Early patterns of Andean diversification, genomic and color patterns variation across the *Metallura tyrianthina* complex of the Northern Andes

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SUMMARY

The highly variable topography, the climatic history and the great variety of niches of the Andes mountain range have had major contributions to the formation and maintenance of the genetic and morphological diversity of tropical birds. The main causes of divergence promoted by the Andes are attributed to the accumulation of mutations by genetic drift in populations that are reproductively isolated by geographic accidents, and rapid divergence events mediated by adaptation to ecological niches. Studying early evolutive patterns of diversification has shown promise for understanding the role of the Andes mountain behind speciation. Therefore, I propose to study the evolution of the *Metallura tyrianthina* subspecies which had a rapid diversification in the Andes that resulted in seven subspecies with variable coloration. Here, we corroborate and extend previous studies on the evolution of the Tyrian Metaltail in the northern Andes, using reduced genome (NextRAD) and quantitative coloration assessments for finding phyllogenetic, genetic, demographic and morphologic evidence of two early divergence with gene flow processes in the same species. First, evidence showed that Quaternary glacial cycles may have played a major role in the recent foundation of the Sierra Nevada mountain and a posterior rapid divergence event between populations of *Metallura tyrianthina districta,* suggesting a new subspecies for the complex. Also, evidence points towards another divergence pattern between eastern and western populations of *Metallura tyrianthina tyrianthina* that was subtle and not explained by the topography nor isolation by distance.

RESUMEN

La topografía altamente variable, la historia climática y la gran variedad de nichos de la cordillera de los andes ha contribuido a la formación y mantenimiento de la asombrosa diversidad genética y morfológica de las aves tropicales, promoviendo dos mecanismos de especiación. El primero consiste en la acumulación de mutaciones por deriva genética en poblaciónes aisladas reproductivamente por la geografía y/o por su ecología. El segundo patron consiste en eventos de divergencia rápida, mediada por la adaptación a sus diversos nichos ecológicos. No obstante, poco se sabe sobre los procesos que ocurren en estadíos tempranos de la especiación. De tal forma que propongo estudiar la evolución del complejo de subspecies de *Metallura tyrianthina,* ya que ejemplifica un evento de diversificación rápida en los Andes que resultó en siete subespecies con coloración variable. En este estudio corroboramos y ampliamos los hallazgos de estudios previos sobre evolución del colibrí Metallura Tyria en Colombia, mediante un muestreo reducido del genoma (NextRAD) y valoraciones cuantitativas de coloración. Esto nos permitió encontrar evidencia significativa de procesos de divergencia temprana en presencia de flujo genético. En primer lugar, la evidencia mostró que los ciclos glaciales del Cuaternario pudieron haber jugado un papel importante en la reciente colonización de la Sierra Nevada y un posterior evento de divergencia rápida entre las poblaciones de *Metallura tyrianthina districta*, hallando una nueva subespecie para el complejo. Además, la evidencia apunta hacia otro patrón de divergencia en el mismo sistema, entre las poblaciones oriental y occidental de Metallura tyrianthina tyrianthina que fue sutil y no se explica por la topografía ni el aislamiento por distancia.

INTRODUCTION

One of the most striking areas of research in evolutionary biology is the understanding of the natural mechanisms that promote population divergence, and how new species arise. Speciation has been widely studied across the tree of life, and strong evidence supports the mutation order speciation mechanism, where the accumulation of novel mutations by genetic drift or selective pressures causes divergence of geographically and/or ecological distant populations over time (1,2). In some cases, topographical or ecological factors, or both, burst the formation of several lineages in short periods of time associated with adaptation to different habitats, forming species via isolation by adaptation (3,4). In tropical birds of the new world, altitudinal and latitudinal geographic isolation across the Andean cordillera is correlated with phylogenetic splits, suggesting that topography has an important role in species formation (5). These ideas are not new; several authors such as Frank Chapman in 1917, thought about the importance of Andean cordillera in shaping and producing diversity (6). Since then, fruitful research has been done for establishing the role of geographical accidents in species formation suggesting that strong genetic structure and plumage differences are a consequence of allopatric speciation with dispersal and colonization events (7–12). Nevertheless, geological and palynological data suggest that the Andes mountain range could be older than the dates obtained with molecular phylogenies (13). Consequently, past climate oscillations and colonization via dispersion could be major factors in the formation of andean species (9,10) but it remains debatable (14).

Most studies tested the importance of topographical/geographical events in diversification at the interspecific level, ignoring the effect of such events in early stages of speciation (i.e subspecies) (15,16). Therefore, it is fundamental to study how the Andes mountain chain itself, or the differential adaptation to its niches, casts the genetic and morphologic variation at the intraspecific level. Here, I conducted a genetic and morphologic study on the Colombian subspecies of the hummingbird *Metallura tyrianthina* to establish the role of the Andes in its diversification. This striking bird is endemic to the Andes and show sexual dimorphism where the females are green, white and ocre, while males are almost uniformly vibrant green. Amazingly, male tail plumage (rectrices) show a range of colors that vary geographically between a palette of metallic green, blue, purple, rufous and blue (17). There are seven subspecies occurring on a broad altitudinal range (2100 to 3500 mamsl) described along the Andes, and two of them are distributed in Colombia where they populate all three cordilleras (EC: Eastern Cordillera, CC: Central cordillera and WC: Western Cordillera) and inhabit the Sierra Nevada de Santa Marta mountain (nomenclature follows 18).

Andean topography's effect on the evolutive patterns of *Metallura tyrianthina* has been assessed before on Ecuadorian, Bolivian and Peruvian populations using a qualitative approach , where the importance of this species as a model was highlighted for understanding diversification in the Andes, due to its striking color variations along a wide altitudinal and latitudinal distribution on this mountain complex (17). Later, descriptions of the biogeographic history of *Metallura* genus found recent (1.5 mya) and wide diversification of the 7 subspecies of *Metallura tyrianthina* (19,20). Nevertheless, the nuclear and mitochondrial markers they used could not resolve entirely the phylogenetic relationships within Metallura and, consequently, *Metallura iracunda* remained part of the *Metallura tyrianthina* complex. However, based on this phylogenetic hypothesis, the authors suggest that dispersion and migration events promoted by the Quaternary climatic oscillations before the last glacial maxima, shaped the evolution of *Metallura*.

Here, NextRad SNP data and quantitative measures of color were used to answer the following questions: Are the Colombian subspecies discrete geographic entities as suggested by their morphology? How much gene flow exists between them? Do sympatric populations have higher divergence than allopatric (or the contrary)? What is the role of Andes Cordillera in the formation of these subspecies? And finally, is there evidence of novel speciation events in the system?

MATERIALS AND METHODS

Genetic sampling

I extracted total DNA products from 86 muscle tissue subsamples from Instituto Alexander von Humboldt (IAvH), covering 77 individuals of *M. tyrianthina*, 6 for *M. iracunda* and 3 *M. williami* outgroup that some of the tissues were resampled from Colombian tissues included in (20). For DNA extraction, we followed the magnetic beads protocol (21) and the product was quality verified using fluorometry (Qubit, thermofisher) after electrophoresis on agarose 1% gel. Posteriorly, Genomic DNA was first fragmented with Nextera reagent (Illumina, Inc), which also ligates short adapter sequences to the ends of the fragments. The Nextera reaction was scaled for fragmenting 30 ng of genomic DNA, although 45 ng of genomic DNA was used for input to compensate for degraded DNA in the samples and to increase fragment sizes. Fragmented DNA was then amplified for 27 cycles at 74 degrees, with one of the primers matching the adapter and extending 10 nucleotides into the genomic DNA with the selective sequence GTGTAGAGCC. Thus, only fragments starting with a sequence that can be hybridized by the selective sequence of the primer will be efficiently amplified. The nextRAD libraries were sequenced on a HiSeq 4000 with one lane of 150 bp reads (University of Oregon).

The genotyping analysis used custom scripts (SNPsaurus, LLC) that trimmed the reads using bbduk (BBMap tools, http://sourceforge.net/projects/bbmap/):

```
bbmap/bbduk.sh in=reads/run_4447/CATACGTA-CTAAGCCT_S96_L007_R1_001_subset.fastq.gz
out=reads/run_4447/CATACGTA-CTAAGCCT_S96_L007_R1_001_t.fastq.gz ktrim=r k=17 hdist=1 mink=8
ref=bbmap/resources/nextera.fa.gz minlen=100 ow=t qtrim=r trimq=10
```

Next, a de novo reference was created by collecting 10 million reads in total, evenly from the samples, and excluding reads that had counts fewer than 7 or more than 700. The remaining loci were then aligned to each other to identify allelic loci and collapse allelic haplotypes to a single representative. All reads were mapped to the reference with an alignment identity threshold of .95 using bbmap (BBMap tools). Genotype calling was done using callvariants (BBMap tools) (`callvariants.sh list=ref_hummingbird_rm.txt.align_samples out=hummingbird_total.vcf ref=ref_hummingbird.fasta ploidy=2 multisample=t rarity=0.05 minallelefraction=0.05 usebias=f ow=t nopassdot=f minedistmax=5 minedist=5 minavgmapq=15 minreadmapq=15 minstrandratio=0.0 strandedcov=t`). The vcf was filtered to remove alleles with a population frequency of less than 3%. Lastly, we applied VCFtools commands for three additional filters, in order to extract only biallelic variants with 70% representation on all samples and removed samples with missing data over the 50% threshold.

