Nevertheless, several specimens were posteriorly analyzed, none turn to be a different species but some seems to have intermediate morphology traits.

2.2 Color analyses and definition of morphologic units

I took standardized pictures from 493 specimens selected based on distribution, preservation, and coloration with the objective to maximize the amount of geographic and coloration space included in the analyses. I photographed all specimens under standard conditions, controlling as many variables as possible (Fig. 5). I photographed all specimens in three different positions (back, ventral, and side views), with emphasis on five body regions: crown, back, chest, lesser coverts, and distal border of the primary feathers. The colors of those areas were standardized using Smithe (1975). I used the standardized colors to group plumages with

color homogeneity at these five areas. For each type of plumage, I pick up a picture and equalized the white balance of it. For this, I used the free trial of the software Photoshop® and the standard-grey in each picture. Later, I catalogued the color on the same five areas for each plumage, using the HTML notation. Also, I rank the amount of white on the external greater coverts from 0 to 5, based on figure 6.

Figure 5. Picture assembling for standardized pictures. I took pictures with a camera Nikon D800, lens AF-S NIKKOR 28-300mm f/3.5-5.6G ED VR, a tripod, a black background and a grey standard. Pictures were standardized by shooting them with the following settings: Shutter speed 1/250, F 10.0, ISO 200, built-in flash -2.0 and white balance for flash +3.0 B6.

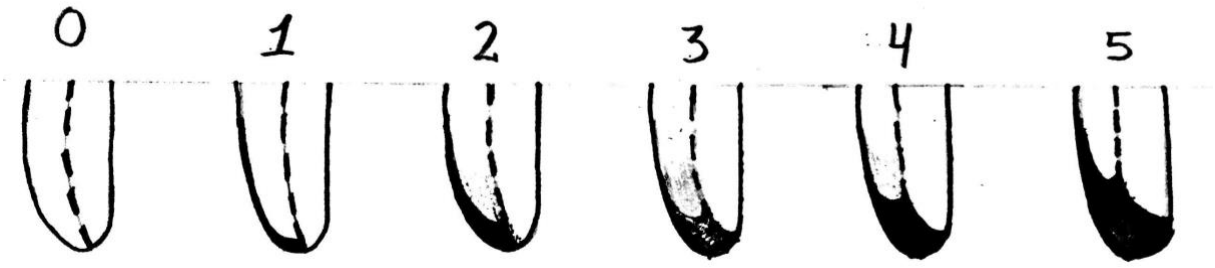

Figure 6. Scale of amount of white in the greater wing-coverts in *T. episcopus* at the Andes east slope. Shaft (dashed line). Amount of white on greater wing-covertes (black).

I used the coordinates to plot each specimen on a map, using the free software Qgis v.2.18.7 and the HTML notation code to color the species symbols with the color of each part of the body. The results are summarized in five maps, one for each body region, with symbols colored as similarly as the true coloration of the specimens. Then, I grouped in polygons those specimens with similar coloration within a defined geographical area. The map for amount of white on the greater coverts only included specimens with values higher than zero (*T. episcopus*), and they were plotted with circles with different sizes related to the amount of white on the greater coverts. Based on the resulting maps I compared the polygons across them and stablish morphological units (MU). In order to find congruence between color homogeneity and geographic distribution. MU were later used in morphometric analyses.

2.3 Morphometric analyses

I measured a total of 1.104 individuals using a digital caliper to the nearest 0.01 mm. Specimens with characteristics of young birds such as lower density body feathers and greenish coloration (similar across all species in the complex) were not included. Taken measurements were: total culmen (CT), culmen from the distal border of the nares (CN), tarsus (TS), wing chord (Wing) and tail length (Tail) (Baldwin, Oberholser and Worley, 1931; Ralph *et al.*, 1996). Additional information such as body weight was recorded when available on the specimen label.

All morphometric analyses were run in RStudio v.1.0.143. (RStudio, 2016). I assessed normality for each trait and each MU using a Kruskal-Wallis test and a quantile-quantile plot (QQ plot).

Before subsequent analyses data were natural log-corrected. I ran a MANOVA test with an α=0.05 to find significance difference within the MU. To avoid having missing-data in CT, CN, TS, Wing and Tail, I used the arithmetic mean of each measurement within each MU. Because weight was the measurement with more missing data, I generated a linear regression model for each MU of the weight as a function of all other morphometric traits. Then, I used the corresponding linear model to predict the weight of each individual. In two special cases, where all individuals from a single MU were lacking weight information, I used the linear model from the closest taxonomic/geographic MU (e.g. For *T. sayaca obscura* I used the linear model of *T. sayaca sayaca* to predict the weight of the individuals of *T. sayaca obscura*)(Little and Rubin, 2002). I used the packages "devtools" (Wickham and Chang, 2017), "githubinstall" (Makiyama, 2016) and "ggbiplot" (Vu, 2011) to perform a principal component analysis (PCA) in R. First, I ran the PCA of the complete data set with all MU. Posteriorly I ran individual PCAs including only sympatric MU. Finally, I ran a linear discriminant analysis (LDA) to assess diagnosibility of MU via numerical functions summarizing all the observed variation in all measured traits.

2.4 DNA extraction, PCR amplification, and sequence editing and alignment.

I performed DNA extractions from ~20 mg of pectoral muscle using the the Qiagen DNeasy kit, following the manufacture's protocol (Qiagen, Valencia, CA, USA). I amplified two mitochondrial genes, cytochrome β (Cyt-β, 1143 bp) and nicotinamide dehydrogenase subunit 2 (ND2, 1041 bp). Furthermore, I amplified three non-coding nuclear introns: the transforming growth factor-beta 2 (TGFB2, 534 bp), ß-fibrinogen intron 5 (bF5, 486 bp), and the Z-linked muscle-specific tyrosine kinase receptor (MUSK, 519 bp; see Table 2 for information about primers). Amplification protocols used for each marker are summarized in Table 3.

Table 2. Used primers in the PCR process. Each group of primers was used separately on each sample to get five sequences of five molecular markers for sample.

Marker	Primer	Reference
Cyt-β	L14990	(Helm-Bychowski & Cracraft 1993)
Cyt-β	H16055	(Helm-Bychowski & Cracraft 1993)
Cyt-β	L15496	(Helm-Bychowski & Cracraft 1993)
Cyt-β	H15496	(Helm-Bychowski & Cracraft 1993)
ND2	L5215	(Hackett 1996)
ND2	H6313	(Johnson & Sorenson 1998)
ND2	L5758	(Brumfield *et al.* 2007)
ND2	H5766	(Brumfield *et al.* 2007)
MUSK	MUSK-13F	(Kimball *et al.* 2009)
MUSK	MUSK-13R	(Kimball *et al.* 2009)
TGFB2	TGFB2.5F	(Primmer *et al.* 2002)
TGFB2	TGFB2.6R	(Primmer *et al.* 2002)
Bf5	FIB5L	(Brumfield *et al.* 2007)
Bf5	FIB5H	(Brumfield *et al.* 2007)

For PCR quality control, I ran amplification products on a 1.2% agarose gel together with a molecular-weight size ladder. Amplified samples were sent to the Louisiana State University Genomics Facility for clean-up, cycle-sequencing, and sequencing.

Chromatograms were imported into the software Geneious v.9.1.8 (Kearse *et al.*, 2012) for edition and alignment. First, I assembled the parts of a single specimen and marker, individual by individual, using the tool "map to reference". Reference sequences were downloaded from GenBank website (Benson *et al.*, 2012).

I manually removed low-quality fragments. For nuclear sequences, I used the plug in "Find heterozygotes" with a peak similarity value of 60% and the automatically option to change bases per ambiguities on the molecular markers. Subsequently, sequences of the same molecular marker were aligned with the option "Pairwise/Multiple Alignment" using the algorithm MUSCLE. Alignments of Cyt-β and ND2 were concatenated since they belong to the mitochondrial locus. I phased nuclear sequences with heterozygotes sites, using the open-access software PHASE v.2.1.1 (Stephens, Smith and Donnelly, 2001) to select the two more probably sequences for each nuclear marker. For the input data for PHASE I used the web tool SeqPHASE (Flot, 2010).

Table3. PCR protocol for the used molecular markers. Values under the name of each marker are amount of DNA / total volume of the reaction. Touch down per cycle (↓).

Marker	Thermocycler program	Master mix
Cyt-β 1 µl / 24 µl	94ºC per 2’ x1 94ºC per 30”, 50ºC per 30”, 72ºC 60” x34 72ºC per 7’, 10ºC per ∞ x1	buffer 1x, primer 0.4µM each, MgCl2 1.6mM, dNTPs 0.2µM, BSA 0.2x, Taq 0.02U
ND2 1 µl / 25 µl	94ºC per 2’ x1 94ºC per 30”, 50ºC per 30”, 72ºC 60” x34 72ºC 7’, 10ºC ∞ x1	buffer 1.1x, primer 0.4µM each, MgCl2 1.5mM, dNTPs 0.2µM, BSA 0.2x, Taq 0.02U
TGFB2 2 µl / 20 µl	94ºC per 5’ x1 95ºC per 25”, 65ºC↓-0.5ºC per 30”, 72ºC per 90’’ x30 95ºC per 25’’, 48ºC per 30’’, 72ºC per 90’’ x15 72ºC per 10’’, 10ºC per ∞ x1	buffer 1x, primer 0.5µM each, MgCl2 2mM, dNTPs 0.4µM, Taq 0.1U
MUSK 2.5 µl / 25 µl	94ºC per 2’ x1 94ºC per 30”, 50ºC per 30”, 72ºC 50” x35 72ºC 10’, 10ºC ∞ x1	buffer 1.1x, primer 0.4µM each, MgCl2 1.5mM, dNTPs 0.2µM, BSA 0.2x, Taq 0.1U
BF5 3 µl / 25 µl	95ºC per 5’ x1 95ºC per 60”, 58ºC per 60”, 72ºC 60” x35 72ºC 5’, 10ºC ∞ x1	buffer 1x, primer 0.4µM each, MgCl2 1.5mM, dNTPs 0.2µM, Taq 0.1U

2.5 Gene trees, haplotype networks and species tree reconstruction

To build gene trees, I ran independent RAxML v.8 (Stamatakis, 2014) analysis for each locus: mitochondrial locus (concatenated ND2 and Cyt-β), TGFB2, MUSK and BF5. For the mitochondrial locus I used the program Partition Finder2 v.2.1.1 (Lanfear *et al.*, 2016) to choose the best partition scheme. Besides this parameter, I applied the nucleotide substitution model GTR + Γ for all loci. To assess nodal support, I conducted a rapid-bootstrap analysis using 999 replicates. For the RAxML analyses I used the Cipres Science Gateway site v.3.2 (Miller, Pfeiffer and Schwartz, 2010).

Also, for each locus I constructed a haplotype network using the option “Median Joining Network” in the software PopART (Leigh and Bryant, 2015). Based on the RAxML

trees and the haplotype networks, I grouped the samples into genetic units (GU), which not necessarily coincided with the MUs.

To infer a phylogenetic hypothesis of the genus *Thraupis*, I performed multispecies coalescent species tree analyses using Taxonomic Units (TU) defined *a priori* based on the comparison between MU and GU. Birds with obvious intermediate genetic profiles (i.e., haplotypes of different ancestry across loci) were excluded because they represent a violation of the assumption of no migration between species in the multispecies coalescent model. I prepared the input data for the species tree using the graphical interface program BEAUTI (Drummond *et al.*, 2012) within BEAST 2 (Bouckaert *et al.*, 2014). I performed a species tree analysis under a coalescent Bayesian framework calibrated with a molecular clock using *BEAST (Drummond and Rambaut, 2007; Heled and Drummond, 2010) on the Cipres Science Gateway website (Miller, Pfeiffer and Schwartz, 2010) . The best partition scheme within each locus was selected with Partition Finder 2 (Lanfear *et al.*, 2016). The best substitution model was GTR + Γ +I for all loci, except for the one of ND2 that the GTR + Γ model was the best fit. In *BEAST I ran three independent analyses with unlinked substitution models within partitions. For the mitochondrial locus, I used a strict clock with a clock rate of 0.0105 (Weir and Schluter, 2008). The other three loci had a relaxed clock Log - normal and the clock rate was open to be estimate. I used the Yule model for tree shape. 200 million generations, sampling every 10,000 and a burning of 25%. I used de LogCombiner v.2.4.6 from BEAST2 (Bouckaert *et al.*, 2014) to combine the log files and the species tree from the three independent runs. I used the software Tracer v.1.6.0 (Rambaut, Suchard and Drummond, 2013) to analyze log file. I opened the log file using a 25% burning. I checked all ESS values were greater than 200. I used LogCombiner v.2.4.6 and TreeAnnotator v.2.4.6 from BEAST2 (Bouckaert *et al.*, 2014) to fusion the three species tree files and combine all the posterior probability into a single maximum clade credibility tree. I ran TreeAnnotator with a burnin of 25% and a posterior probability limit of 0.5. All trees figures were edited with FigTree v.1.4.3 (Rambaut, 2016).

2.6 Species concept

The species concept used to determine and delimit species is the unified species concept (de Queiroz, 2007). It considers as species each unit that evolves separately from other linages. Thus, characteristics as sexual isolation, phenotypic differences, ecological differentiation or reciprocal monophyletic, are secondary-not defining properties of a species. It means that a species is not defined by a single of the characteristics described before, but each characteristic represents evidence in favor of the hypothesis of two (or more) different evolving units. Also, those characteristics not necessarily appear with an intrinsic order across the speciation process.

3. RESULTS

3.1 Color traits and taxonomic units

Based on the characters' maps (Fig. 7-12), I classified specimens into a total of 10 MU (Fig. 13). Most of the MUs correspond to recognized taxa (Table 4). The most informative character was the color of the lesser-coverts (Fig. 7), which allows identification of seven of out of the ten MU, followed by the color of the crown and chest (Fig. 8 and 9 respectively), both characters identified five of the ten MU. The back and primary feather coloration (Fig. 10 and 11 respectively) identified three and two of the MU respectively, and were the traits with more color variation, especially in Amazon Moist Forest and at west of the Andes. The least informative character was the amount of white in the greater coverts. This character shows a clinal distribution (Fig. 12). Individuals with the highest amount of white are distributed around the Brazil-Colombia-Peru border in Amazonia. The amount of white decreases as the distance to this point increases, especially those specimens from eastern Amazonia, where individuals exhibit almost not white on the greater coverts.

The MU 1 (*T. cyanoptera*) is recovered by the five most informative color characters (Fig. 7-11). It is distributed in the Atlantic forests of Brazil and it has contrasting colors with the overlapping MU 2 (*T. s. sayaca*). MU 2 and MU 3 (*T. s. obscura*) show no differential colors in most of characters, nevertheless, they present different coloration on the crown. MU 2 has a Brownish Olive crown and MU 3 a Dark Grayish Brown (Fig. 8). Because this character represents the difference that divide them into subspecies, *T. sayaca sayaca* and *T. sayaca obscura* respectively (Fig 13 b, c), both were maintained as separate MUs for morphometric analyses. Both occur in the southern dry areas of South America: Caatinga, Cerrado and Chaco, with MU 3 the one distributed in the Chaco Dry Forest at the west of the distribution. The MU 4 (*T. glaucocolpa*) is recovered by three of the characters (Fig. 7-9). It is distributed in a Deserts/Shrublands biome in the extreme north of South America. It exhibits a Turquoise Green chest color and a Turquoise Blue lesser-coverts clearly differentiate it from another MU. MU 5 (*T. e. quaesita*) and MU 6 (*T. e. cana*) are distributed on the west side of the Andes. Both have similar colors with a high variance, however, they are the only groups with dark blue lesser-coverts. MU 5 has a Cobalt color (Fig. 13e) and it is distributed on the

pacific side of the Andes. Both were maintained as separate MU due to the original description of *T. e. quaesita* that draws attention to its "lesser and middle wing coverts *much* darker blue" than those of *T. e. cana* (Bangs and Noble, 1918). On the other hand, MU 6 shows greater color variation. Examined individuals by pictures of the types of *T. episcopus cumatilis* and *T. episcopus caesitia* (distributed in islands of Panamá) did not allow establishing color difference with *T. e. cana*. MU 7 and MU 8 form another compound group than correspond to various subspecies of *T. episcopus* (see Table 4). These MUs are the only ones with white lesser-coverts. They are distributed in the Amazon Moist Forest. The characters that differentiate the two groups of birds with white lesser-coverts are the crown and chest color.

Specimens around the Colombian–Brazilian–Peruvian border show Turquoise Green color on these body areas (Fig. 13h). The last two MU, 9 (*T. e. nesophila*) and 10 (*T. e. nesophila* and *T. e. berlepschi*), are recognizable because the presence of different tones of purple in the lesser-coverts. This color may vary from Lavender to Smalt Blue (Fig. 13i, j). The specimens with more variation are the ones collected from the continental part in the Llanos of Colombia and Venezuela. The reason they are considered as two different MU is that individuals from Trinidad and Tobago, and the states of Sucre, Monagas and Delta Amacuro in Venezuela, show an iridescent Sky Blue in the chest (Fig. 13j) and back that differs from other specimens with purple lesser-coverts. There were some individuals with intermediate characteristics that did not allow certain ascription to specific MU (Fig. 14) and they were marked as intermediates or potential hybrids in the character maps (Fig 7-12). The specimen H2, H3 and H4 belong to the skins photographed on Figure 13a, 13b and 13c respectively.

