Genetic and morphometric divergence in the Garnet-Throated Hummingbird *Lamprolaima rhami* (Aves: Trochilidae)

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Cloud forests are one of the most endangered ecosystems in the Americas, as well as one of the richest in biological diversity in the world. The species inhabiting these forests are susceptible to environmental changes and characterized by high levels of geographic structure. The Garnet-Throated Hummingbird, *Lamprolaima rhami,* mainly inhabits cloud forests, but can also be found in other habitats. This species has a highly restricted distribution in Mesoamerica, and five disjunct regions have been delimited within the current geographic distribution of the species from Mexico to Honduras. According to variation in size and color, three subspecies have been described: *L. r. rhami* restricted to the Mexican highlands and Guatemala, *L. r. occidentalis* distributed in Guerrero (Mexico), and *L. r. saturatior*, distributed in the highlands from Honduras and El Salvador. We analyzed the levels of geographic structure in *L. rhami* and its taxonomic implications. We used mitochondrial and nuclear DNA to analyze genetic variation, demographic history, divergence times, reconstructed a multilocus phylogeny, and performed a species delimitation analyses. We also evaluated morphological variation in 208 specimens. We found high levels of genetic differentiation in three groups, and significant variation in morphological traits corresponding with the disjunct geographic populations. *L. rhami* presents population stability with the highest genetic variation explained by differences between populations. Divergence time estimates suggest that *L. rhami* split from its sister group around 10.55 million years ago, and the diversification of the complex was dated ca. 0.207 Mya. The hypotheses tested in the species delimitation analyses validated three independent lineages corresponding to three disjunct populations. This study provides evidence of genetic and/or morphometric differentiation between populations in the *L. rhami* complex where four separate evolutionary lineages are supported: 1) populations from the Sierra Madre Oriental and the highlands of Oaxaca (*rhami*), 2) populations from the highlands of Guerrero (*occidentalis*), 3) populations from the highlands of Chiapas and Guatemala (this is a non-previously proposed potential taxon: *tacanensis*), and 4)

populations from the highlands of Honduras and El Salvador (*saturatior*). The main promoters of the geographic structure found in the *L. rhami* complex are likely the Isthmus of Tehuantepec as a geographic barrier, isolation by distance resulting from habitat fragmentation, and climatic conditions during the Pleistocene.

1   Genetic and morphometric divergence in the Garnet-Throated Hummingbird *Lamprolaima*

2   *rhami* (Aves: Trochilidae).

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15 **Abstract**

17 Cloud forests are one of the most endangered ecosystems in the Americas, as well as one of

18 the richest in biological diversity in the world. The species inhabiting these forests are

19 susceptible to environmental changes and characterized by high levels of geographic

20 structure. The Garnet-Throated Hummingbird, *Lamprolaima rhami,* mainly inhabits cloud

21 forests, but can also be found in other habitats. This species has a highly restricted

22 distribution in Mesoamerica, and five disjunct regions have been delimited within the

23 current geographic distribution of the species from Mexico to Honduras. According to

24 variation in size and color, three subspecies have been described: *L. r. rhami* restricted to

25 the Mexican highlands and Guatemala, *L. r. occidentalis* distributed in Guerrero (Mexico),

26 and *L. r. saturatior*, distributed in the highlands from Honduras and El Salvador. We

27 analyzed the levels of geographic structure in *L. rhami* and its taxonomic implications. We

28 used mitochondrial and nuclear DNA to analyze genetic variation, demographic history,

29 divergence times, reconstructed a multilocus phylogeny, and performed a species

30 delimitation analyses. We also evaluated morphological variation in 208 specimens. We

31 found high levels of genetic differentiation in three groups, and significant variation in

32 morphological traits corresponding with the disjunct geographic populations. *L. rhami*

33 presents population stability with the highest genetic variation explained by differences

34 between populations. Divergence time estimates suggest that *L. rhami* split from its sister

35 group around 10.55 million years ago, and the diversification of the complex was dated ca.

36 0.207 Mya. The hypotheses tested in the species delimitation analyses validated three

37 independent lineages corresponding to three disjunct populations. This study provides

38   evidence of genetic and/or morphometric differentiation between populations in the *L.*

39   *rhami* complex where four separate evolutionary lineages are supported: 1) populations

40   from the Sierra Madre Oriental and the highlands of Oaxaca (*rhami*), 2) populations from

41   the highlands of Guerrero (*occidentalis*), 3) populations from the highlands of Chiapas and

42   Guatemala (this is a non-previously proposed potential taxon: *tacanensis*), and 4)

43   populations from the highlands of Honduras and El Salvador (*saturatior*). The main

44   promoters of the geographic structure found in the *L. rhami* complex are likely the Isthmus

45   of Tehuantepec as a geographic barrier, isolation by distance resulting from habitat

46   fragmentation, and climatic conditions during the Pleistocene.