Phylogenetic reconstruction and Population Analyses

We estimated a phylogenetic hypothesis using all samples from *M. tyrianthina* and *M. iracunda*, with *M. williami* as an outgroup. The reconstruction was made implementing the IQ-Tree plugin (22), which established the most probable relationships among clades with maximum likelihood. The parameters were the following: node support was evaluated over 20000 bootstraps, auto detect threads option for optimization, the most probable molecular evolution model using the Modelfinder option and last, I corrected for Ascertainment bias adding the option to the best model found (i.e: MFP+ASC).

In order to delimitate populations for these analyses, we first applied the FineRADstructure tool (23) to obtain the coancestry matrix and an ad-hoc tree for reviewing the genetic structure and ancestry relationships between clades. Posteriorly, using VCFtools and PLINK we sampled one random SNPs per tag and removed SNPs outside the HW equilibrium for identifying clade clustering from shared ancestral haplotypes using the assignment method ADMIXTURE (24). Summary population statistics per loci were calculated using BCFtools commands (25). We obtained estimations for nucleotide diversity (**π**), Tajima's D (**D**) per loci, that were posteriorly summarized by populations and pairwise relative divergence (**Fst**) between populations.

Genetic differentiation is usually consistent with a pattern of isolation by geographic distance (IBD). However selective processes, idiosyncratic ecology, geographical accidents or a combination between the three, may accelerate divergence while impeding genetic exchange between nearby populations. For this, I ran a Mantel test for the calculated geographic distance between populations, corrected by altitudinal variation using the topoDistance R package (26), and tested for correlations with the genetic distance from the FST values. Complementary to the Mantel test, I used the EEMS method (27) for illustrating the effect of geography on effective migration rates relating to the genetic dissimilarities between *M. tyrianthina* samples.

Demographic modelling and Coalescence parameters

Based on the genetic clusters obtained from the phylogenetic tree and structure analyses, we performed demographic model comparisons between pairs to estimate divergence times, rate and strength of migration between them using the composite-likelihood method fastsimcoal2 (28) using the folded joint Site frequency spectra (SFS) from four *Metallura tyrianthina* populations (SNSM districta, Perijá districta, Eastern tyrianthina, Western tyrianthina). In order to obtain unbiased results on our SFSs, the VCF file was previously pruned for highly linked SNPs and from loci with missing data; the scripts were made by Joana Meier and are available on GitHub: Speciation & Population Genomics: a how-to-guide. Keeping in mind our main research question of how the Andes shaped the Colombian populations, we tested three pairs of populations (i.e: *districta* SNSM vs Perijá, Eastern *tyrianthina* vs Perijá *districta* and Eastern vs western *tyrianthina*) on 5 demographic scenarios (see Fig. 1) which may give insight on how (and when) the andean highlands and lowlands acted as barriers or passes affecting the diversification patterns of this hummingbird. The models are described as follows: Strict divergence (no gene flow at all, Fig. 1A), Constant gene flow over time (Fig. 1B), early gene flow (Fig. 1C), Recent gene Flow (secondary contact scenario; Fig. 1D), Divergence under asymmetric genetic flow (unequal number of migrants; Fig. 1E). To improve the performance of parameter estimates from the SFS, the parameter files were established following the fsc27 manual and other studies with similar approaches as (29,30). Complementary to models, we included an estimate for substitution rate value, assuming two broods per year (4.5×10^{-9} ss/year), taking in consideration a study on reproductive ecology of hummingbirds (31) and the other on the evolution of whole avian genomes (32), and set a median value of 0.00225 site substitutions per million years for vocal learning birds such as hummingbirds. Posteriorly, the best runs of each model were selected evaluating the fittest of the observed likelihoods, and then, we were able to select the best model from the lowest AIC values calculated. Finally, bootstrapping was necessary to obtain confidence intervals of the estimated parameters on the best models.

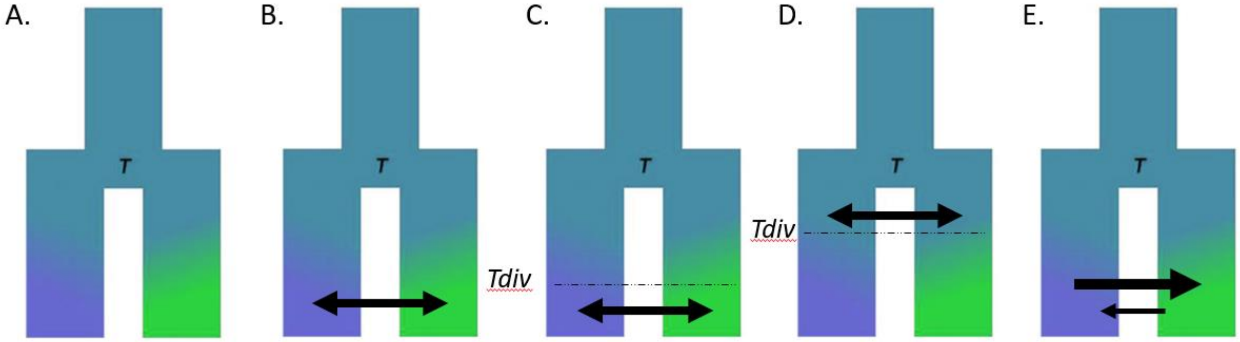

Figure 1. Demographic scenarios tested on FastSimcoal2 for each pair of populations

Colorimetric Evaluation

Color was measured quantitatively from individuals deposited on the IAvH and natural sciences institute (ICN) biological collections (n=210). Our sampling covered both males and females from most of the national territory, including the same Species and individuals sampled in the genomic analyses. Our approach for measuring color on the iridescent plumage of hummingbirds in this study was an adaptation of a protocol designed and implemented by Parra (33), where selected feathers were measured upon three different angles of incident light (45°, 90° y 105°). We used a Deuterium-Halogen induced light source (Ocean optics, DH-2000 BAL), paired with a spectrophotometer (Ocean optics, FlameS) which was connected to a pc and viewed with the Software OceanView. The software extracted reflectance values over the electromagnetic spectrum of three feather patches selected for having reported social selection importance (rectrices, mantle, and throat). We took three measures for each plumage patch corresponding to the incident light angles, resulting in 9 curves of reflectance measures for each specimen. Although the protocol suggested using individual feathers on a flat Surface, we had to extract the color features from the complete feather patches of the specimen to preserve the integrity of the skins and due to museum constraints. For those purposes, we designed a device with a flat Surface that could be adjusted into the desired angles for sampling, enabling consistent measuring between specimens and reducing overall experimental error.

Computational analyses were made in R where spectral data of all specimens was organized by the clades suggested by genetic analyses and consecutively processed with the package PAVO (34). Spectral data was filtered selecting the angle with the highest reflectance values on each plumage patch, for posterior normalization and softening of the curves following the protocol described by (33). Aligned to our hypothesis, we tried to detect discrete morphological groups from the visual system of birds under their habitat normal light conditions. Consequently, we obtained the quantum catches for each photoreceptor implementing the average visual system model of birds under two sets of light conditions described as 1)

Forest shade and 2) Sky blue in order to reduce bias of implementing an artificial light source and detect changes on variation under different habitat light conditions, since *Metallura tyrianthina* is seen mostly on forest edges (35). Quantum catches were posteriorly analysed by calculating the bootstrapped distances between pairs of individuals also known as just noticeable differences (JNDs). For visualizing these results, we extracted the point estimates and 95% confidence intervals of JNDs and plotted a threshold of 1 JND as the theoretical discrimination threshold for a bird visual system. Data was organized by four types of clade comparisons taking sex into account so we would not compare sexually dimorphic males with morphologically distinct females. The comparisons between clades were 1) Northern populations for subspecies *Metallura tyrianthina districta* (SNSM and Perijá) 2) Western and Eastern individuals of *Metallura tyrianthina tyrianthina* 3) *M.t. tyrianthina* and *M.t. districta* subspecies and 4) *Metallura iracunda* vs *M. tyrianthina tyrianthina.*

RESULTS

Phylogenetic reconstruction

My ML hypothesis recovered *Metallura iracunda* as an independent species sister to *Metallura tyrianthina* (BS=100). Both species were reciprocally monophyletic (Fig. 2). We could not recover *Metallura tyrianthina tyrianthina* (green and purple, Fig. 2) as a monophyletic subspecies because *Metallura tyrianthina districta* (blue, Fig. 2) is derived from *M. t. tyrianthina* and was recovered as monophyletic (Fig. 2). This subspecies was closely related with *M. t. tyrianthina* lineages from Norte de Santander (indicated by a star, Fig. 2) although this node is not well supported (BS <90; Fig. 2). Interestingly, *M. districta* individuals fell into two well supported lineages (BS >90, Fig. 2), one of them corresponding to the eastern cordillera population (Perijá) and the other to Sierra Nevada de Santa Marta mountain (SNSM). Finally, populations from Central and Western Andes constitute a clade (BS>90; purple, Fig. 2) sister to the eastern individuals from Caquetá (BS>90, Fig. 2, ★). Based on our phylogenetic reconstruction, assuming this western clade as a single subspecies might be misleading for two reasons 1) The sister lineage of our western clade comes from eastern populations (✱) and 2) Short branch lengths show almost no substitutions per site differentiating eastern and western divergence.