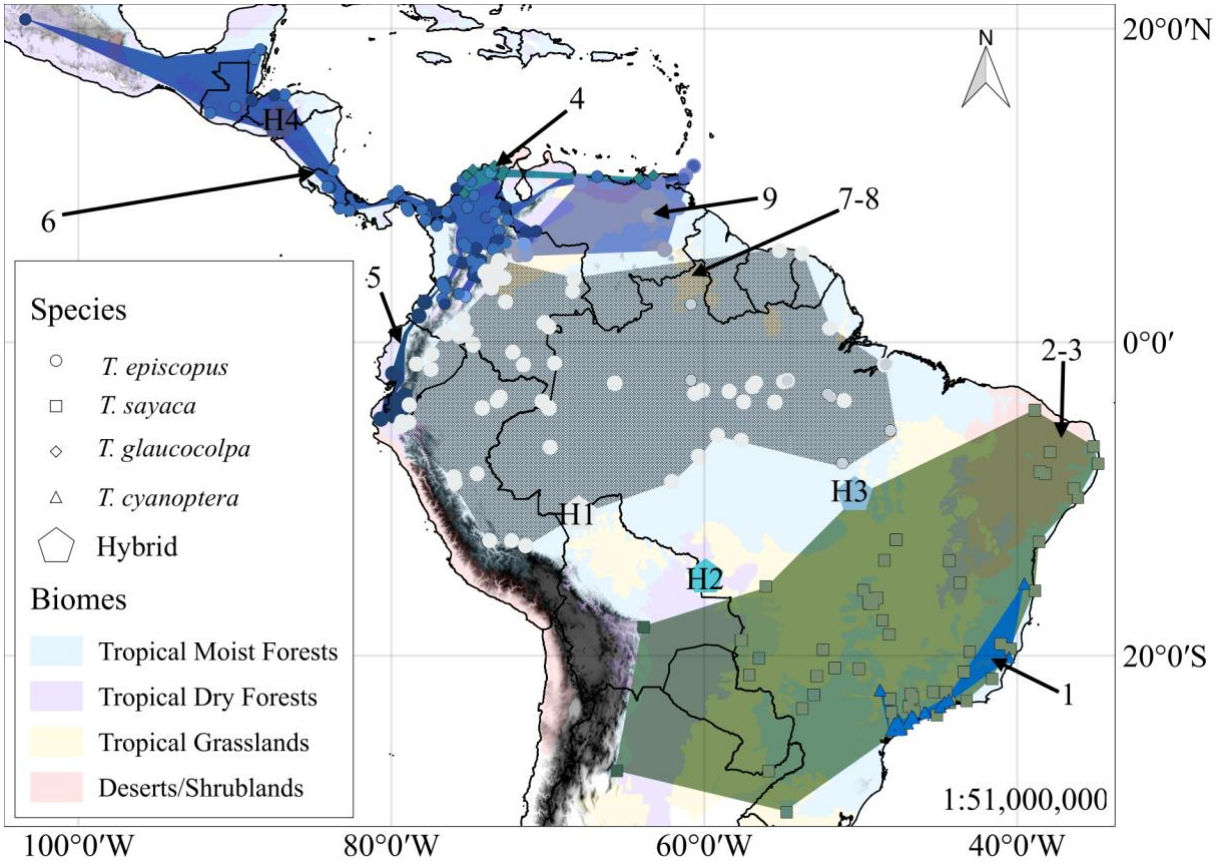

Figure 7. Color from lesser-coverts area. Symbols' and polygons' colors represent the colors from specimens after equalizing white balance. Numbers represents the respective Morphological unit. H1, H2 and H3 are skins with intermediate traits between *T. episcopus* and *T. sayaca*. H4 is a possible hybrid between *T. episcopus* and *T. abbas.*

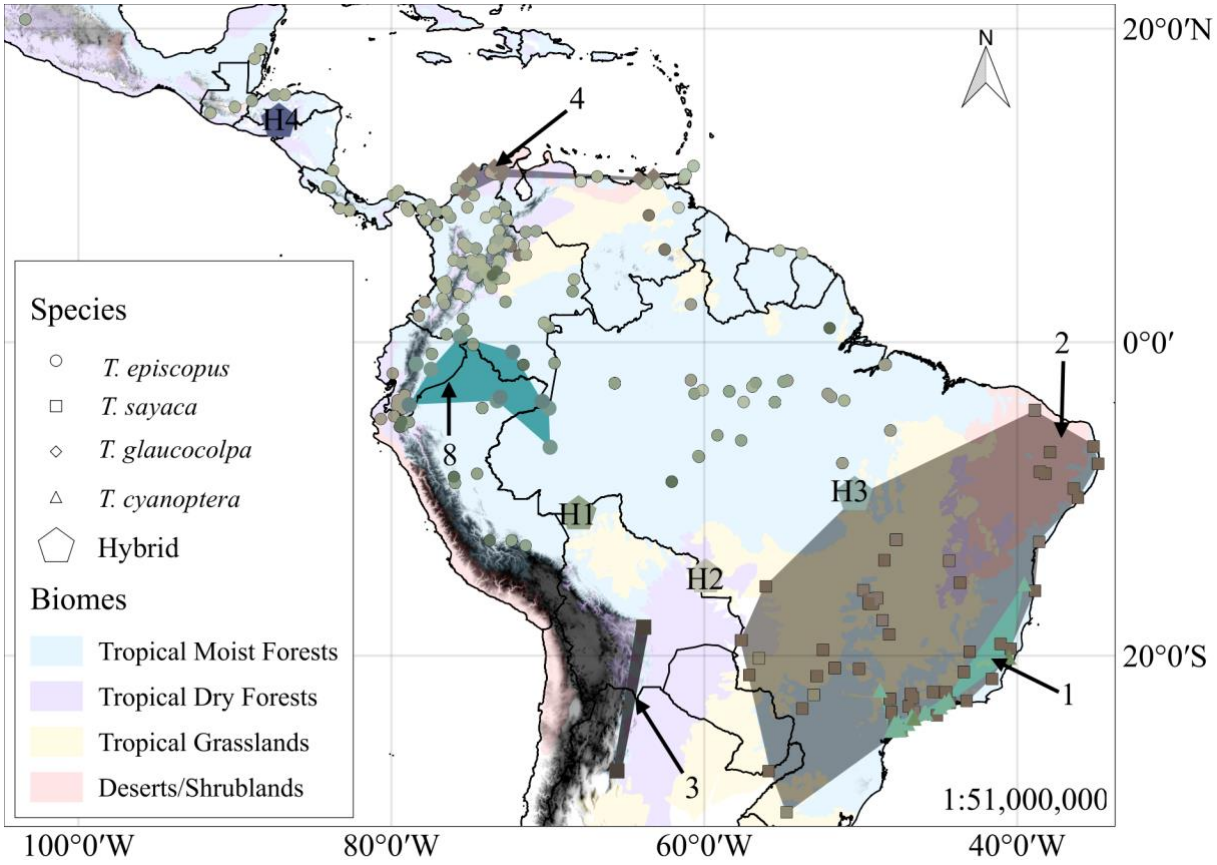

Figure 8. Color from crown area. Symbols' and polygons' colors represent the colors from specimens after equalizing white balance. Numbers represents the respective Morphological unit. H1, H2 and H3 are skins with intermediate traits between *T. episcopus* and *T. sayaca*. H4 is a possible hybrid between *T. episcopus* and *T. abbas.*

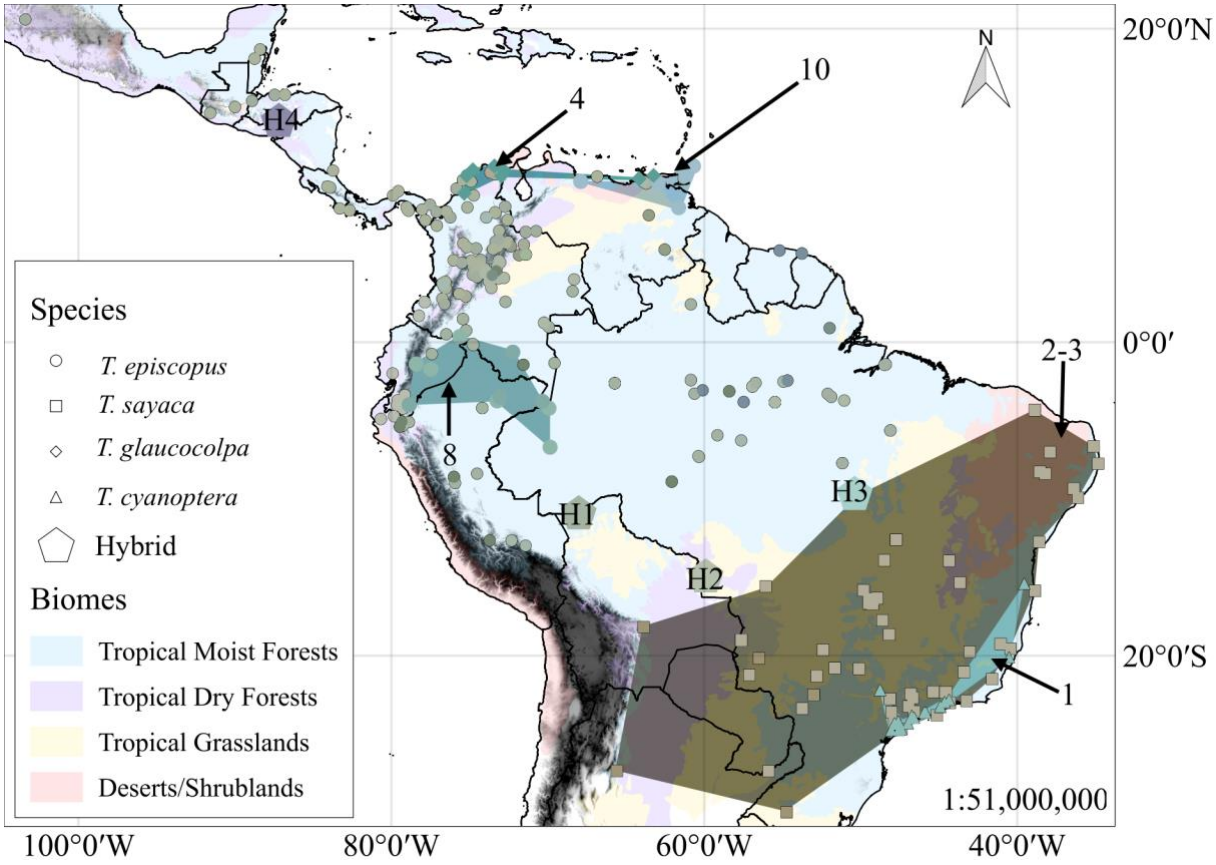

Figure 9. Color from chest area. Symbols' and polygons' colors represent the colors from specimens after equalizing white balance. Numbers represents the respective Morphological unit. H1, H2 and H3 are skins with intermediate traits between *T. episcopus* and *T. sayaca*. H4 is a possible hybrid between *T. episcopus* and *T. abbas.*

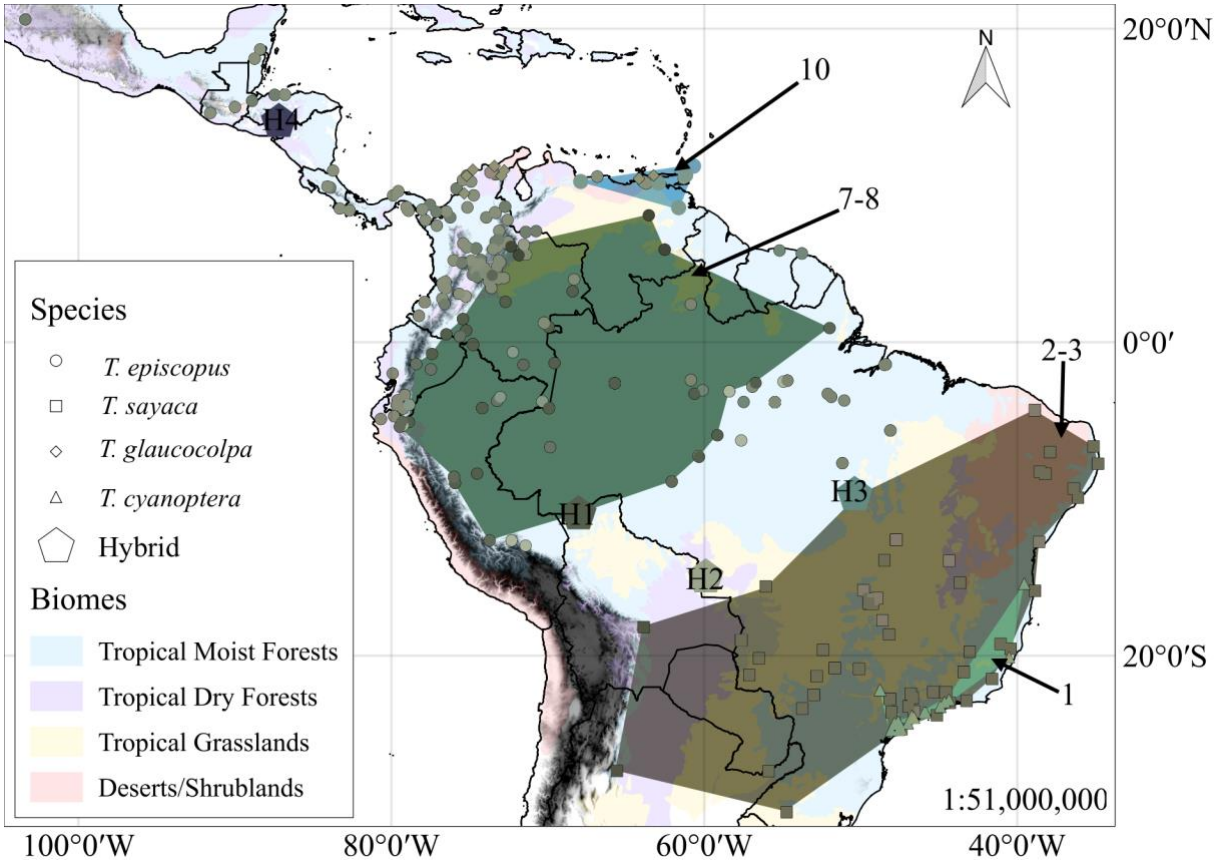

Figure 10. Color from back area. Symbols' and polygons' colors represent the colors from specimens after equalizing white balance. Numbers represents the respective Morphological unit. H1, H2 and H3 are skins with intermediate traits between *T. episcopus* and *T. sayaca*. H4 is a possible hybrid between *T. episcopus* and *T. abbas.*

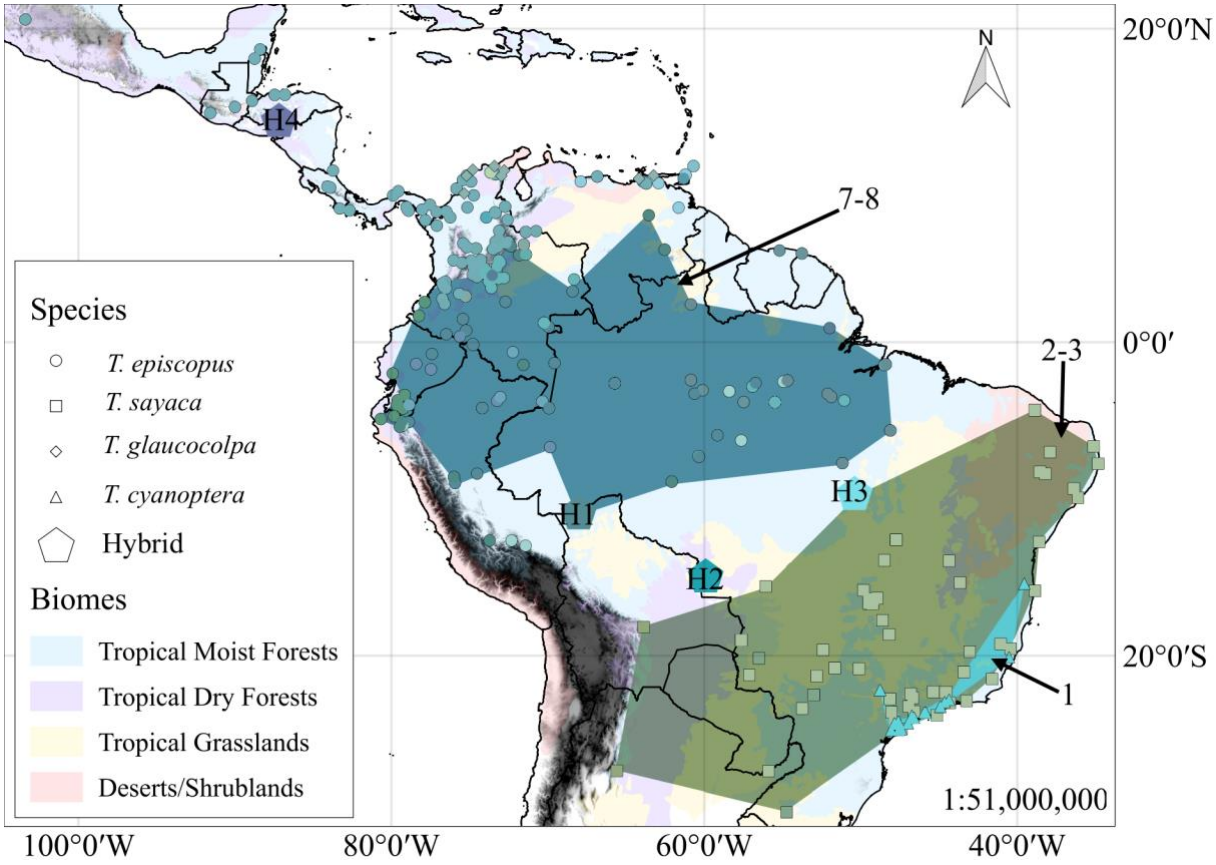

Figure 11. Color from primary feathers area. Symbols' and polygons' colors represent the colors from specimens after equalizing white balance. Numbers represents the respective Morphological unit. H1, H2 and H3 are skins with intermediate traits between *T. episcopus* and *T. sayaca*. H4 is a possible hybrid between *T. episcopus* and *T. abbas.*

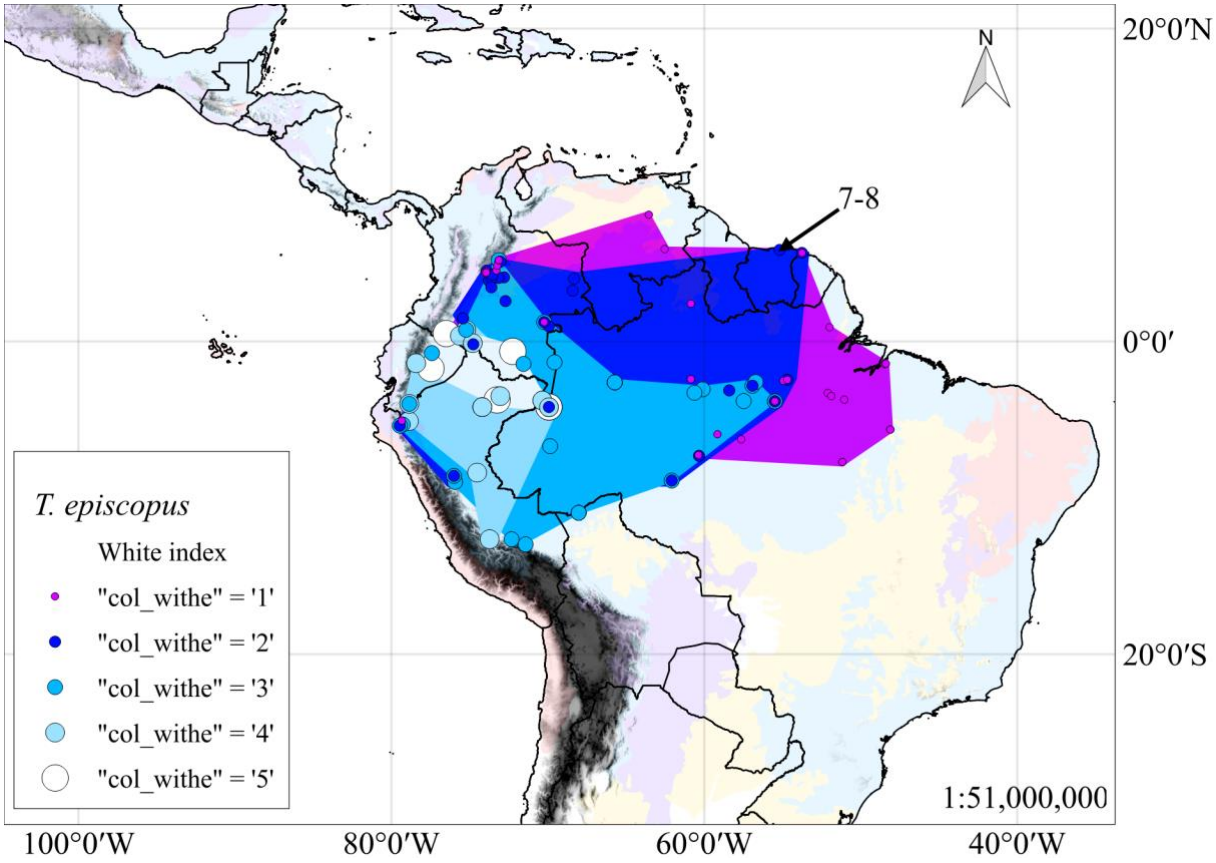

Figure 12. White amount in greater coverts. Different sizes of the circles represent are correlated with the amount of white on the greater coverts. The amount of white was categorized following the Fig. 6.

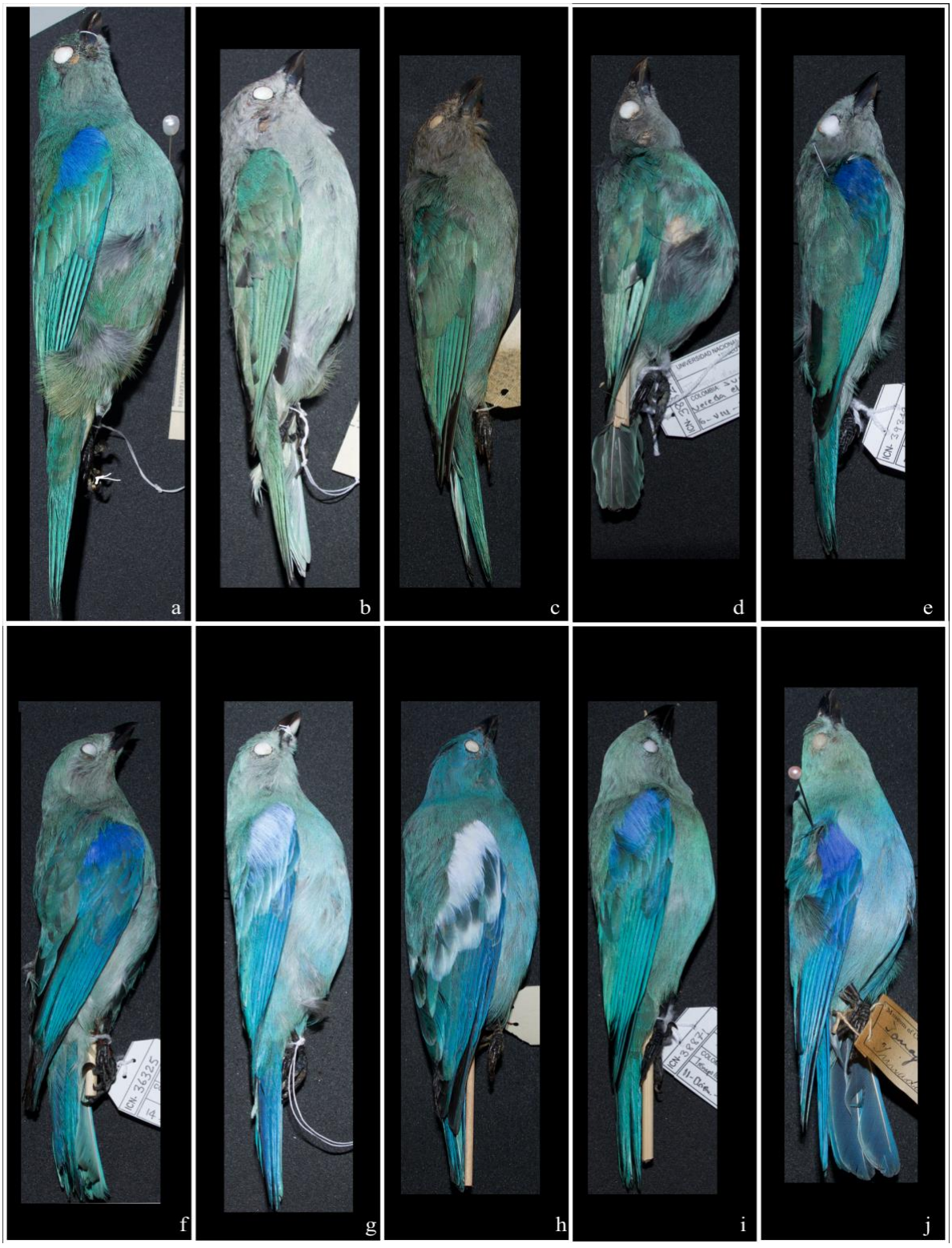

Figure 13. Taxonomic Units in order 1-10 from left to right from top to down. Photographed museum specimens: MZUSP 91046, MZUSP 98620, MCZ 96766, ICN 38321, ICN 39349, ICN 36325, MZUSP 95739, MCZ 299657, ICN 38871 and MCZ 32412 respectively.

Table 4. Summarize of the MU found after color classification. Related taxa refer to the current taxa that match with the distribution of the different MUs, next to it the geographic regions were teach MU is distributed.

MU	Related taxa	Geographic distribution
1	*T. cyanoptera*	Brazilian Atlantic Forest
2	*T. s. sayaca*	Catinga, Cerrado, Atlantic Forest
3	*T. s. obscura*	Dry forest on the east slope of Bolivia and Argentina Andes
4	*T. glaucocolpa*	Dry areas in north Colombia and Venezuela
5	*T. e. quaesita*	West slope of Colombia, Ecuador and Peru Andes
6	*T. e. cana*	Central America, north Colombia and valleys within the north Andes
7	*T. e. episcopus, T. e. mediana, T. e. leucoptera, T. e. major, T. e. urumbabae*	Amazon forest
8	*T. e. coelestis, T. e. caerulea*	Extreme west of amazon. From Colombia, Brazil and Peru border, to Colombia, Peru and Ecuador border.
9	*T. e. nesophila*	Colombian and Venezuelan llanos
10	*T. e. nesophila, T. e. berlepschi*	Trinidad and Tobago, and extreme north east of Venezuela.

Figure 14 Presumed hybrids specimens. *T. episcopus* x *T. sayaca* (a, b) and *T. cana* x *T. abbas* (c). Photographed museum specimens: MZUSP 107246, MZUSP 98621, MCZ 163160

3.2 Morphometric analyses

Kolmogorov-Smirnov tests show that the distribution of all characters fit a normal distribution (Table 5; Figs 15 - 20). The MANOVA test show a significant difference in mean values across all MU ($P < 0.001$). The PCA analysis including all MU (Fig. 21) shows MU 1 and 4 as the most distinctive but without clear separation from the other MU. The PCA analysis including only the sympatric Atlantic Forest MU (MU 1, MU 2, MU 3; Fig. 22) shows a clear separation among them. Conversely, other PCA including other MU than come into geographic contact (Fig. 23 – 25) show no differences among groups. Finally, the LDA analysis (Fig. 27 and 28) groups apart the MU 1 from all other MU, corroborating the result from the PCAs. MU 4 is not completely separated, but it groups near the borders of the cloud of points, overlapping with a few individuals of others MU. Other MU are mix in the middle of the cloud of points and is impossible to differentiate anything else. In general, these results

show that most MU are not diagnosable morphometrically with the only exception of the largest form (*T. cyanoptera*).

Table 5. Kolmogorov-Smirnov test p-values. NA represents not enough data. Lower values (red). Higher values (green).

TU	Weight	CN	CT	TS	Wing	Tail
1	0.68	0.33	0.64	0.32	0.36	0.76
2	0.94	0.27	0.53	0.13	0.80	0.78
3	NA	1.00	1.00	1.00	1.00	1.00
4	0.77	0.63	0.96	0.96	0.86	0.68
5	0.74	0.86	0.87	0.82	0.72	0.95
6	0.94	0.14	0.41	0.26	0.58	0.48
7	0.72	0.24	0.42	0.23	0.70	0.85
8	1.00	0.41	0.53	0.87	0.99	0.99
9	0.97	0.92	0.78	0.98	0.63	0.96
10	NA	0.94	1.00	0.91	0.91	0.53

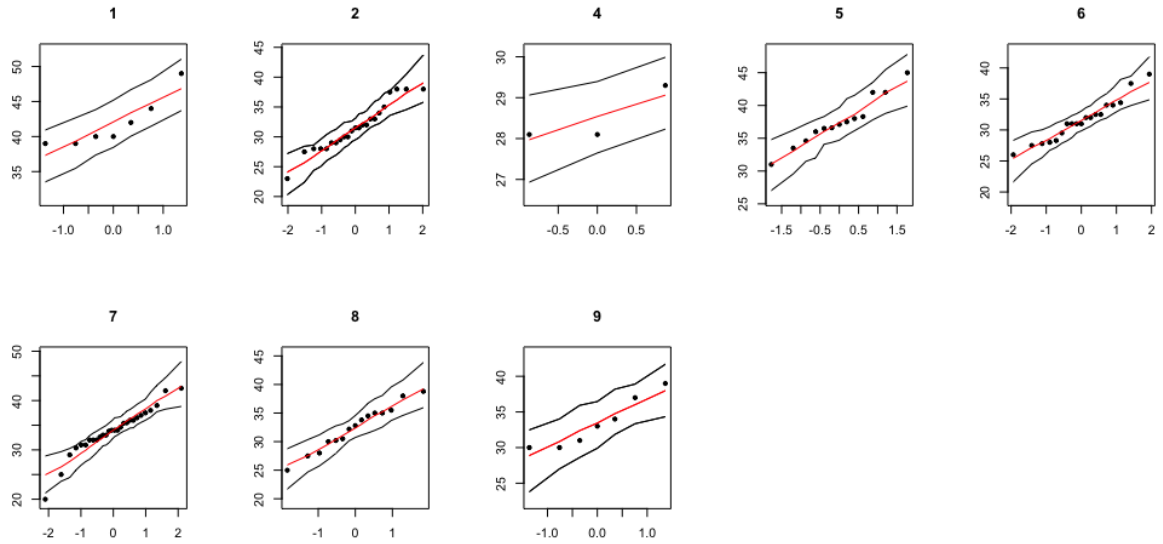

Figure 15. QQplot of weight by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).MU 3 and MU 10 did not have any individual with weight information.

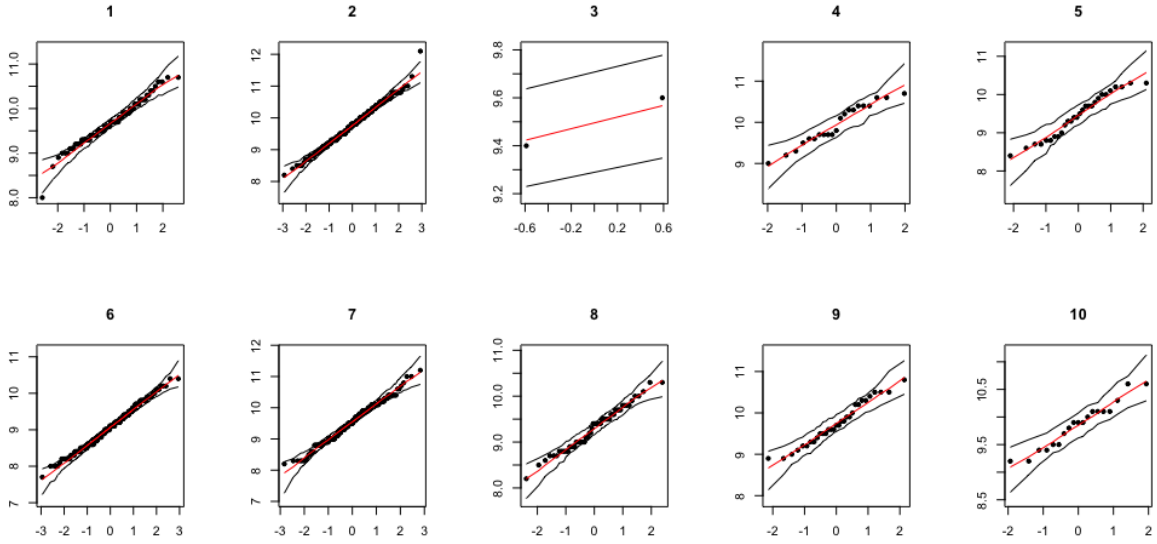

Figure 16. QQplot of culmen from nares by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).