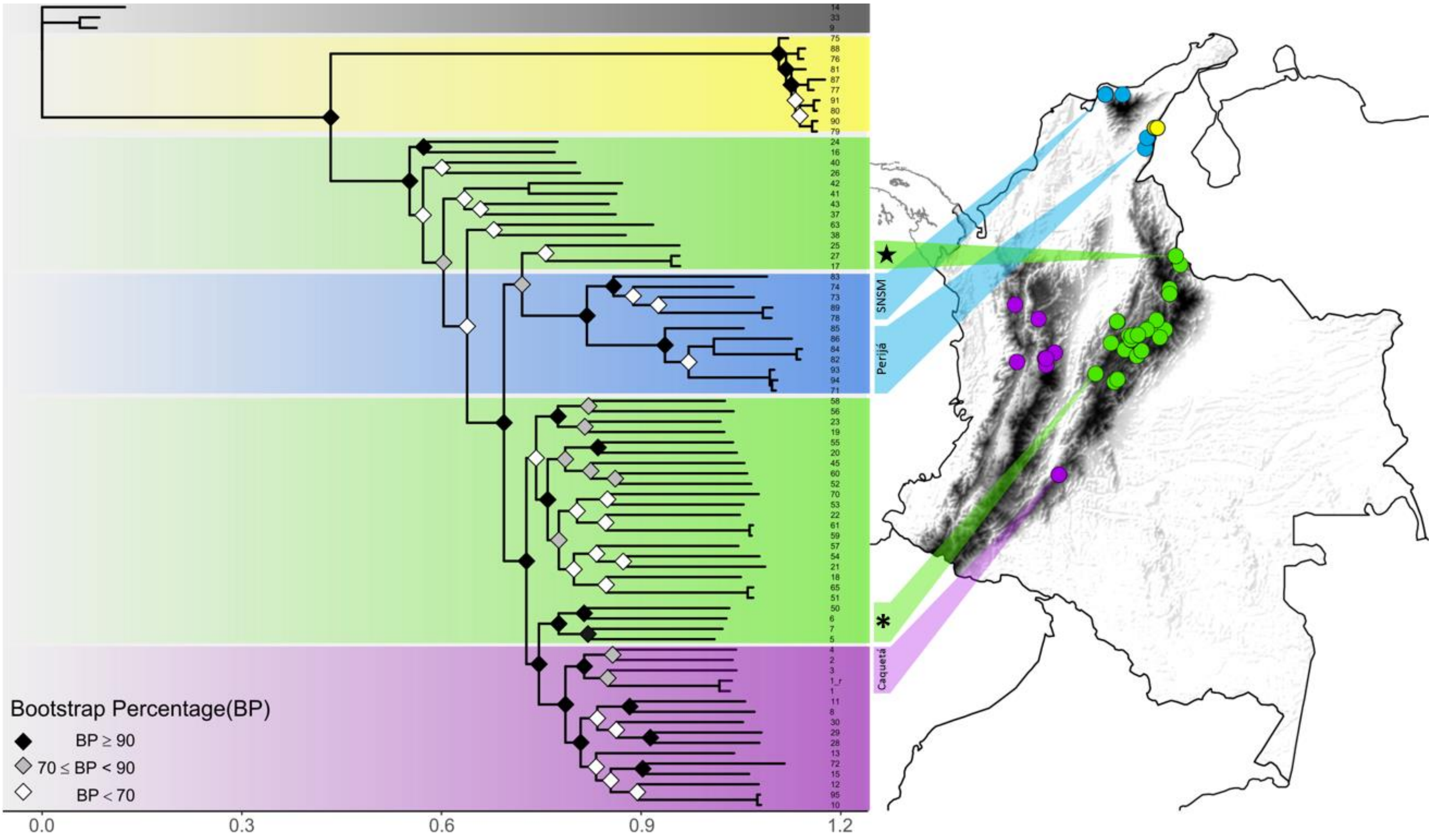

Figure 2. Geographic distribution of our sampling (right) and ML phylogenetic reconstruction of M. tyrianthina Colombian subspecies, M. iracunda and M. williami species. Colored nodes represent intervals for Bootstrap values, being white, 0-35; gray=35-0; black=75-100). Clades of interest are highlighted right to the tips of the topology in reference to Caquetá, Perijá and SNSM regions, as well as two eastern M. t. tyrianthina subpopulations of Norte de Santander (★) and Guasca (✱).

Population Genetic Analyses

The coancestry matrix obtained with fineRADstructure is informative about the degree in which individuals share ancestry (23). This approach showed that *Metallura iracunda* is the most genetically structured group and has no shared ancestry with *Metallura tyrianthina* individuals (see red group, Fig. 3). Likewise, it detected structured subpopulations within *Metallura tyrianthina*. The highest structure within the clade was obtained from the two populations of *M. t. districta* subspecies SNSM and Perijá (see blue, Fig. 3). Nevertheless, even though the eastern and western populations of *M. t. tyrianthina* were clustered by the ad-hoc tree; they showed almost no structure between them (see purple and green groups, Fig.3).

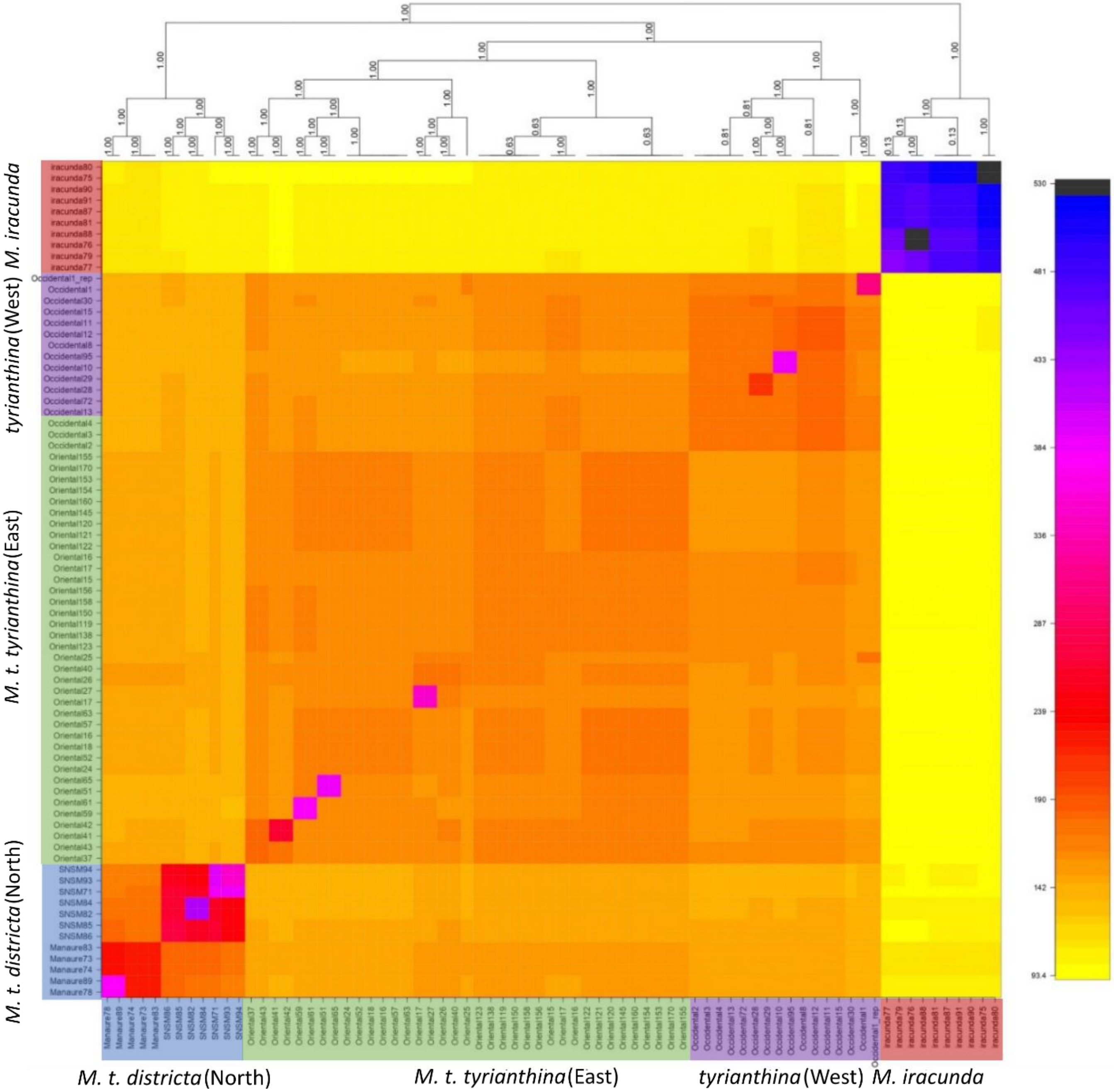

Figure 3. Coancestry matrix from FineRADstructure from 75 hummingbirds grouped by the ad-hoc topology clades. The color scale indicates the coancestry values of population averages representing chunk numbers from donors (column) to recipients (row), and the trees show the subpopulations and their clustering relative to each other.