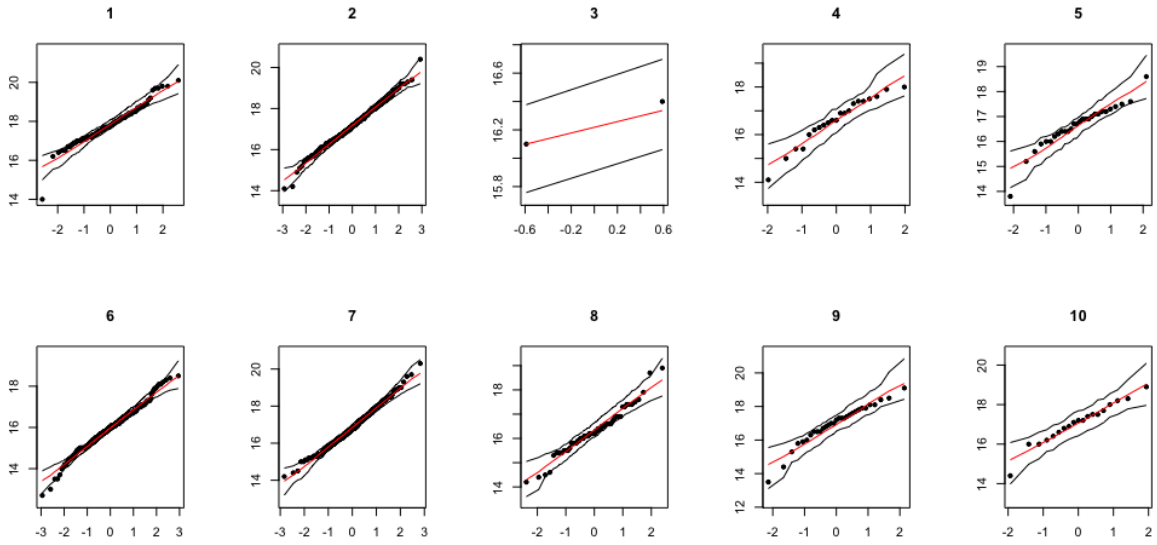

Figure 17. QQplot of total culmen by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).

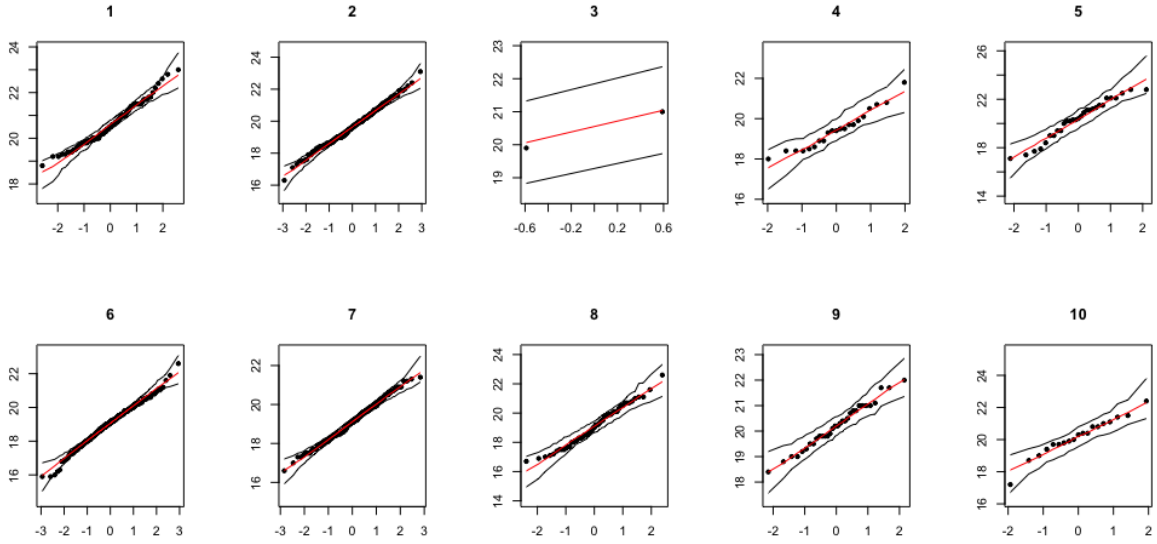

Figure 18. QQplot of tarsus length by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).

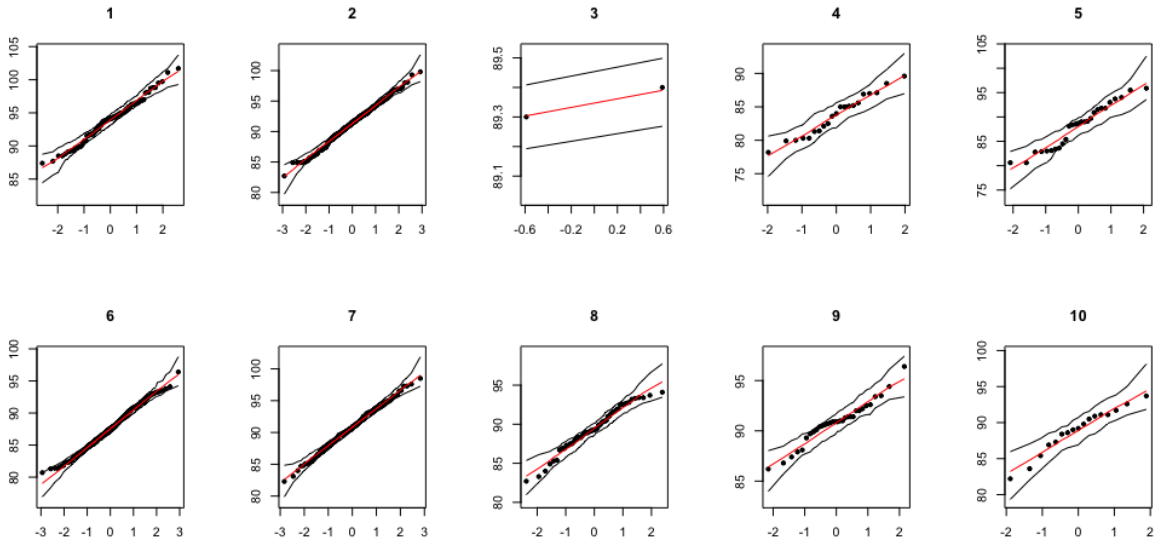

Figure 19. QQplot of wing chord by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).

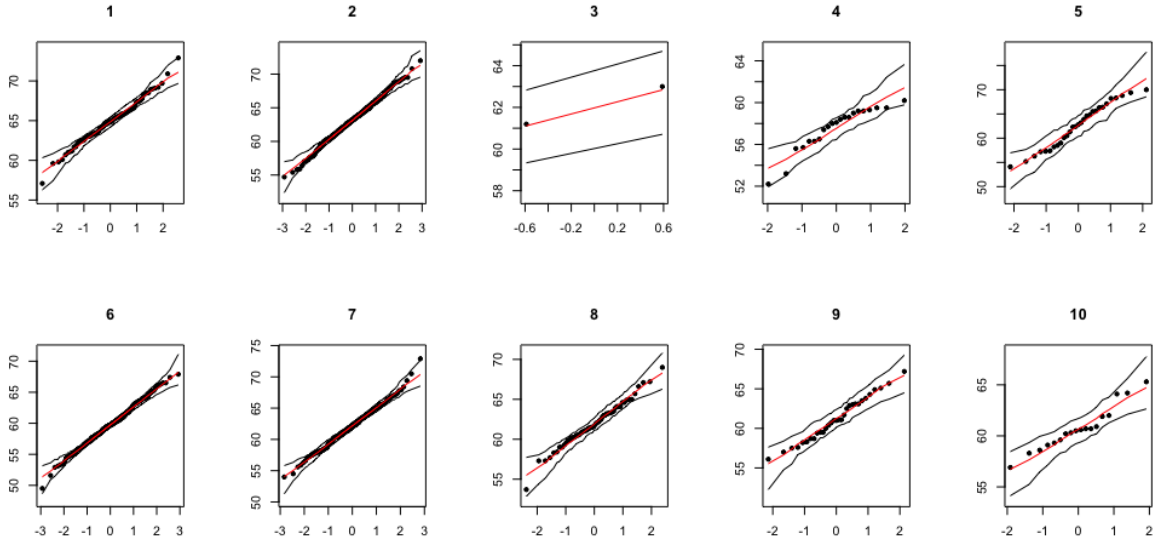

Figure 20. QQplot of tail length by MU. Samples (black points), normal distribution (red line), superior and inferior limits (black lines).

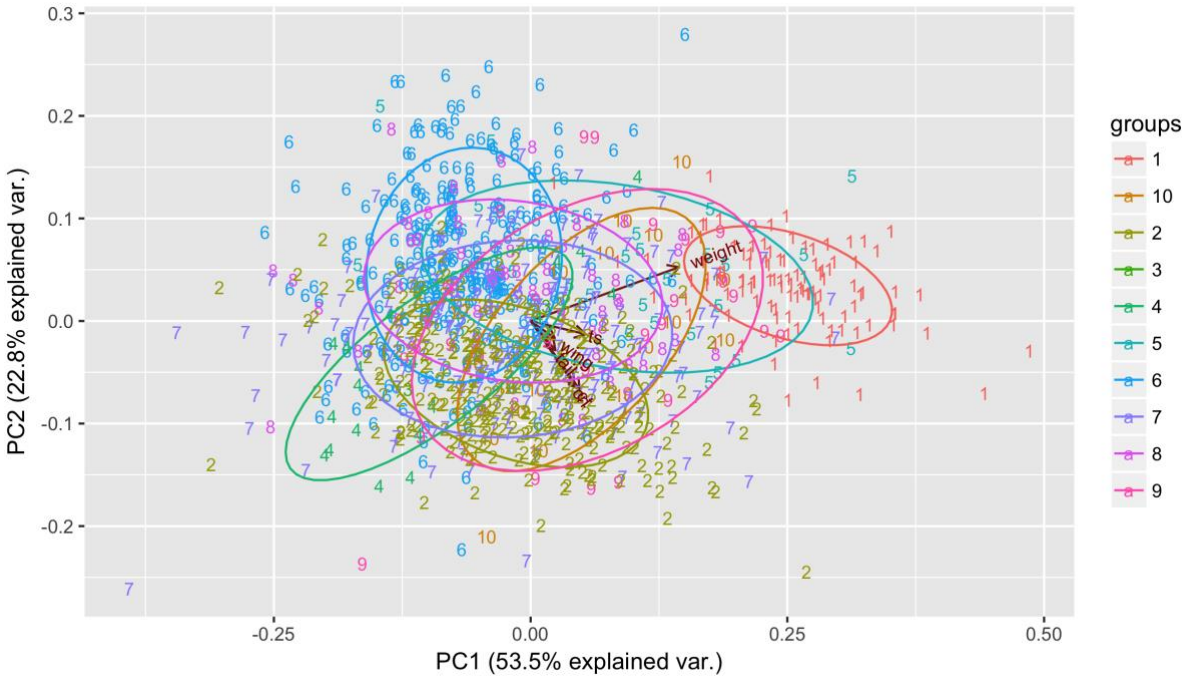

Figure 21. PCA of all MUs. MUs are represented with the corresponding number. Ellipse probability is 0.65. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

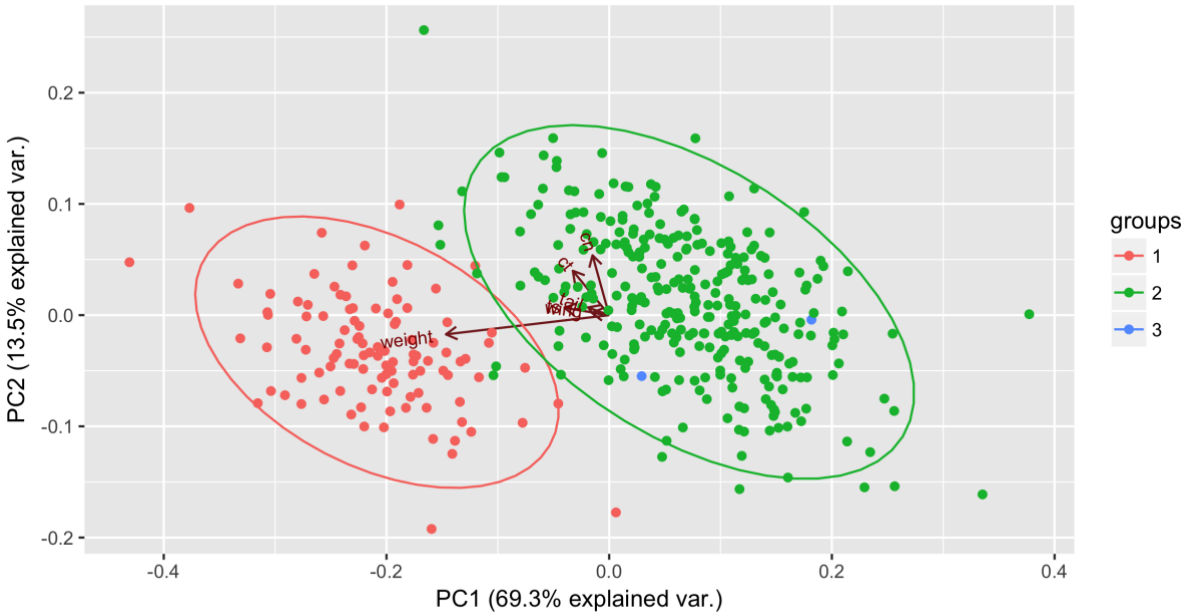

Figure 22. PCA comparing MUs 1, 2 and 3. MUs are represented with the corresponding number. Ellipse probability 0.95. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

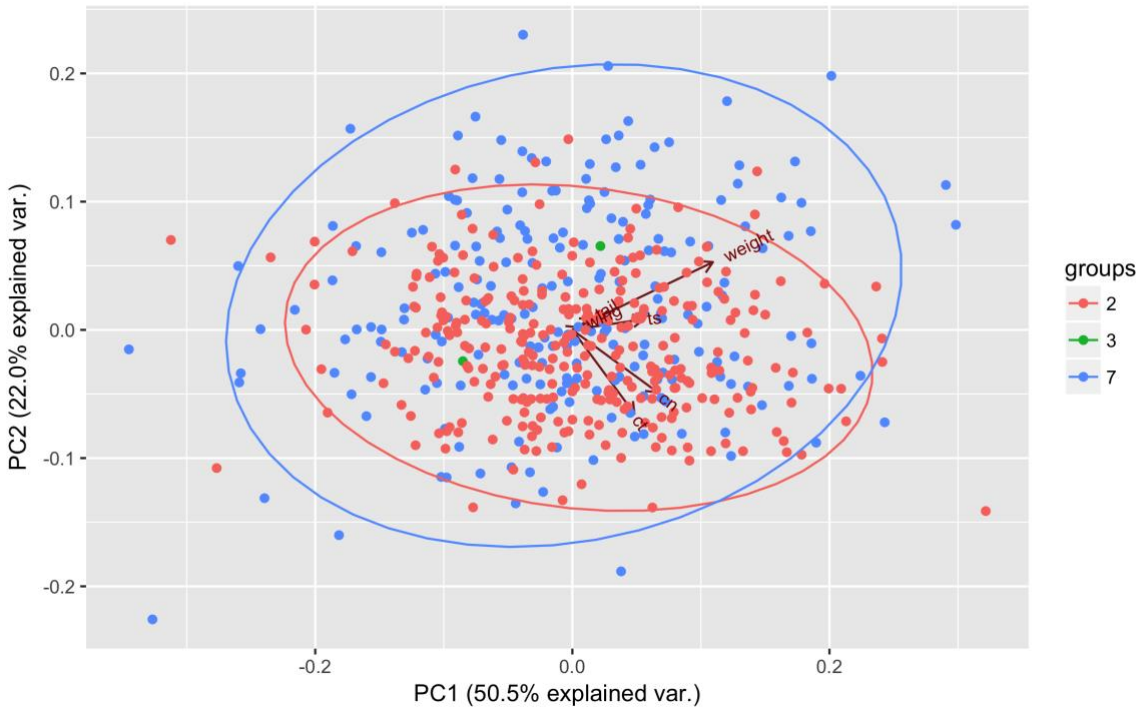

Figure 23. PCA comparing MUs 2, 3 and 7. MUs are represented with the corresponding number. Ellipse probability 0.95. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

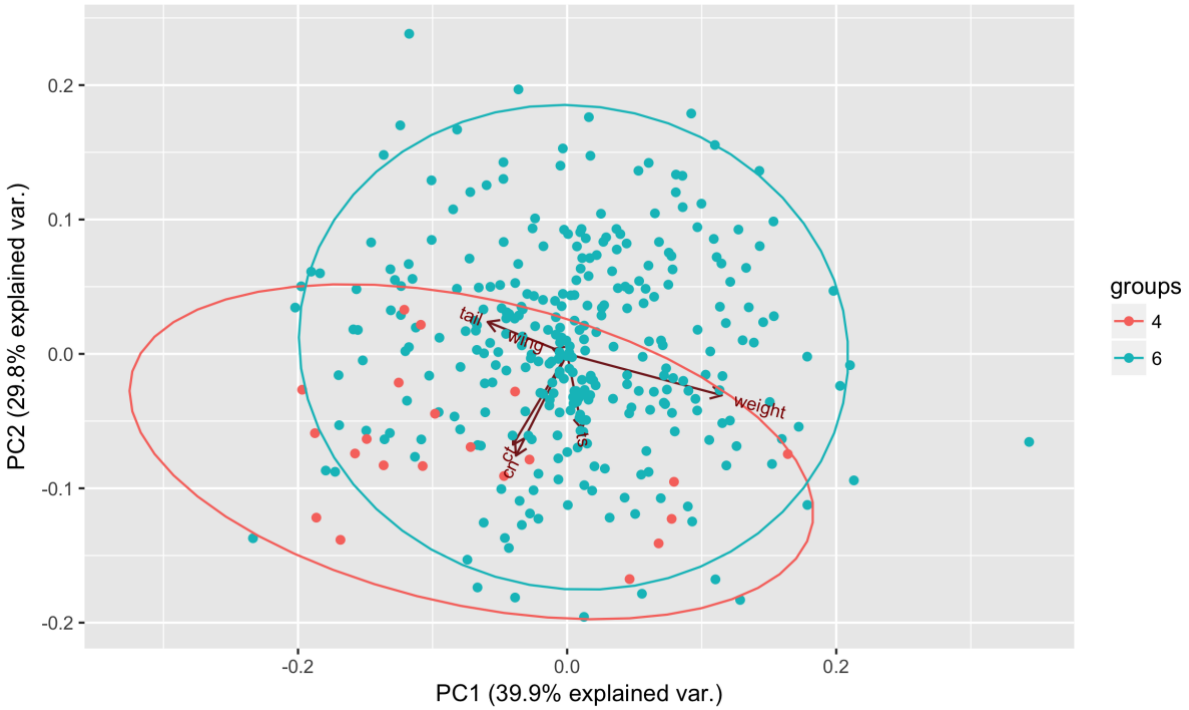

Figure 24. PCA comparing MUs 4 and 6. MUs are represented with the corresponding number. Ellipse probability 0.95. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

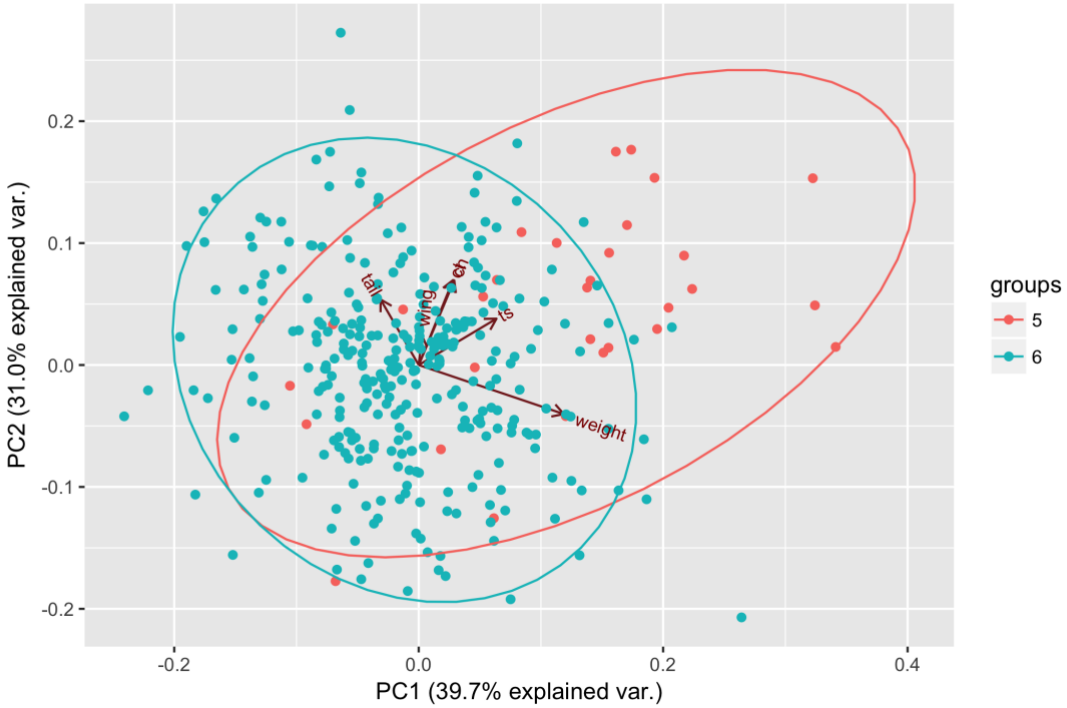

Figure 25. PCA comparing MUs 5 and 6. MUs are represented with the corresponding number. Ellipse probability 0.95. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

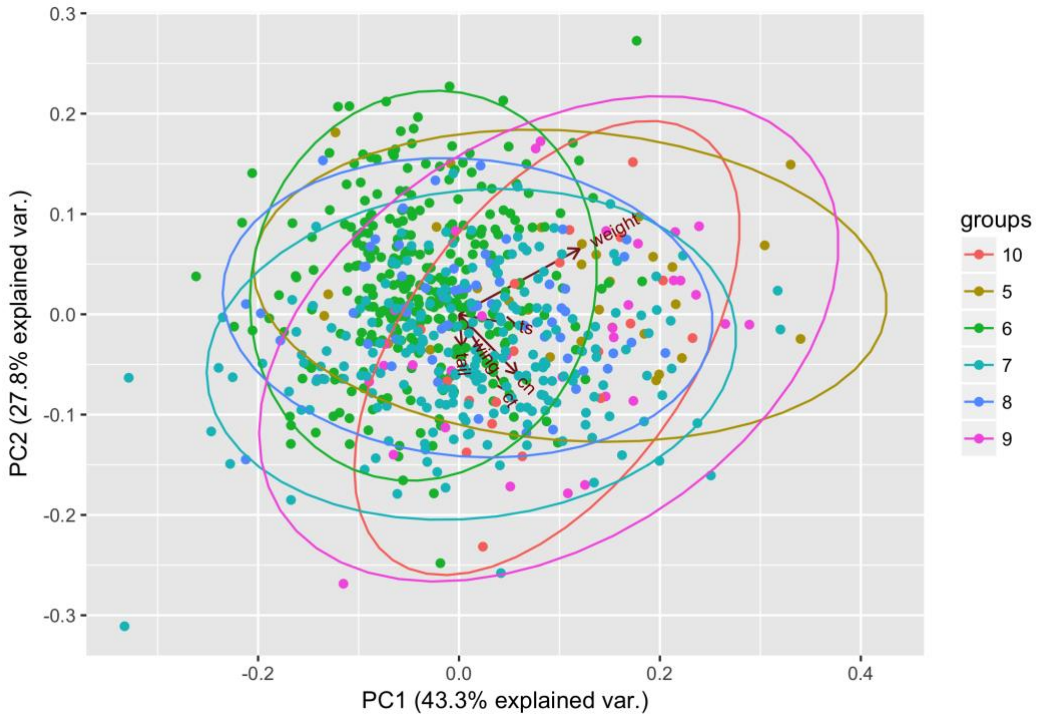

Figure 26. PCA comparing MUs 5, 6, 7, 8, 9 and 10, MUs classified as a subspecies within *T. episcopus*. MUs are represented with the corresponding number. Ellipse probability 0.95. Vectors represents the loadings for each variable. For more information about the loadings see Table 6.

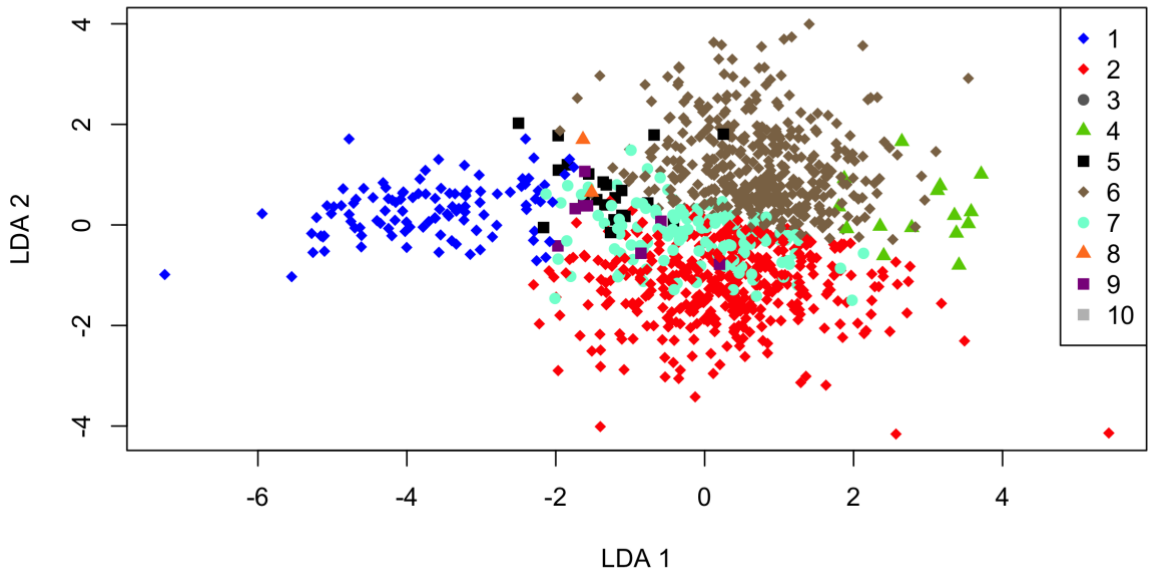

Figure 27. LDA made with base on the morphometric measurements (Weight, CN, CT, TS, Wing and Tail) within the MUs. Numbers represent the respective MU.

Table 6. PCA loadings of the Fig. 21-26. The values are organized in all boxes in the order: Weight, CN, CT, TS, Wing and Tail.

	PCA 1	PCA 2
PCA Fig. 21	0.60443389 0.54563850 0.44595998 0.34099588 0.09423913 0.11357285	0.731146353 -0.543766135 -0.393979022 -0.108389121 -0.005768629 -0.052368436
PCA Fig. 22	-0.92728264 -0.09464000 -0.20647443 -0.25354421 -0.09872035 -0.12053263	-0.24750611 0.77022145 0.57091600 0.09416311 0.02958266 0.09905929
PCA Fig. 23	0.73633233 0.44719964 0.32507231 0.37548978 0.02996622 0.10131447	0.53951780 -0.49750054 -0.66720547 0.08563575 0.02036868 0.09220763
PCA Fig. 24	0.81265639 -0.26912618 -0.28443013 0.07772185 -0.07359975 -0.41809399	-0.25193021 -0.61933525 -0.55257444 -0.45696124 0.02941272 0.19477634
PCA Fig. 25	0.82402891 0.20180727 0.19488117 0.44155428 0.01815114 -0.21672996	-0.3160029 0.5449375 0.5470358 0.2986529 0.1908602 0.4222748
PCA Fig. 26	0.78633043 0.38061839 0.30075208 0.37355711 0.08002428 0.02033440	0.53398542 -0.48741794 -0.61558541 -0.08652131 -0.14402289 -0.26478178

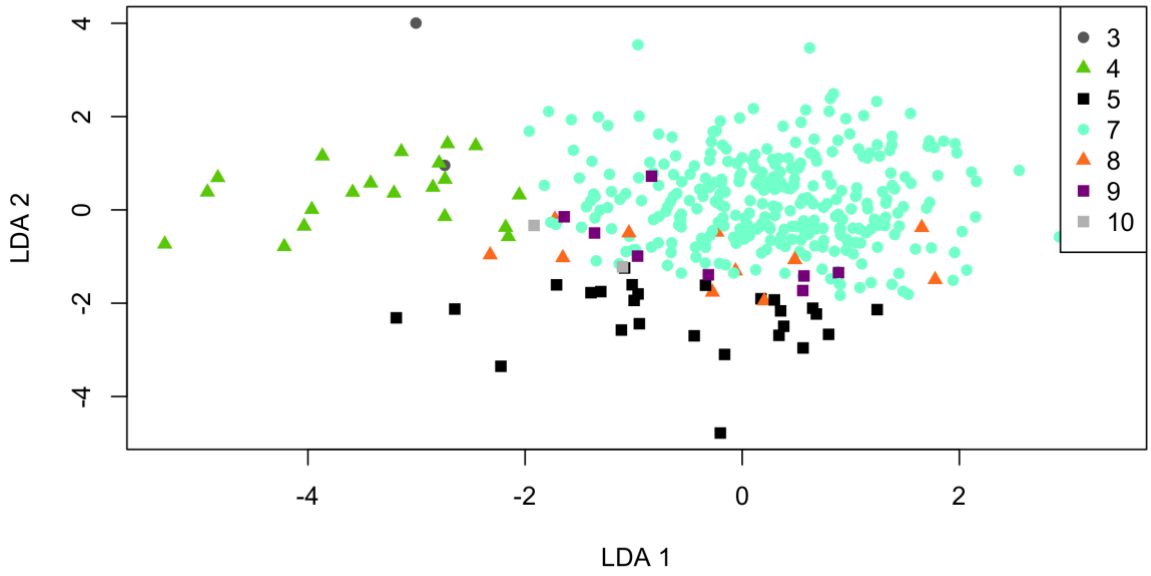

Figure 28. LDA made with base on the morphometric measurements (Weight, CN, CT, TS, Wing and Tail) within the MUs. Numbers represent the respective MU. Here were excluded the MUs with more individuals, MU 1, MU2 and MU 6

3.3 Molecular analysis

3.3.1 Gene trees and haplotype networks

The BF5 RAxML gene tree and haplotype network (Fig. 29 and 30) show little genetic divergence among samples, branches with low support, and no clear geographic or taxonomic pattern. The MUSK gene tree and haplotype network (Fig. 31 and 32) show more structure and better support for several branches, with some values higher than 75. It is important to highlight that one of the specimens classified as *T. sayaca* was recovered within the *T. episcopus* clade (zoom in Fig. 30), also visible in the haplotype network. This specimen (MZUSP 98621) was collected in Vila Bela da Santisima Trinidad, MT (14º59'S, 59º55'W), it was originally classified as *T. sayaca* by MZUSP but it was one of the specimens catalogued as a hybrid in the morphological analyses because of its intermediate plumage

characteristics (Fig. 14b). Another specimen collected in the same locality and date was recovered as *T. sayaca* and has the normal coloration of *T. sayaca* (Fig 13b).

TGFB2 exhibits a similar structure than that of MUSK. It shows two individuals of *T. sayaca* within a large clade composed by *T. episcopus* (Fig. 33). The two birds are deposited at MZUSP (90269 and UFG4362). They were collected in Santana do Araguaia, PA (9º47'S, 50º13'W) and Carolina, MA (7º14'S, 47º08'W) in close proximity to the ecotone between Tropical Moist Forest and Tropical Grasslands. These specimens do not have external characteristics that permit differentiation from other *T. sayaca* individuals. This fact is also evident in the haplotype network (Fig. 34). Furthermore, the sample of *T. abbas* is recovered within the haplotypes corresponding to *T. episcopus*. Finally, the mitochondrial tree and haplotype network are the most structured (Fig. 35 and 36). Almost all branches have support values above 75 and the network shows several mutations steps in the middle of haplotypes groups.

This tree recovers *T. glaucocolpa* as the most divergent lineage within *Thraupis*. Moreover, it recovers a clade formed by *T. abbas*, *T. ornata* and *T, pamarum*, with the two latter as sister taxa, also visible in the haplotype network. Another recovered clade is the one of *T. sayaca* and *T. episcopus,* that shows an evident structure within *T. episcopus*. This subdivision, conformed by three groups, largely reflects current subspecies. The first one corresponds to *T. episcopus quaesita* (pacific coast, Fig. 36a), the second one to *T. episcopus cana* (north inter-andean valleys, north of south America and central America, (Fig. 36b), and the third one to all other *T. episcopus* subspecies (Fig. 36c). The mitochondrial haplotype network shows that *T. sayaca* shares haplotypes with the population of *T. episcopus* that get in contact, but not with the others. It is important to highlight that the *T. sayaca* specimen that appears within the *T. episcopus* clade in the mithocondrial tree is the same individual that appears in the *T. episcopus* clade in the MUSK tree (MZUSP 98621). It was collected in Vila Bela da Santíssima Trinidad, MT (14º59'S, 59º55'W). This individual, suggest and hybridization process where the parental species are a female *T. episcopus* and the male a *T. sayaca*. The other specimens of *T. episcopus* that appears in the *T. sayaca* clade are: KU 115635, MZUSP 101479, LSU B-9554, MSB 27433, MSB 36846, MPEG T-11969, MZUSP 107246, collected in San Juan del Oro, Puno (14º12'S, 69º11'W), Alta Mira, PA (3º17'S, 52º07'W), Nicolas Suarez, Pando (10º57'S, 68º02'W), San Pedro, Cusco (14º11'S, 71º20'W), Cadena, Cusco (13º24'S, 70º43'W), Oriximiná, PA (1º45'S, 56º13'W) and Santana do Araguaia, PA (9º43'S, 50º24'W) respectively.

All hybrid specimens were classified as *T. episcopus* because their white lesser-coverts. However, the width and amount of white is somewhat variable. Without molecular information, it is hard to tell if they are hybrids, because can be confused with a juvenal a molting bird or maybe a female.