I calculated population genetics summary statistics for *M. iracunda* and the four geographic groups of *M. tyrianthina*: **Northern SNSM** and **perijá** *M. t. districta*, **Western** *M. tyrianthina* and **Eastern** *M. tyrianthina*. M. iracunda and both of *M. t. districta* populations (SNSM and Perijá) exhibited higher nucleotide diversity than Eastern and Western populations of *M. t. tyrianthina* (see table 1, Fig.). Tajimas D values were close to cero in all populations, but positive for the Sierra Nevada (SNSM), Perijá and *M. iracunda*, and negative for the east and west *M. t. tyrianthina* populations.

Lineage	**Population**	**Mean Nuc. Div. (∏)**	**Median Tajima's D**	**SS**
M. t. tyrianthina				
	West	0.001660653	-0.234982	16750
	East	0.001512463	-0.0860577	19825
M. t. districta				
	SNSM	0.002677618	0.821586	7995
	Perijá	0.002677072	0.1876625	9164
M. iracunda		0.002410665	0.722614	3165

Table 1. Summary statistics for *Metallura iracunda* and two subspecies of *Metallura tyrianthina* (i.e: *M. t. districta* and *M. t. tyrianthina*), where the subspecies calculations were made from their populations.

Ancestry analysis conducted with Admixture showed a better fit for two clusters (AIC=0.349). However, I cannot discard the presence of three clusters (AIC= 0.367; see Fig. 3C). Therefore, both clustering scenarios were considered. From this analysis we observed a clear separation of the *M. t. tyrianthina* and *M. t. districta* subspecies on both K values. Admixture K=2 was not able to distinguish the two populations of *M. t. districta* nor the western and eastern populations of *M. t. tyrianthina* (Fig. 3C). Additionally, for K=3 *M. t. tyrianthina* was divided into west and east clusters with some admixture between them, and between the eastern group with *M. t. districta*. Remarkably, two eastern samples from 'Norte de Santander' locality were classified entirely as a *M. t. districta* for K=2 but was successfully grouped with eastern individuals on K=3, suggesting gene flow between *M. t. districta* and eastern *M. t. tyrianthina*. When *M. iracunda* was included, the best K was four, recovering this species and the same groups for *M. tyrianthina* (see Fig. 3C). F_{ST} values were consistent with the level of structure revealed by Admixture (Fig. see Fig. 4B). Estimated effective migration surface (EEMS) analysis showed low migration rates between Perijá and SNSM populations and a subtle barrier to gene flow in the Magdalena River valley, isolating east and west populations of *M. t. tyrianthina* (see Fig. 3B). Likewise, migration rates were higher between the *M. t. tyrianthina* individuals from Caquetá and the western individuals from this subspecies, consequently with Admixture analysis (see Fig 3 B and C).

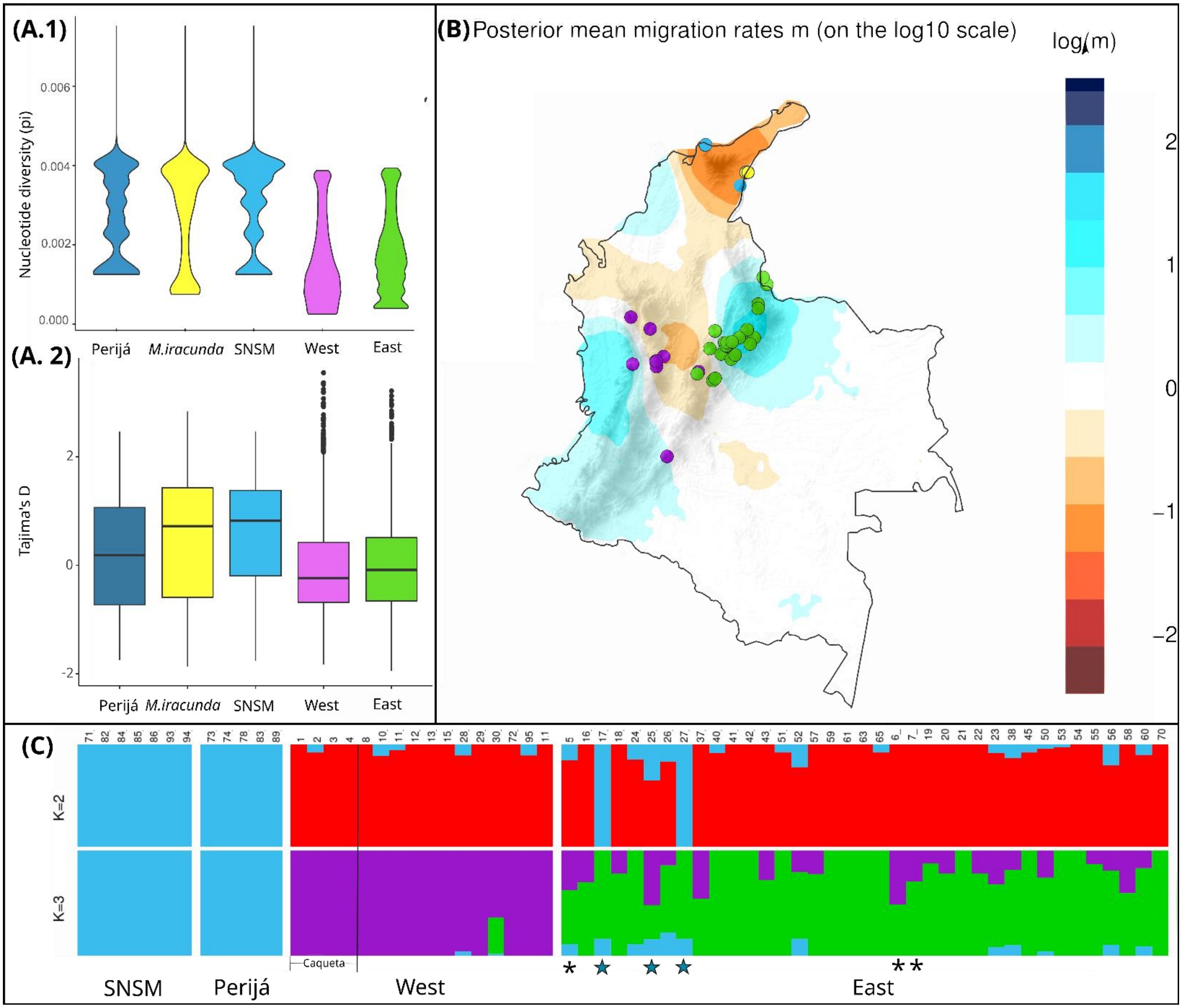

Figure 3. Genetic diversity and structure plots. A) Summary genetic statistics per loci and per populations (1) Nucleotide diversity and (2) Tajima's D; B) Estimated effective migration surface plot (EEMS) from the spatial configuration of genetic distance between populations showing positive rates of migration in a blue scale and negative rates in a red scale; C) Ancestral clustering method by ADMIXTURE for the two clustering methods with better fit (K= 2, 3).

Moreover, PCA analysis recovered a clear separation between *M. t. tyrianthina* and *M. t. districta,* and recovered the two populations (SNSM and Perijá) from the latter subspecies (see Fig 4C). A subtle level of differentiation was found in the first two principal components between east and west populations of *M. t. tyrianthina*. As seen on previous analyses, *M. t. tyrianthina* individuals from Caquetá grouped with the western individuals of the same subspecies (Fig 4C). Eastern *M. t. tyrianthina* individuals from Guasca (see Fig. 2, ✱) were intermediate between west and east groups of *M. t. tyrianthina,* in contrast to the phylogenetic analysis where they are sister to the western *M. t. tyrianthina* clade. On the other hand, the *M. t. tyrianthina* individuals from Norte de Santander that grouped with a low support in the phylogeny with *M. t. districta* (BS< 90, Fig 2) were grouped in the PCA with the other eastern *M. t. tyrianthina* individuals (Fig. 4C).