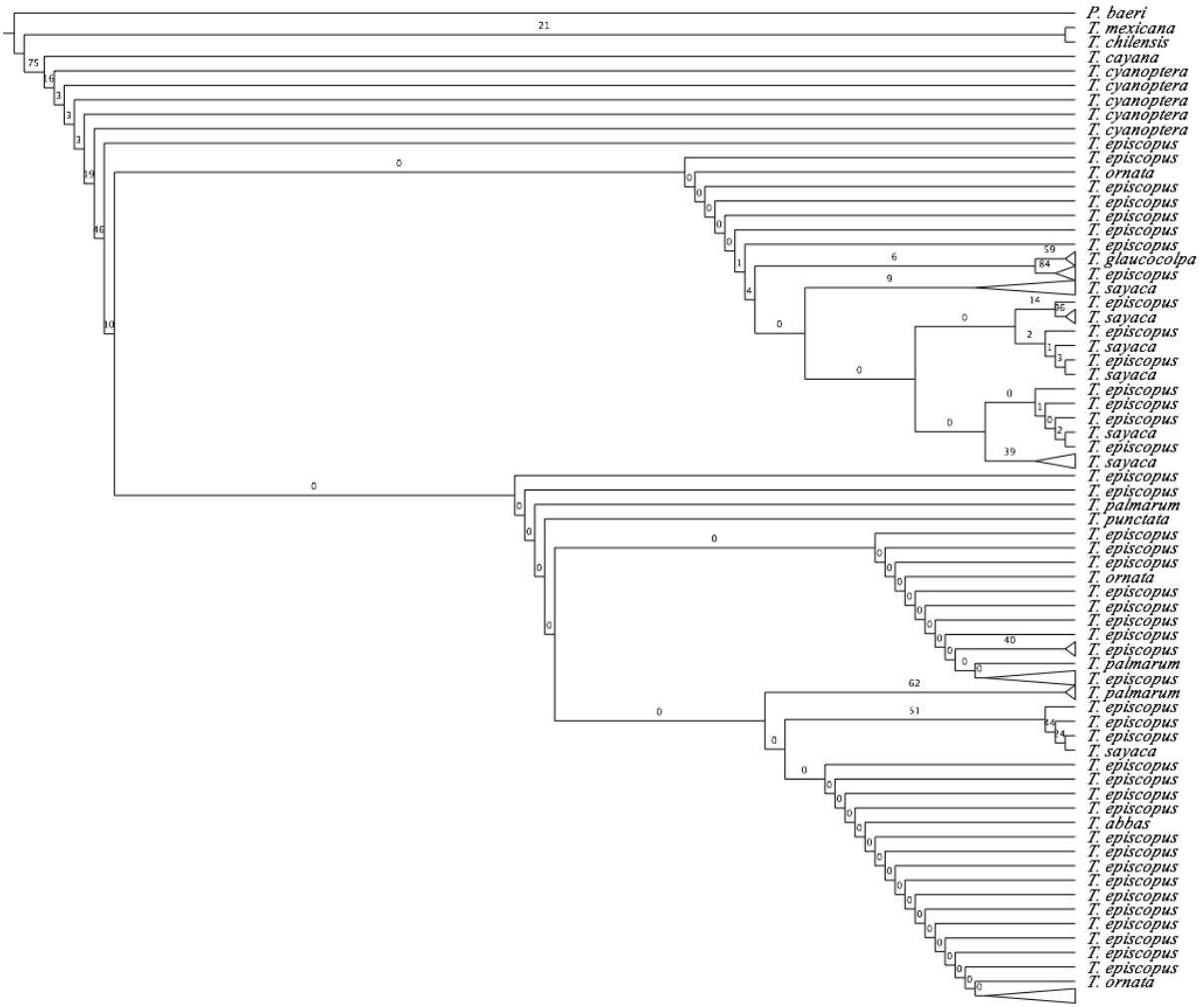

Figure 29. Intron BF5 RAxML tree, of genus *Thraupis*. Bootstrapping values over each branch.

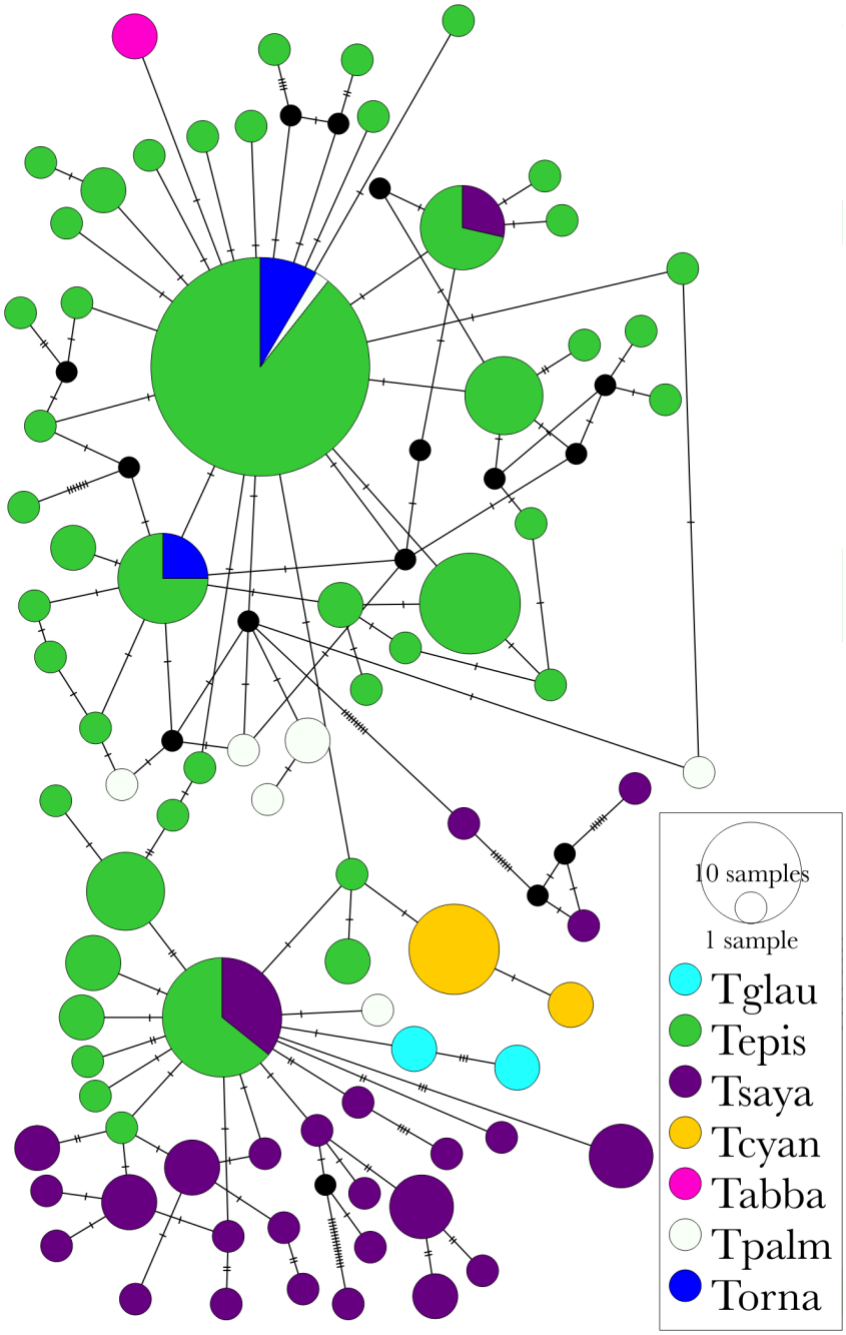

Figure 30. BF5 haplotype network. *T. glaucocolpa* (Tglau), *T. episcopus* (Tepis), *T. sayaca* (Tsaya), *T. cyanoptera* (Tcyan), *T. abbas* (Tabba), *T. palmarum* (Tpalm), *T. ornata* (Torna).

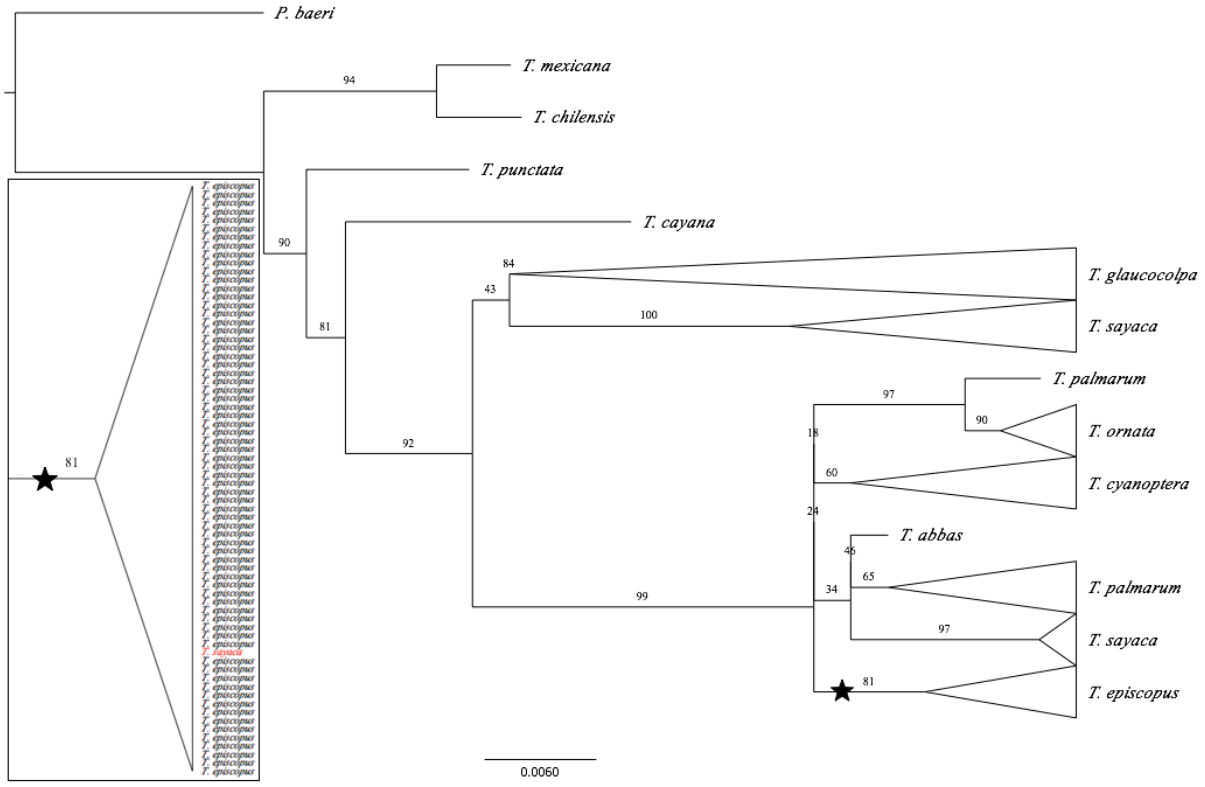

Figure 31. MUSK RAxML tree, of genus *Thraupis*. Bootstrapping values over each branch. The black start marks the clade zoomed in the left. *T. sayaca* within the *T. episcopus* clade (red).

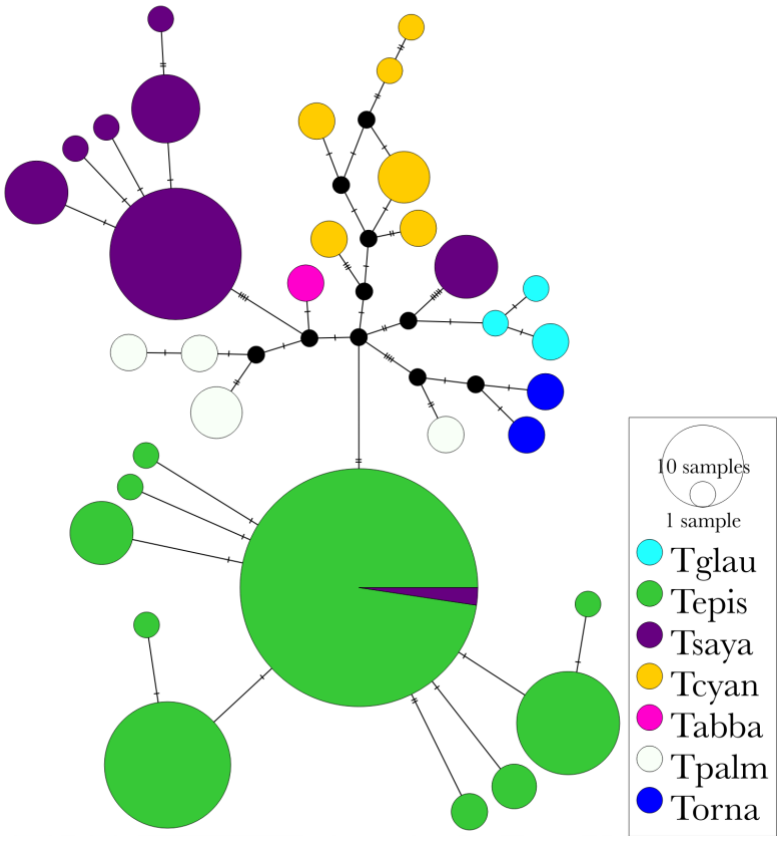

Figure 32. MUSK haplotype network. *T. glaucocolpa* (Tglau), *T. episcopus* (Tepis), *T. sayaca* (Tsaya), *T. cyanoptera* (Tcyan), *T. abbas* (Tabba), *T. palmarum* (Tpalm), *T. ornata* (Torna).

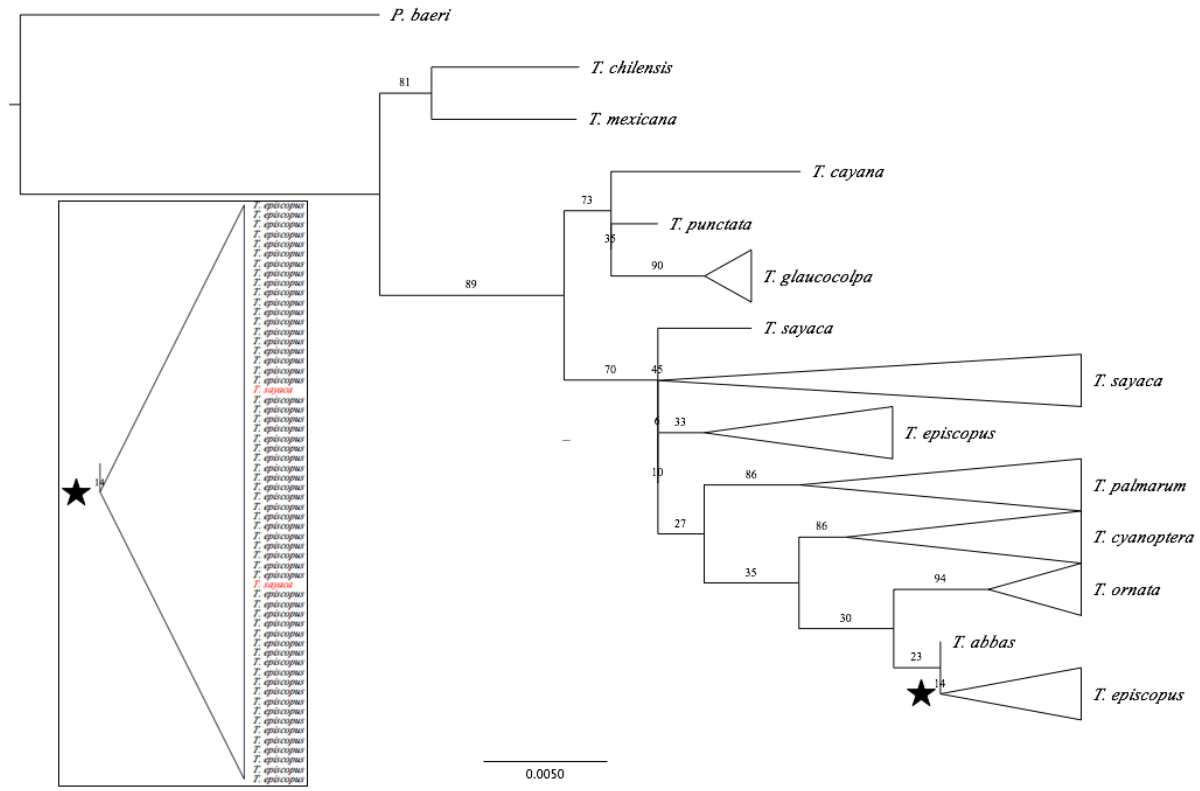

Figure 33. TGFB2 RAxML tree, of genus *Thraupis*. Bootstrapping values over each branch. The black start marks the clade zoomed in the left. *T. sayaca* within the *T. episcopus* clade (red).