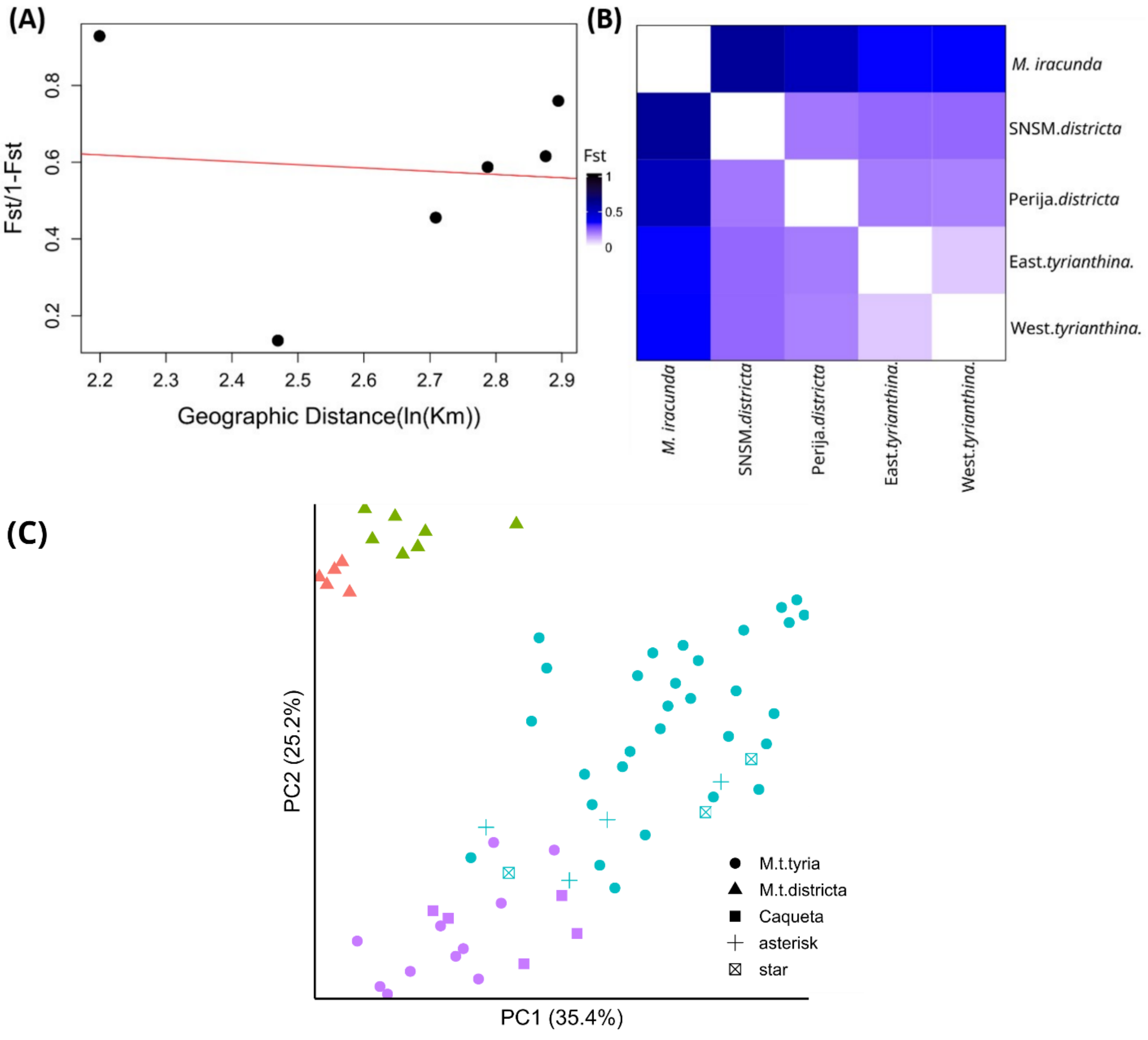

Figure 4. Genetic population structure analyses of A) Isolation by distance plot between all population pairs where the corrected F_{ST} was regressed over the base 10 logarithm of the altitudinal corrected distances; B) Pairwise F_{ST} between all population pairs including *M. iracunda*; and C) PCA from SNP data where asterisk and star shapes represent the individuals from the ML phylogeny (Fig. 2).

Demographic modelling and Coalescence parameters

Modeled scenarios from paired populations (i.e.: *districta* SNSM vs Perijá, Eastern *tyrianthina* vs Perijá *districta* and Eastern vs western *tyrianthina)* showed that divergence with gene flow had better fit than the strict divergence scenario in all cases (see table 2). Demographic scenarios gave insight on divergence times and proportion of migration depending on the pair of taxa evaluated. Point estimates and confidence intervals for time of divergence were all placed on mid Pleistocene before the last glacial maxima (i.e., always >40 kya; Table 3) indicating recent events of diversification for all three pairs of populations after the last uplift of the Eastern Cordillera. The oldest divergence (318,5 kya) is reported on east and west populations of *M. t. tyrianthina* under secondary contact or recent bidirectional geneflow (RGF) with a rate of 2 migrants per generation. Posteriorly, the second older divergence was found between the populations of *M. t. districta* (i.e: SNSM and Perijá) under an asymmetric single pulse migration model (SPM) around 174,4k years ago, where the highest migration occurred near the time of divergence and moved directionally from the eastern cordillera to the SNSM population (Table 3). Lastly, the divergence between *M. t. tyrianthina* and *M. t. districta* was found to occur more recently (9 kya aprox.) under a constant gene flow scenario with low symmetric migration rates (Table 3). However, AIC values were almost equal for recent gene flow and constant gene flow scenarios on west vs east tyrianthina, and Perijá districta vs eastern tyrianthina pairs (Table 2); confirming that gene flow was present during divergence.

	Model	AIC
M. t. tyrianthina (East vs. West)	**RGF**	**3,174.86**
	CGF	3,175.01
	EGF	3,182.83
	NOGF	4,425.02
M. t. districta (SNSM vs. Perijá)	**SPM**	**710.96**
	CGF	844.57
	RGF	846.42
	NOGF	936.48
	EGF	844,26
M. t. tyrianthina vs. M. t. districta (East vs Perijá)	**CGF**	**429.29**
	EGF	429.56
	RGF	431.67
	SPM	1,921.75
	NOGF	634.45

Table 2. Measures of fit of alternative models applied for each pairwise comparison. Bold values represent the most probable model.

	M. t. tyrianthina (East vs. West)	M. t. tyrianthina vs. M. t. districta (East vs Perijá)	M. t. districta (SNSM vs. Perijá)
Model	***RGF***	***CGF***	***SPM***
Parameter			
NM1 (Ne)	113837	14493	767028
	(125.3 - 49403.45)	(1796.65 - 65032.85)	(549.44 - 954803)
NM2 (Ne)	1250	918	3078578
	(199.45 - 60276.2)	(106.45 - 1774.95)	(683.35 - 8508853)
MIG1-2 (m)	8.48E-04	1.00E-04	0.1202688
	(0.161E-4 – 2.179E-4)	(0.16E-4 – 7.161E-4)	(0.0660 - 0.672)
MIG2-1 (m)	1.27E-06	1.35E-06	0.0294122
	2.3095E-6 - 0.0021	(1.2845E-6 – 3.5415E-6)	(0.0215 - 0.3205)
Tadm (gen)	2586	NA	348913
	(40.025 - 35550.8)	NA	(20021.38 - 84835.88)
TDIV (gen)	637630	17918	367112
	(66.05 - 689074.02)	(1613.55 - 11831.9)	(17715.38 - 589769.62)
MaxEstLhood	-36	91.117	-284.472
MaxObsLhood	-34	78.508	-221.638

Table 3. Point estimates and confidence intervals of demographic parameters for each pair of populations compared, over the more probable model of divergence obtained with FASTSIMCOAL27 (28). Migration index (m) represents the number of genes leaving a population per generation, Ne and gen estimates are in units of haploid individuals.

Colorimetric Evaluation

We extracted JNDs from quantum catches distances between 4 pairs of hummingbird lineages tested on this study in the following order 1) Northern populations for subspecies *Metallura tyrianthina districta* (SNSM and Perijá); 2) Western and Eastern individuals of *M. t. tyrianthina;* 3) *M. t. tyrianthina* and *M. t. districta* subspecies and 4) *Metallura iracunda* vs *M. t. tyrianthina* (see Fig. 5A). Almost all JND values were far greater than the theoretical discrimination threshold of 1 JND, with the only exception of western and eastern populations of *M. t. tyrianthina* on tail and back plumage patches (Fig. 5A). JND values from different modeled light sources (i.e: forest shade and Sky-blue) showed a similar pattern, but error bars were wider under the sky-blue model (Fig. 5A).

Based on our results, the chromatic contrast between SNSM and Perijá *M. t. districta* populations was different on both plumage patches and different sexes (Fig. 5A). Although each patch is highly variable, all the error bars are above the discrimination threshold and point estimates reveal that the back plumage patch showed the highest value of noticeable differences of this pair, whilst females were more different and variable than males, but the lowest difference was seen on tail plumage (Fig. 5A). Contrasts between *M. iracunda* and eastern *M. t. tyrianthina* revealed significant differences in color contrast for every patch, but the female tails and male back plumage patches showed the highest values for all comparisons, with 52 JNDs and 45, respectively (Fig. 5A). The lowest differences between *M. iracunda* and eastern *M. t. tyrianthina* were found on the throat patches for both sexes; however, they remained significantly different to each other. My analysis between western and eastern populations of *M. t. tyrianthina* showed that the throat patch represented the only noticeable different patch under the avian vision model. In this comparison, male throat patches overcame the discrimination threshold under all light conditions, but female throats were significantly different, only under sky blue light conditions. Finally, between *M. t. districta* and *M. t. tyrianthina* subspecies, all patches were significantly different and female throat and back plumage patches showed the lowest distance values of this last comparison. The higher distance values on this last comparison were on tail plumages for both sexes, as expected, since *M. t. districta* tails are blue-purple colored while *M. t. tyrianthina* tails are rufous.

When a PCA analysis was conducted with the quantum catches modeled, the first two components represented 77.8% of the data variability and showed a clear discrimination of the tail plumage between *M. t. districta* and *M. t. tyrianthina* on both sexes (see Fig. 5B and 5C). The male throat plumage patch for these subspecies formed a single cluster. Likewise, on females there is a visible segregation between the throats of *M. t. districta* and *M. t. tyrianthina* (Fig. 5C). Finally, the back plumage patches did not show a clear segregation but *M. t. districta* males and females showed more variation than *M. t. tyrianthina* (Fig. 5B and 5C).

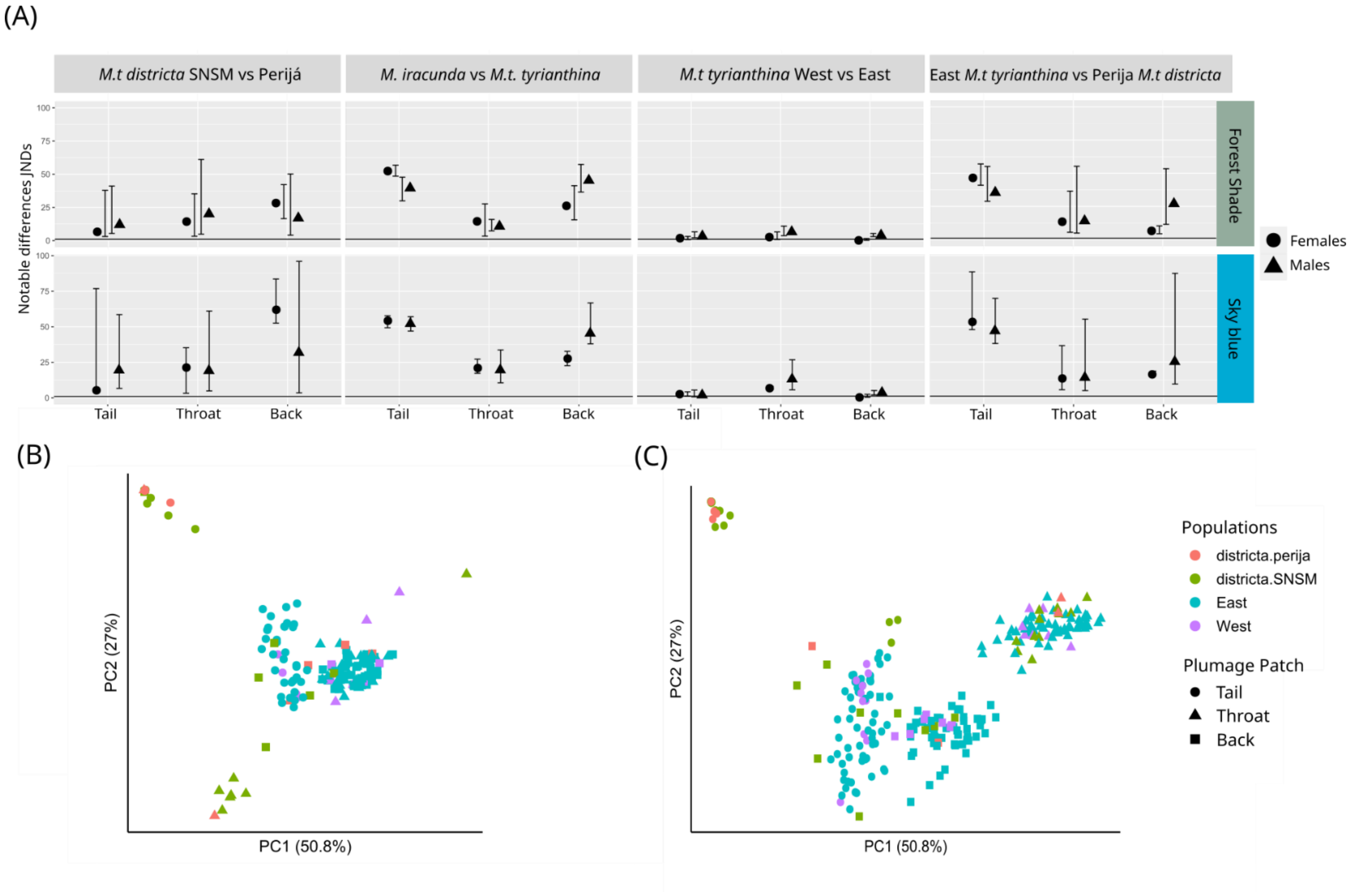

Figure 5. Color contrast of quantum catches values extracted from n = 157 test subjects A) Just Noticeable Differences between the feather patches of three population pairs, where 'Sky blue' and 'Forest shade' represent modeled light conditions. The theoretical discrimination threshold of 1 JND was used and error bars indicate standard deviation. B) PCA of quantum catches from the tree plumage patches, extracted from Females; and C) same as B, but for Males.

DISCUSSION

The effect of the Andes topology driving animal evolution has been widely studied on different tetrapod taxa including birds, (8,36), reptiles (37), amphibians (38,39), bees (40), butterflies (41), and arachnids (42) describing the altitudinal depressions acting as barriers to gene flow on highland species, promoting vicariant divergence. However, as interesting as these studies are, they focus on ancient divergence events that may obscure the true history of speciation due to long accumulation of unwitnessed change (i.e.: undocumented extinctions (43), replacement of competitors (44), or ancient changes of distribution (45)). On the contrary, recently divergent hummingbird lineages have demonstrated high promise as models for describing the main causes of lineage divergence (8,46,47).

Metallura tyrianthina subspecies have recently diverged (>1.5 kya) into all 7 subspecies inhabiting the Andes. For the Colombian individuals, our demographic analyses situated *M. t. tyrianthina* divergence after the final uplift of the Andes mountains, and supported the importance of Pleistocene glacial cycles on the evolution of tropical avifauna (48,49). Climatic oscillations promoted repeated cycles of range fragmentation and expansion that resulted in the replacement of related lineages with a strong positive correlation between high altitudinal distributions and rates of diversification (17,20,50). All genetic assessments of Admixture (K=2 and K=3), coancestry, phyllogenetic reconstruction, and color quantification analyses show support for the latitudinal segregation of two lineages within Tyrian Metaltails: 1) *M. t. districta* subspecies occurring on northern Colombian mountains and 2) *M. t. tyrianthina* on west-central and eastern cordilleras. Additionally, the effect of the Andes topography on the evolution of *M. tyrianthina* was not explained by a correlation between genetic structure and geographic distance (Mantel test: r= 0.1429, p=0.5). Therefore, the isolation and genetic structure of populations does not follow an isolation by distance pattern. Below, I describe the genetic and demographic patterns for *M. tyrianthina* subspecies, reflecting that *M. t. districta* and *M. t. tyrianthina* had different evolutive histories, but both were consistent with the divergence with gene flow (51).