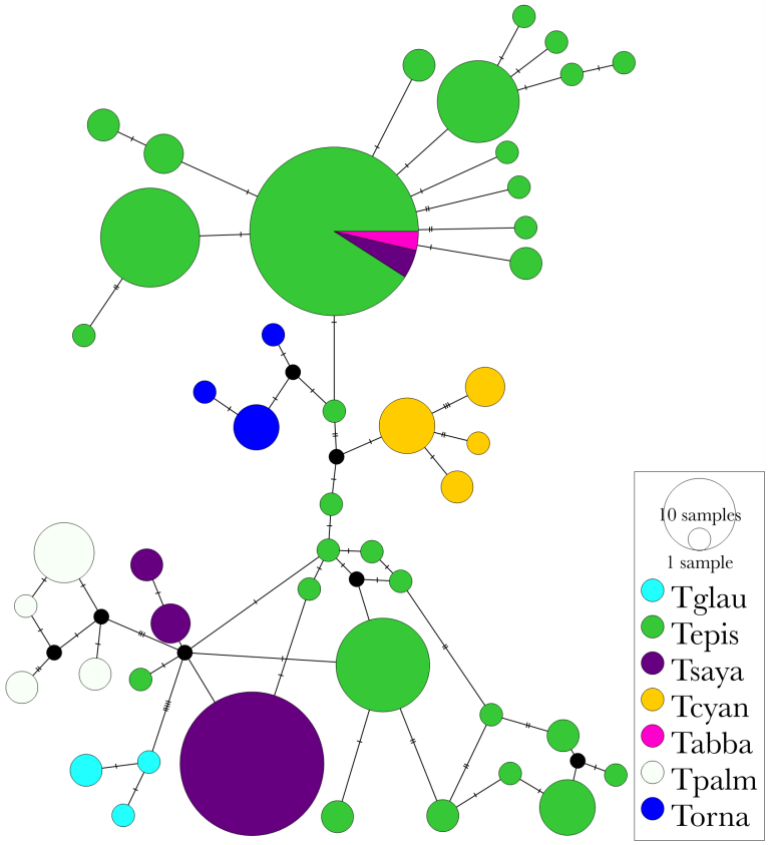

Figure 34. TGFB2 haplotype network. *T. glaucocolpa* (Tglau), *T. episcopus* (Tepis), *T. sayaca* (Tsaya), *T. cyanoptera* (Tcyan), *T. abbas* (Tabba), *T. palmarum* (Tpalm), *T. ornata* (Torna).

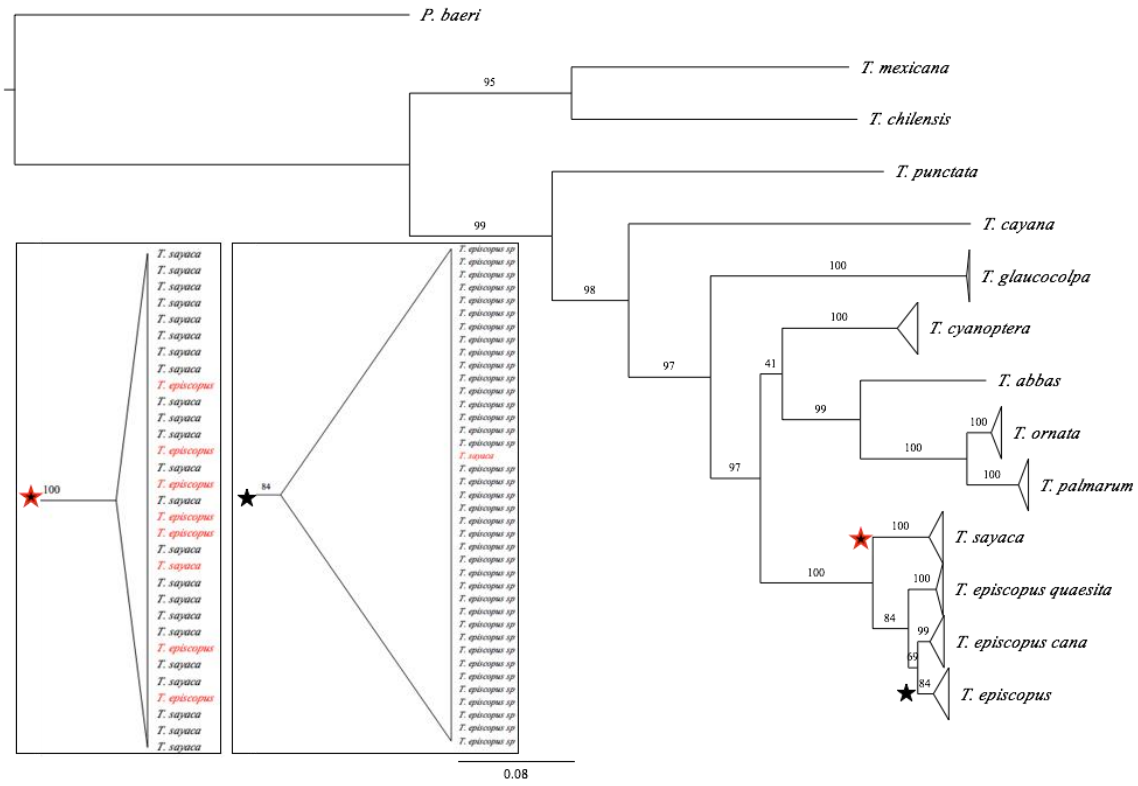

Figure 35. mitochondrial (ND2 AND Cyt-β) RAxML tree, of genus *Thraupis*. Bootstrapping values over each branch. The starts mark the clade zoomed in the left. Taxa within a different clade as expected (red).

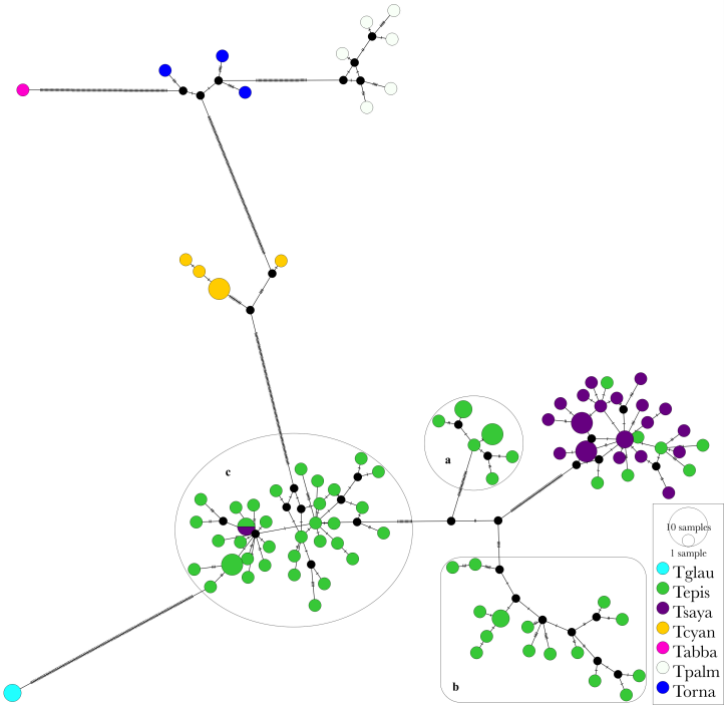

Figure 36. Mitochondrial (ND2 and Cyt-β) haplotype network. a) *T. episcopus quaesita, b) T. episcopus cana* and c) *T. episcopus* sp. *T. glaucocolpa* (Tglau), *T. episcopus* (Tepis), *T. sayaca* (Tsaya), *T. cyanoptera* (Tcyan), *T. abbas* (Tabba), *T. palmarum* (Tpalm), *T. ornata* (Torna).

3.3.2 Species tree inference

I delimited the Taxonomic Units (TU) for the multilocus species tree, mostly based on the suggested information by RAxML gene trees and haplotypes networks and those MU matching this information. Thus, I decided to lump MU 2 and MU 3 into *T. sayaca* and MU 7 and MU 8 into a broad *T. episcopus* because of the small phenotypic variation and the lack of genetic structure. Finally, *T. e. quaesita* and T. e. cana were maintained separate given the supported structure showed in the mitochondrial data.

The resulting tree (Fig. 37) estimates the origin of *Thraupis* between 5.5 and 7.5 million years before present (MYBP) in the Messinian age at the end of the Miocene epoch. It strongly supports *T. glaucocolpa* as the earliest diverging lineage of the genus *Thraupis*, originating about 5.5 MYBP. The clades composed by *T. abbas–T. ornata–T. palamarum* and *T. sayaca–T. episcopus* do not have high posterior probability support (0.89). Thus, *T. cyanoptera* may be closer to any of those two clades, and its phylogenetic position remains unresolved. Additionally, the posterior probability support value for the clade formed by *T. abbas* with *T. ornata–T. palmarum* is also low, suggesting a polytomy between the clades named above, *T. abbas* and *T. cyanoptera*. *Thraupis sayaca* and *T. episcopus* were recovered as sister taxa, and the relationships within *T. episcopus* is congruent with the morphological groupings based on lesser-covert coloration. Both groups *T. episcopus quaesita* (MU 5) and *T. episcopus cana* (MU 6) have a blue color in this area, whereas in other subspecies the coloration is predominantly white.

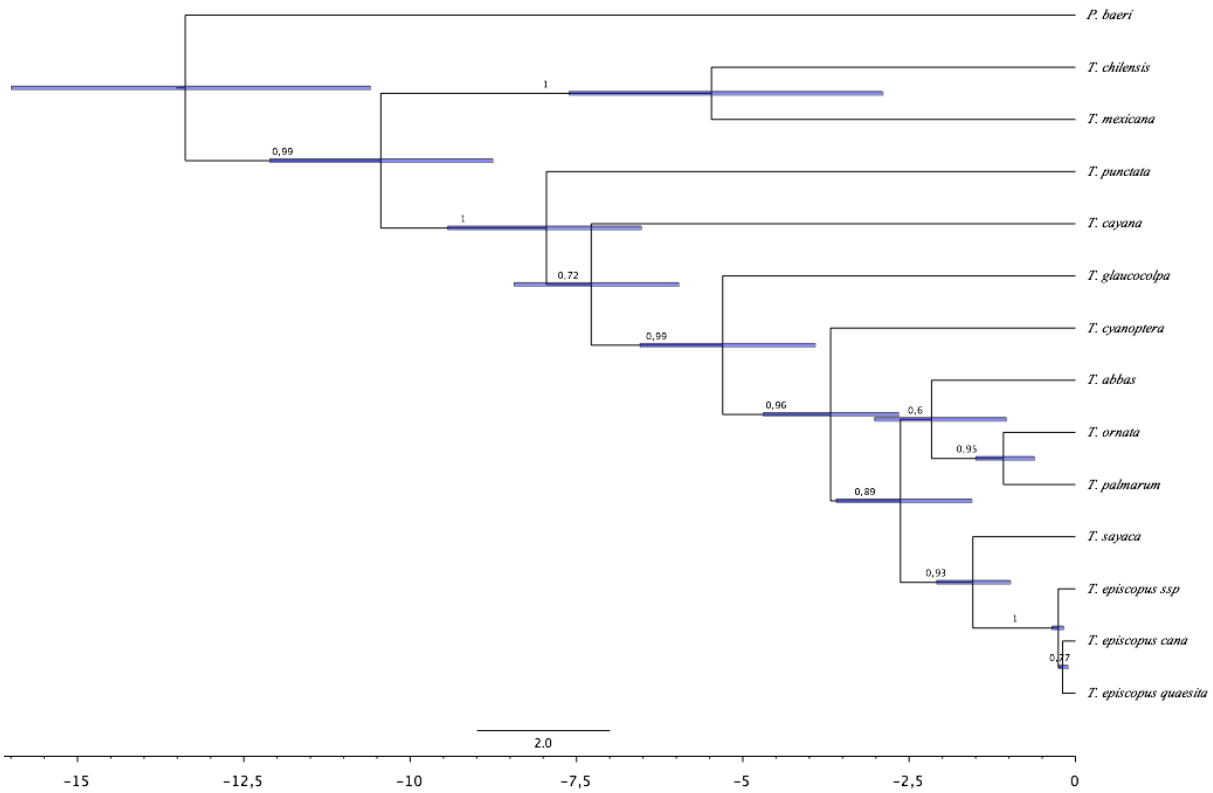

Figure 37. Multilocus time-calibrated species tree using TU base on RAxML gene trees. Posterior probability (above branches), time scale in million years before present and 95% time confidence-interval at each node.

4. Discussion

4.1 Phenotypic variation and Morphometric units

Thraupidae is a family with diversification rate that is 40% greater than the average of the 9-primaried oscines (Barker *et al.* 2013, Burns *et al.* 2014). A lot of this external variance is represented by plumage coloration, sometimes with little genetic divergence (Burns *et al.*, 2014; Campagna *et al.*, 2016). Within *Thraupis* I did not find much morphometric variation, as showed by the PCA and LDA. Morphometric variation is small and the most distinctive group was *T. cyanoptera* (Fig. 27), which was expected due to its bigger size and distinctive appearance (Naumburg, 1924; Hilty, 2011). The other groups are not diagnosable. I consider that exploring more measurements as bill width (nares), bill height (nares), 1st and 9th primary feathers, 1st secondary feather, external and internal tail feathers and include new/alternative technologies, will permit to separate and diagnose others MUs/TUs. Besides, it will increase the number of variables for linear model to estimate the weight, probably decreasing the error within the estimation.

On the other hand, coloration is really informative and within the genus *Thraupis* the most informative color character is the coloration of the lesser coverts. As mentioned above, it allows recognition of seven out of the ten MU and permits the diagnosis of almost all TU used in the species tree with the exception of *T. episcopus cana* that has a really similar color, when compared with *T. episcopus quaesita*. It is possible that the color of the patch formed by the lesser-coverts has an importance in reproductive communication. It has been described the importance of colors for communication in birds and how it may become a sexual barrier depending on the females preferences, pushing rapid differentiation and a partial isolation (Barrera-guzman *et al.*, 2017). In a group with a high color diversity as Thraupidae, it is expected that color characters are related with speciation process (Hilty, 2011). I consider important to highlight quantitative methods. Color catalogues can be an important method but in cases as *Thraupis*, in which some areas show iridescent colors, it becomes difficult and ambiguous. There are new techniques that permit us to have a wider view of bird's colors and its evolution. Some of them are spectrometry, scanning electron microscope and transmission electron microscope. It will permit us not only to quantify colors but study structural colors as

the ones in hummingbirds and some tanagers. Together with next generation sequencing will highly improve our knowledge about tropical birds (e.g. Barrera-guzman *et al.* 2017).

4. 2 Molecular analyses

The gene trees, haplotypes networks and species tree had interesting results. Maybe the most surprising one is the phylogentic relationship of *T. glaucocolpa* with the genus. It is the most divergent lineage in *Thraupis*. *Thraupis glaucocolpa* shares some similarities with *T. sayaca* as biome selection and overall plumage coloration (eventhough their colors are not the same, they are similar in some degree). Also, they are the only TU with gray-brown crowns. Because of similarities both species were considered as conspecific (Hellmayr, 1936) or they were believed to be sister taxa (Sedano and Burns, 2010; Burns *et al.*, 2014; Burns, Unitt and Mason, 2016). The fact that these species exhibit similar colors and inhabit comparable biomes suggest several hypothetical scenarios. First, color convergence, color adaptation to similar environmental conditions such as solar radiation, temperature, and predature pressure (e.g. Monge-Najera & Hernandez 1994). Second, it might represent a plesiomorphic trait. It is possible that if we consider glaciations and climate oscillations during the Pliocene and Pleistocene (Potts *et al.*, 1992), expansion and contraction of biomes as the Moist Forests and Grasslands might have favor the maintenance of this plumage coloration pattern and so currently observed colors and biome selection may be the legacy of a *Thraupis* common ancestor. A widely-distributed *Thraupis* in an expanded savannah and posteriorly split and isolated because the expansion of the Moist Forests. Having genomic sampling might prove useful to test these scenarios. Also, this will allow finding conserved-loci related to coloration in both species and assess how they had change in the other species of the genus.

Besides the phylogenetic position of *T. glaucocolpa*, other relationships such as *T. sayaca* with *T. episcopus* and *T. ornata* with *T. palmarum* were expected, because of previous results from other researchers (Burns *et al.*, 2014). However, the phylogenetic relationships of *T. cyanoptera* and *T. abbas* is not well supported within the genus. It is necessary a larger dataset to resolve the phylogenetic position of this species.

The other taxon with low support is *T. abbas*. When using the mitochondrial markers, this species is clearly the sister taxon of the clade *T. palmarum–T. ornata*. But in the species

tree that includes nuclear introns, that support is low. I consider, that this discordance is related with the fact that mitochondrial DNA is only heredity from the female parent and it does not recombine as nuclear DNA. Nuclear introns may be influenced by the gene flow between species. The TGFB2 haplotype network (Fig. 34) and the skin MCZ 163160 (Fig. 14c) suggests that introgression between *T. abbas* and *T. episcopus* is an option. It may happen in a lower rate that within *T. episcopus* and *T. sayaca* (this issue will be discussed later), but gene flow between *T. episcopus* and *T. abbas* will affected the results, certainly.
Even some phylogenetic relationships were found and others confirmed. It is necessary to increase the number of samples but specially the markers level sample. Probably with the use of genome-reduction technologies such as RADseq (Peterson *et al.*, 2012) we can improve the phylogenetic hypothesis within *Thraupis* and have a better knowledge about introgression process and its effects on evolution and speciation.