Colonization and rapid divergence

Metallura tyrianthina districta individuals were recovered on our phylogenetic hypothesis as a monophyletic clade derived from *M. t. tyrianthina*. Additionally, coancestry matrix, PCA and genetic structure supported a pattern of diversification within its populations on SNSM and the north part of eastern cordillera (Perijá). Confidence intervals for both, time of divergence and time of admixture overlapped with the ending of the last glacial maxima (1.8 ky and 1.7 kya, respectively), with an impressive amount of migration from Perijá towards the SNSM populations. At that time, it is thought that mountain fauna extended their distribution range as the low temperatures of the glacial periods moved the forest line downwards, therefore, improving the connectivity between mountain fauna (Table 3, Fig. 6). Diversity estimates also suggests a recent event of colonization by dispersion, as sudden population contractions (founder effect) result in positive Tajima's D values and higher nucleotide diversity than *M. t. tyrianthina* (Table 1, Tajima's D=0.82) (52). Divergence between SNSM and Perijá have been reported on other avian organisms and that could suffer a similar population event (20,46,53). Although Tajima's D is not sufficiently high, genetic and morphologic data are consistent with a pattern of rapid divergence mediated by a founder effect (20); for two reasons that are not mutually exclusive. 1) The effect of genetic drift increase the probability of allele fixation on fewer generations, favouring allopatric speciation (54) and 2) The evolution of sexual selected traits on hummingbirds, such as feathers, is fast (46), but it occurs faster on shorter wavelength colors (55) such as the blue and purple tail feather patches and green throats of *M. t. districta*. Therefore, after the colonization of the SNSM, evidence show that populations structured rapidly, and migration was interrupted as suggested by the EEMS analysis. It is possible for *M. t. districta* purple tail and brighter green throat to be rapidly fixated by genetic drift and posteriorly distanced genetically by assortative mating with the Perijá individuals. Additionally, gene flow between SNSM and perijá populations during the colonization of the SNSM, could affect the resolution of the site frequency spectra in detecting bottleneck.

SNSM and Perijá individuals of *M. t. districta* have the highest amount of genetic structure within the Tyrian Metaltails and display significant morphological disparity on all three plumage patches considered (Fig 5A, 5B and 5C).

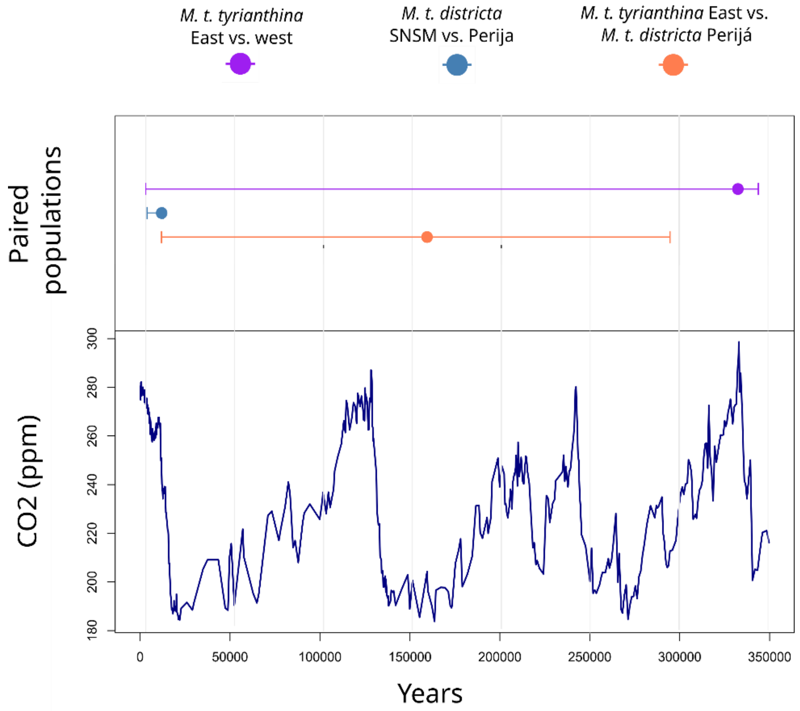

Figure 6. Contrast between the historic record of Carbon dioxide concentrations since 350 kya, Point estimates and confidence intervals for the times of divergence for each pair of populations tested, estimated from each more probable demographic model of FASTSIMCOAL2.

Subtle divergence with gene flow

Metallura tyrianthina tyrianthina was expected to display a marked genetic effect from topography, since this subspecies has the widest distribution occurring on all three Colombian cordilleras. Previous phylogenetic hypothesis using nuclear and mitochondrial loci, suggested a clear divergence between an eastern and western populations of *M. t. tyrianthina* (20). Studies in other bird species showed that the Magdalena river's altitudinal depression could act as a barrier to gene flow between Colombian cordilleras (7,9,56) and could explain the presence of the two eastern and western populations of *M. t. tyrianthina*. In the previously published amplicon phylogeny, the eastern clade of *M. t. tyrianthina* grouped with *M. t. districta* and the western was sister to southern Peruvian populations of *M. t. tyrianthina* (20). However, our phyllogenetic reconstruction recovered a western clade that is derived from the eastern individuals. Also, small branch lengths on our tree, low genetic structure and a high degree of shared ancestry between west and eastern populations (Figs. 2 and 3C) suggest a scenario of subtle divergence in the presence of gene flow. Demographic models were not able to differentiate between a secondary contact scenario and a constant gene flow pattern of divergence between *M. t. tyrianthina* eastern and western populations. Nevertheless, the less probable model was divergence without gene flow. This result was consistent with the EEMS pattern with higher migration rates found on the southern parts of the Colombian Andes where the three cordilleras converge, near Caquetá and the Las Cruces altitudinal depression. Climatic history of the Andes was not completely explicative for divergence times between the eastern and western populations of *M. t. tyrianthina*, as confidence intervals were broad (Table 3. Fig. 6). Consistently, JND's analysis showed poor degree of visual recognition between the individuals of eastern and western populations, based on plumage color (Fig. 5A).

Taxonomic implications

Morphologic assessments determined that birds could identify significant noticeable differences between all plumage patches of the individuals from SNSM and Perijá *M. t. districta* populations. Additionally, genetic analyses support a rapid divergence and genetic structuration scenario between these populations. Therefore, a prompt study is conducting an integral evaluation of Perijá individuals of *M. t. districta* for defining the taxonomic status as a new Colombian endemic subspecies, whilst leaving the SNSM individuals as nominal *M. t. districta* (Mongui; Cuervo; Styles and Salazar, in prep.). Genetic analyses also revealed a subtle differentiation between the individuals from eastern and western populations of *M. t. tyrianthina*. Individuals occurring on the western and central cordilleras formed a single genetic cluster that may be a homogeneous lineage with Peruvian and Bolivian individuals of *M. t. tyrianthina* which are continuously distributed towards south. Consequently, the divergence of the of *M. t. tyrianthina* occurring in the northern Andes has yet to be described. Further studies with broader sampling and additional data could be able to determine if the individuals occurring on the Colombian eastern cordillera should be treated as another subspecies or even as a new species (Mongui; Cuervo; Styles and Salazar, in prep.).

CONCLUSIONS

In conclusion we can observe two patterns of divergence acting differentially over a single topographic accident on the two Colombian subspecies of the Tyrian Metaltail. First, *M. t. districta* showed a dispersion and colonization pattern of divergence, promoted by the climatic cycles of the Pleistocene. Whereas *M. t. tyrianthina* showed a subtle divergence pattern that is not consistent with the Andes acting as a barrier or with climatic oscillations in the Pleistocene shaping the population divergence of *M. t. tyrianthina*.

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CONTRIBUTIONS

Juan Pablo Mongui did laboratory procedures and analysed the data. JP, Andres Cuervo and Camilo Salazar designed the study, provided the samples and Camilo Salazar funded the molecular markers.

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Museum ID	Sample-Num	State	Latitude	Longitude	Species	Population
IAvH-CT-18714	1	Caqueta	2.6317306	-75.1024278	M.t.tyrianthina.aff	West
IAvH-CT-18713	2	Caqueta	2.6317306	-75.1024278	M.t.tyrianthina.aff	West
IAvH-CT-18712	3	Caqueta	2.6317306	-75.1024278	M.t.tyrianthina.aff	West
IAvH-CT-18709	4	Caqueta	2.6317306	-75.1024278	M.t.tyrianthina.aff	West
IAvH-CT-19717	5	Cundinamarca	4.7605	-73.7807222	M.t.tyrianthina.aff	East
IAvH-CT-02559	6	Cundinamarca	4.7036111	-73.8511111	M.t.tyrianthina.aff	East
IAvH-CT-19716	7	Cundinamarca	4.7605	-73.7807222	M.t.tyrianthina.aff	East
IAvH-CT-04116	8	Antioquia	6.1	-75.55	M.t.tyrianthina	West
IAvH-CT-01655	9	Caldas	5.2296111	-75.3839444	M.t.williami	out
IAvH-CT-01737	10	Caldas	5.2198333	-75.4040278	M.t.tyrianthina	West
IAvH-CT-01946	11	Caldas	5.2198333	-75.4040278	M.t.tyrianthina	West
IAvH-CT-01822	12	Caldas	5.2198333	-75.4040278	M.t.tyrianthina	West
IAvH-CT-04095	13	Caldas	5.3522222	-75.1861111	M.t.tyrianthina	West
IAvH-CT-01717	14	Caldas	5.2296111	-75.3839444	M.t.williami	out
IAvH-CT-01911	15	Caldas	5.2198333	-75.4040278	M.t.tyrianthina	West
IAvH-CT-19290	16	Santander	6.6654722	-72.6176111	M.t.tyrianthina.aff	East
IAvH-CT-20063	17	Santander	6.0426111	-73.7879444	M.t.tyrianthina.aff	East
IAvH-CT-23037	18	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-23622	19	Boyaca	5.39191	-73.25009	M.t.tyrianthina.aff	East