4.3 Hybrids, speciation and extinction

Here I present compelling evidence of introgression at different levels. The first level is between subspecies with different morphologies: *T. episcopus cana* and *T. episcopus* (several subspecies from Amazon Moist Forest). They hybridize in the Grasslands of Colombia and Venezuela. Birds from this area has different tones of purple. The second level is between sister species: *T. episcopus* and *T. sayaca* along the Amazonia-Cerrado ecotone in the Brazilian Shield. The hybrids from this area have little white or lighter Turquoise Blue colors on the lesser-coverts (Fig. 14a, b) Finally, I found evidence of *T. cana* with *T. abbas*. A complete purple individual (Fig. 14c) that also shows back-plumage marks similar to *T. abbas*.

From all those levels, the one I want to highlight is the one between *T. episcopus* and *T. sayaca*. Both species have a genotypic and phenotypic structure and differentiation. When plotting the individuals recovered in gene trees recovered in different clades from the "conspecifics" (Fig. 30-32, red), it is clear that most of the points (except two) are located along the ecotone between Amazonia and drier biomes (Cerrado, Caatinga, Chaco) and the overlapping area between the two-species distribution (Fig. 38a). The two northern points in the middle of Amazonia are located in places with obvious marks of deforestation (Fig. 38b).

The habitat had been turn into artificial grasslands, a habitat that resemblance in some level the natural Grasslands, where *T. sayaca* distributes naturally.

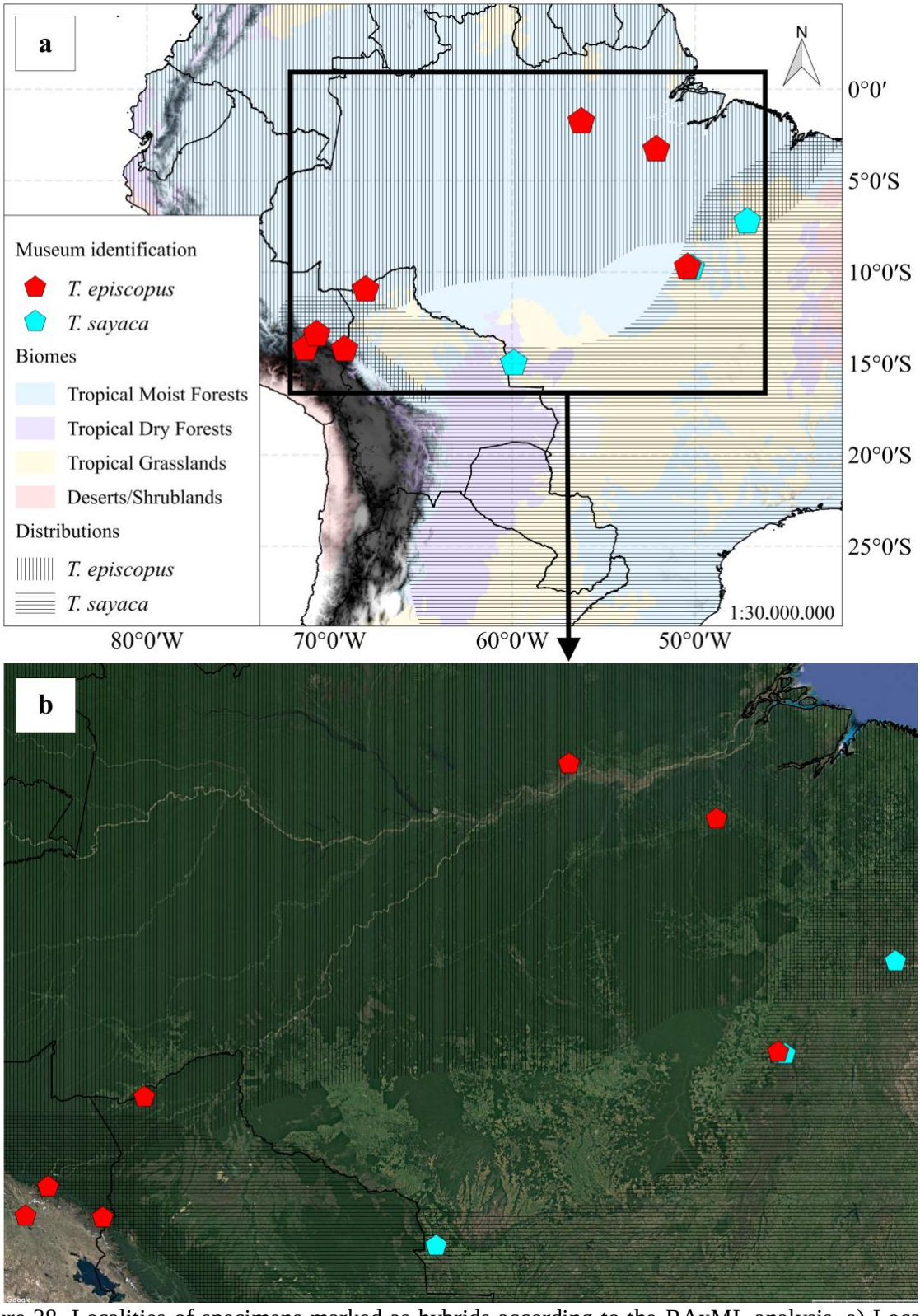

Figure 38. Localities of specimens marked as hybrids according to the RAxML analysis. a) Location of the points related to ecotone and species contact area. b) Satellite image showing current vegetation and the relation of the northern points with deforestation.

According to the biological species concept, hybridization is thought to be the antagonist of speciation, by merging the parental species and permitting the homogenization of gene pools of different populations (Wolf and Ellegren, 2016). Anyway, several recent publications agree that isolation with migration (horizontal gen flow) may occur and even accelerate the speciation process by increasing the genetic material for later selection or by sharing important traits that increase the fitnets (Dasmahapatra *et al.*, 2012; Martin *et al.*, 2013; Barrera-guzman *et al.*, 2017). Hybridization has generated a new research field in genomic biology. We used to believe most of species evolve by allopatric speciation. But recent researches present strong evidence that support speciation process with gen flow (Grant *et al.*, 2004; Dasmahapatra *et al.*, 2012; Martin *et al.*, 2013; Abbott, Barton and Good, 2016). Also, there is evidence of completely new species produced by hybridization process, with a posterior isolation from the parental species.

This isolation may be a geographic barrier, a genetic barrier or behavioral, as sexual selection (Presgraves, 2010; Barrera-guzman *et al.*, 2017; Lamichhaney *et al.*, 2017). Probably, introgression between *Thraupis* populations responsible for the conflicts in the phylogenetic relations within the genus, but at the same time it may be one of the engines driving rapid diversification of the genus. Other vectors that may contributed to the speciation process within *Thraupis* are geographic barriers and Pleistocene refuges (Behling, 1998). Abiotic phenomena as decreasing temperature generates the retraction and extension of the forests and dry areas. For existing animals as *Thraupis*, conquering new biomes may permit ecological isolation between populations that hybridize only in the ecotones areas. Also, the expansion of Moist Forests or Grasslands may act as barriers separating expanded populations. Another barrier are the Andes. When rising, they partially cut the gen flow and may permit that a faster selection than introgression selects a different morphotypes at different slopes, as *T. episcopus cana* and *T. episcopus* (MU 7).

Finally, I want to discuss the two northern individuals marked as hybrids that appear in the middle of Amazon Tropical Mist Forest (Fig. 38b). As shown in the image, the two points belong to deforested areas that permit entrance of several factors not native from the Amazon. I believe one of this factor is *T. sayaca*. Some individuals may reach this regions by following the deforestation path. This is the most probable reason to find hybrids this far within Amazonia. Hybridization can not only increase speciation, but also extinction risk. The introgression equilibrium between *T. episcopus* and *T. sayaca*, seems to be given by the ecological differentiation in the ecotone (Burkle, Wolf and Rieseberg, 2003). Nevertheless,

deforestation has allowed human-induced hybridization breaking this equilibrium (Allendorf *et al.*, 2001). Fortunately, *Thraupis* species are currently widely distributed birds with large populations far from any extinction risk. But, species with smaller and restricted distributions merging it within another species or by breeding infertile chicks that will consume energy but will not add anything to the population dynamics, decreasing the fitness. Hybridization is an interesting phenomenon to explore within *Thraupis* and as I show, it may have evolutionary, ecological and conservation repercussions. Nevertheless, is require jumping to a new type of data, in a genomic scale, to explore these new options and use new models that accept migration across taxa.

4.4 Species delimitation and taxonomic proposal.

Here I suggest *T. glaucocolpa*, *T. sayaca*, *T. episcopus* and *T. cana* as valid species. *Thraupis glaucocolpa,* even having some morphological and ecological similarities with *T. sayaca* (grey and turquoise colors and dry biomes selection), is the oldest lineage and there is no evidence of hybridization with any other taxon. Also, it has the most distinctive song among all species in the complex when heard in the field. Song differentiation is a good evidence of phylogenetic differentiation, because of its relationship with reproductive isolation (Mason *et al.*, 2016).

The other three taxa, *T. sayaca*, *T. episcopus* and *T. cana* are less conspicuous due to the continuous hybridization between them. These taxa are still in the gray zone described by de Queiroz (2007). However, despite evidences of gene flow, species limits seem to be maintained due to ecological differences in ecotones and geographic barriers as the eastern Andes and biological competition. *Thraupis episcopus quaesita* is here synonymized in *T. cana* due the similar overall coloration and because the species tree (Fig. 37) recovered closer to *T. cana* than *T. episcopus* (eastern subspecies).

Thraupis sayaca obscura and *T. episcopus coelestis* are also synonymized in their nominate species because their differences seems to be clinal and because both species present a high phenotypical variance. The differentiation of *T. s. obscura* is subtle, and the subspecies is isolated after a big gap without samples in Bolivia and northern Argentina. In *T. episcopus episcopus* the blue in the crown becomes shinier as we move to west, similar to the

amount of white.

The subspecies *T. episcopus major*, *T. episcopus urumbambae* and *T. sayaca boliviana* are described based on specimens collected across the hybrid area located in the north and northwest limits of Bolivia with Brazil and Peru (Fig. 38). The subspecies *T. sayaca boliviana* was originally described as *T. episcopus*, and already show something about the doubts in the identification. Also, the author described other specimens of *T. sayaca obscura* collected in the same region, and he talks about intermediate specimens between *T. sayaca boliviana* and *T. episcopis major*. I analyzed pictures of the type specimen of *T. episcopus urumbambae* and specimens of *T. episcopus major* and together with the information collected here I recognize this subspecies as result of hybridization. Hybrids may show intermediate states, or not and in some cases completely different and new states (Barrera-guzman *et al.*, 2017). Also had been described that intermediates individuals may be larger that both parental species (McCarthy, 2006). Moreover, all this phenotypic variation increases when hybrids hybridize in F2 individuals from F1 hybrids and the parental species. The description of this subspecies is the result of punctual collections in a hybrid zone.

Besides *T. episcopus cana* and *T. episcopus quaestia*, there are two more subspecies that belong to this group. *T. episcopus caesitia* from Escudo de Veraguas island, Panamá and *T. episcopus cumatilis* from Coiba island, Panamá. Unfortunately, I only accessed tissues of *T. episcopus cumatilis.* I did not found genetic differences between the samples from Coiba island and the samples of *T. episcopus cana*. I did not measure specimens from this locality but receive photographs from the type, and I did not found differences between *T. episcopus cana* (closer continental subspecies) and *T. episcopus cumatilis*. Also, I received pictures of the type specimen of *T. episcopus caesitia* seems to have a bigger and longer beak. It is necessary to included morphometric data before taking farther decisions. Finally, the subspecies *T. episcopus nesophila* is also a hybrid between *T. episcopus (*MU 7) and *T. episcopus cana*. The subspecies is located in the llanos, between the distributions of both species. Besides, it is possible to find all degrees of intermediates between birds with white and blue lesser-coverts. The original description also mentioned that it seems to look as an intermediate between the species (Berlepsch, 1880).

To finish, I want to expose a special case I found in Trinidad and Tobago. The hybrid zone between *T. episcopus* (MU 7) and *T. episcopus cana* is located in the llanos of Colombia and Venezuela (Fig. 10d). Front to the hybrid zone are the islands of Trinidad and Tobago. In Trinidad and Tobago was described the subspecies *T. episcopus berlepschi*. Even the

subspecies is genetically (according to the five markers sequenced) identical as the hybrids (*T. episcopus nesophila*) and as the birds to the east slope of the Andes (*T. episcopus episcopus*). It has a clearly differentiate morphotype, with a shiny blue on chest and rump (Fig. 13j). The presence of purple lesser-coverts takes me to think that the island was conquer by hybrids and not for the parental species and the stable morphotype makes me think we have a speciation process by hybridization occurring. Nevertheless, it is a hypothesis and it needs to be tested. We know it is possible to produce a total new species from hybridization, as long presents a type of reproductive isolation (Barrera-guzman *et al.,* 2017; Lamichhaney *et al.,* 2017). I think the distance of the island to the continental grounds may be generating a partial isolation. Are the birds from Trinidad and Tobago descendants of hybrids in Venezuela? Is it a new species forming from hybridization? How much isolation is needed after hybridization to produce a new species? There are several questions I did not have the methods to answer. Hope that with New Sequencing Generation I can address them. Until I get the tools to answer this question I prefer to be conservative and keep this taxa as a subspecies of *T. episcopus*.

Following the unified species concept (de Queiroz, 2007), color traits, morphometric and molecular data. I propose the following taxonomic arrangement for the *Thraupis episcopus – Thraupis sayaca – Thraupis glaucocolpa* species complex:

Thraupis glaucocolpa Cabanis, 1850: Type from Caracas, Venezuela. Housed by the Museum Heineanum Halberstadt, Halberstadt Germany under the catalogue number 863. It is distributed on dry areas in the northern South America. This group is the oldest lineage in the genus *Thraupis* (Fig. 37) and exhibits Turquoise Green plumage on chest and Turquoise Blue lesser on the wing coverts (Fig. 13d).

Thraupis sayaca (Linnaeus, 1766): Suggested type locality: Pernambuco, Brazil (Naumburg, 1924; Hellmayr, 1936) based on “Sayacu” of Marcgrave (Hellmayr, 1936). This taxon is mainly distributed in dry areas in northern Argentina, eastern Bolivia, Paraguay and southern, central and northeastern Brazil. Besides habitat differences from most taxa in *Thraupis*, this taxon has the back and crown Brownish Olive and lesser coverts Smoke Gray chest and Paris Green (Fig. 13b, c).

Thraupis cana (Swainson, 1841): Suggested type locality: Venezuela (Hellmayr, 1936). Type housed by Swainson Collection (Hellmayr, 1936). It is distributed on several biomes on the west of the Andes, including northern Colombia and northern Venezuela. Within the complex, it is the only taxon with blue lesser coverts (Fig. 13e, f).

Thraupis episcopus (Linnaeus, 1766): Suggested type locality: Cayenne (Hellmayr, 1936). Based on "L'Evesque" Brisson (Hellmayr, 1936). Due to geographic differentiation, I propose to synonymize the subspecies *T. episcopus mediana* Zimmer, 1944; *T. episcopus ehrenreichi* Reichenow, 1915; *T. episcopus leucoptera* (Sclater, 1886) into *T. episcopus*. It is distributed in the Tropical Moist-Forest of Amazonia. It is the only taxon with white lesser-coverts and it is one of the subgroups in the mitochondrial gene tree and haplotype network. This taxon has a large phenotypic variation without a geographic structure. Different individuals exhibit different amount of white in the lesser- and greater-coverts. The crown and chest varies from a Light Sky Blue to a Venetian Blue with some iridescent blue tint. (Fig. 13g, h). This species is in contact a there is strong evidence that support introgression with *T. sayaca* and *T. cana*. So, morphotypes with size and color variation may become from the hybridization with those species (McCarthy, 2006).

As mentioned before, several specimens show intermediate states in morphology and genetics, presenting high evidence of introgression. Subspecies as *T. episcopus major*, *T. episcopus urumbabae* and *T. sayaca boliviana* are highly probably a product of this introgression, this issue was mentioned before by several authors, including the original descriptions (Berlepsch, 1880; Bond and de Schauensee, 1941; McCarthy, 2006). It means they are not valid taxa.

5. Conclusions

The *Thraupis glaucocolpa – T. sayaca – T. episcopus* species complex is composed by four species. Adding to the aforementioned taxa we included *T. cana* (Swainson, 1834), elevated from subspecies to the species level.

T. glaucocolpa is the oldest lineage within *Thraupis;* the phylogenetic relationships between *T. cyanoptera, T. abbas, T. palmarum, T. ornata, T. sayaca, T. episcopus* and *T. cana* were investigated in detail.

We found introgression within the representatives of the genus *Thraupis* at different levels and between distinct species.

It is necessary to sample at genome level to clarify the phylogenetic relationships and hybridization process in Cerrado-Amazon ecotone, Colombian–Venezuelan Grasslands, and the possible speciation by hybridization in Trinidad and Tobago.

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