IAvH-CT-26989	20	Boyaca	5.749971	-73.329536	M.t.tyrianthina.aff	East
IAvH-CT-23022	21	Boyaca	5.4222778	-73.6203611	M.t.tyrianthina.aff	East
IAvH-CT-04158	22	Boyaca	5.6944444	-73.4811111	M.t.tyrianthina.aff	East
IAvH-CT-18135	23	Boyaca	5.28333	-73.35	M.t.tyrianthina.aff	East
IAvH-CT-23042	24	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-11470	25	NortedeSantander	7.505947058	-72.4849105	M.t.tyrianthina.aff	East
IAvH-CT-11450	26	NortedeSantander	7.505947058	-72.4849105	M.t.tyrianthina.aff	East
IAvH-CT-11595	27	NortedeSantander	7.505947058	-72.4849105	M.t.tyrianthina.aff	East
IAvH-CT-04056	28	Risaralda	5.1483333	-76.0333333	M.t.tyrianthina	West
IAvH-CT-04055	29	Risaralda	5.1483333	-76.0333333	M.t.tyrianthina	West
IAvH-CT-21315	30	Antioquia	6.4187778	-76.0818333	M.t.tyrianthina	West
IAvH-CT-20144	31	Cundinamarca	4.93875	-74.2458056	M.t.tyrianthina.aff	West
IAvH-CT-20163	32	Cundinamarca	4.8810278	-74.2796111	M.t.tyrianthina.aff	East
IAvH-CT-01712	33	Caldas	5.2198333	-75.4040278	M.t.williami	out
IAvH-CT-04151	34	Boyaca	5.6944444	-73.4811111	M.t.tyrianthina.aff	East
IAvH-CT-04650	35	Caldas	5.3522222	-75.1861111	M.t.tyrianthina	West
IAvH-CT-19157	36	Caldas	5.0745528	-75.3876361	M.t.tyrianthina	West
IAvH-CT-20023	37	Santander	6.0489167	-73.7938333	M.t.tyrianthina.aff	East
IAvH-CT-19294	38	Santander	6.7731111	-72.6223333	M.t.tyrianthina.aff	East
IAvH-CT-19278	39	Santander	6.6644167	-72.6233889	M.t.tyrianthina.aff	East
IAvH-CT-11256	40	NortedeSantander	7.3121667	-72.37545	M.t.tyrianthina.aff	East
IAvH-CT-20044	41	Santander	6.0489167	-73.7938333	M.t.tyrianthina.aff	East
IAvH-CT-20038	42	Santander	6.0426111	-73.7879444	M.t.tyrianthina.aff	East
IAvH-CT-20030	43	Santander	6.0489167	-73.7938333	M.t.tyrianthina.aff	East
IAvH-CT-04186	44	Boyaca	5.72975	-73.4627778	M.t.tyrianthina.aff	East
IAvH-CT-27332	45	Boyaca	5.9526371	-72.9906632	M.t.tyrianthina.aff	East
IAvH-CT-23024	46	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-04175	47	Boyaca	5.72975	-73.4627778	M.t.tyrianthina.aff	East
IAvH-CT-25693	48	Boyaca	5.39191	-73.25009	M.t.tyrianthina.aff	East
IAvH-CT-23599	49	Boyaca	5.39191	-73.25009	M.t.tyrianthina.aff	East
IAvH-CT-27252	50	Boyaca	5.861697	-72.721159	M.t.tyrianthina.aff	East
IAvH-CT-18157	51	Boyaca	5.7272222	-73.5321944	M.t.tyrianthina.aff	East
IAvH-CT-27299	52	Boyaca	5.57206	-73.931986	M.t.tyrianthina.aff	East
IAvH-CT-23044	53	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-04187	54	Boyaca	5.72975	-73.4627778	M.t.tyrianthina.aff	East
IAvH-CT-27300	55	Boyaca	6.077615	-72.918446	M.t.tyrianthina.aff	East
IAvH-CT-23629	56	Boyaca	5.39191	-73.25009	M.t.tyrianthina.aff	East
IAvH-CT-23023	57	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-23598	58	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-23026	59	Boyaca	5.6385556	-73.4773056	M.t.tyrianthina.aff	East
IAvH-CT-26527	60	Boyaca	5.68881	-72.83103	M.t.tyrianthina.aff	East
IAvH-CT-26566	61	Boyaca	5.749971	-73.329536	M.t.tyrianthina.aff	East
IAvH-CT-06967	62	Boyaca	5.7065556	-73.4600556	M.t.tyrianthina.aff	East
IAvH-CT-02276	63	Boyaca	5.64225	-73.4829722	M.t.tyrianthina.aff	East

IAvH-CT-04611	64	Caldas	5.3522222	-75.1861111	M.t.tyrianthina	West
IAvH-CT-04169	65	Boyaca	5.72975	-73.4627778	M.t.tyrianthina.aff	East
IAvH-CT-27300	66	Boyaca	6.077615	-72.918446	M.t.tyrianthina.aff	East
IAvH-CT-17311	67	Boyaca	5.8642778	-73.1304722	M.t.tyrianthina.aff	East
IAvH-CT-11439	68	NortedeSantander	7.505947058	-72.4849105	M.t.tyrianthina.aff	East
IAvH-CT-11423	69	NortedeSantander	7.505947058	-72.4849105	M.t.tyrianthina.aff	East
IAvH-CT-26554	70	Boyaca	5.749971	-73.329536	M.t.tyrianthina.aff	East
IAvH-CT-00472	71	Magdalena1	11.1133333	-74.0527778	M.t.districta	SNSM
IAvH-CT-04672	72	Antioquia	6.1	-75.55	M.t.tyrianthina	West
IAvH-CT-08507	73	Cesar	10.35	-75.9333333	M.t.tyrianthina	Perijá
IAvH-CT-11394	74	Cesar	10.3639722	-72.9473889	M.t.tyrianthina	Perijá
IAvH-CT-11384	75	iracunda	10.3639722	-72.9473889	M.iracunda	iracunda
IAvH-CT-11393	76	iracunda	10.3639722	-72.9473889	M.iracunda	iracunda
IAvH-CT-11379	77	iracunda	10.3639722	-72.9473889	M.iracunda	iracunda
IAvH-CT-11349	78	Cesar	10.3669444	-72.8974722	M.t.tyrianthina	Perijá
IAvH-CT-11348	79	iracunda	10.3669444	-72.8974722	M.iracunda	iracunda
IAvH-CT-11347	80	iracunda	10.3669444	-72.8974722	M.iracunda	iracunda
IAvH-CT-11342	81	iracunda	10.3669444	-72.8974722	M.iracunda	iracunda
IAvH-CT-08462	82	Cesar	10.3639722	-72.9473889	M.t.tyrianthina	Perijá
IAvH-CT-8507	83	Cesar	10.3639722	-72.9473889	M.t.tyrianthina	Perijá
IAvH-CT-00467	84	Magdalena1	11.1133333	-74.0527778	M.t.districta	SNSM
IAvH-CT-11211	85	Magdalena1	11.1058667	-74.06235	M.t.districta	SNSM
IAvH-CT-11208	86	Magdalena1	11.10675	-74.0602833	M.t.districta	SNSM
IAvH-CT-11381	87	iracunda	10.3639722	-72.9473889	M.iracunda	iracunda
IAvH-CT-11393	88	iracunda	10.3639722	-72.9473889	M.iracunda	iracunda
IAvH-CT-11349	89	Cesar	10.3669444	-72.8974722	M.t.tyrianthina	SNSM
IAvH-CT-11348	90	iracunda	10.3669444	-72.8974722	M.iracunda	iracunda
IAvH-CT-11347	91	iracunda	10.3669444	-72.8974722	M.iracunda	iracunda
IAvH-CT-19278	92	NortedeSantander	6.6644167	-72.6233889	M.t.tyrianthina.aff	East
IAvH-CT-11211	93	Magdalena1	11.1058667	-74.06235	M.t.districta	SNSM
IAvH-CT-00472	94	Magdalena1	11.1133333	-74.0527778	M.t.districta	SNSM
IAvH-CT-01737	95	Caldas	5.2296111	-75.3839444	M.t.tyrianthina	West
IAvH-CT-18715	1_rep	Caqueta	2.6317306	-75.1024278	M.t.tyrianthina.aff	West

Supplementary Table 1. Samples used on this study.