48   **Introduction**

49   Cloud forests are one of the most threatened and biodiverse habitats in the world

50   (Hamilton, 1995; Mulligan, 2010). In Mesoamerica, the transition zone between the

51   Nearctic and Neotropical regions (Ríos-Muñoz, 2013; Morrone, 2014), cloud forests are

52   restricted to forest habitats between 600 and 3000 m above sea level (Foster, 2001).

53   Several studies have tried to describe the evolutionary processes that have shaped the

54   enormous diversity observed in cloud forests, concluding that species show high levels of

55   isolation and population differentiation when compared to lowland forest habitats that

56   may be more geographically interconnected (de Barcellos & Voltolini, 1995; Ataroff & Rada,

57   2000; Ornelas et al., 2013). However, studies at the population level, including a large

58   sampling effort, are crucial to describe intraspecific variation more precisely (Bonaccorso

59   et al., 2008; McCormack et al., 2008; Arbeláez-Cortés & Navarro-Sigüenza, 2013).

60   Recently, several studies have focused on describing historical patterns and

61   recognizing new species in cloud forests (González-Rodríguez et al., 2004; Cortés-

62   Rodríguez et al., 2008; Ornelas et al., 2010; González et al., 2011). However, the fast pace at

63   which these forests are disappearing due to anthropogenic causes is one of the multiple

64   reasons to promote the study of the evolutionary processes taking place in this particularly

65   diverse ecosystem (Olander et al., 1998; Martínez-Morales, 2005).

66   The Trochilidae family includes several species complexes that have been excellent

67   models for evolutionary studies (Bleiweiss, 1998a; McGuire et al., 2007; McGuire et al.,

68   2014), though to date, only a few of these studies focus on species inhabiting cloud forests

69   (Bleiweiss, 1998b; Chaves et al., 2007; Cortés-Rodríguez et al., 2008; Chaves & Smith,

70   2011). The Garnet-Throated Hummingbird, *Lamprolaima rhami,* (Lesson, 1839) has a

71   restricted Mesoamerican distribution; it can inhabit tropical upland forests, pine-oak

72   forests and scrub, but primarily occupies cloud forest habitats, within an altitudinal range

73   between 1200 and 3000 m (Howell & Webb, 1995; Schuchmann & Boesman, 2018). It is

74   considered a relatively sedentary species, though individuals do show some seasonal

75   movement to higher elevations during the breeding season (Schuchmann & Boesman,

76   2018).

77   *L. rhami* has a discontinuous distribution with five clearly distinguished geographic

78   areas: 1) Sierra Madre Oriental (Puebla to Veracruz) and the northern highlands of Oaxaca

79   (Mexico), 2) the highlands of Guerrero, in the Sierra Madre del Sur (Mexico), 3) the

80   southern highlands of Oaxaca, in the Sierra de Miahuatlan (Mexico), 4) the highlands of

81   Chiapas (Mexico) and Guatemala, and 5) the highlands of Honduras and El Salvador, in

82   Central America. Three subspecies have been recognized based on differences on size and

83   color: *L. r. rhami*, *L. r. occidentalis*, and *L. r. saturatior*. *L. r. rhami* is restricted to the

84   highlands of central and southern Mexico (in the states of Puebla, Veracruz, Oaxaca and

85   Chiapas) and the Guatemala highlands (Lesson, 1839; Peters, 1945). *L. r. occidentalis*

86   corresponds to populations found in a restricted patch in Guerrero, southwestern Mexico

87   (Phillips, 1966). *L. r. saturatior* is found in the highlands of Honduras and El Salvador

88   (Griscom, 1932; Peters, 1945). Schuchmann and Boesman (2018) considered that *L. rhami*

89   possibly belongs to the genus *Basilinna*, and argued that *occidentalis* and *saturatior* are not

90   suitable for subspecific recognition, proposing them only as races, considering that traits

91   used in their descriptions are either age-dependent or clinal in character (color and size

92   variation).

93   Considering its restricted distribution and frequent occupation of highly fragmented

94   forests with unique bioclimatic characteristics, *L. rhami* represents an interesting model to

95   assess evolutionary hypotheses about geographic structure and populations dynamics with

96   conservation implications. Hence, the main objectives of this paper are to: 1) evaluate the

97   genetic and morphometric variation of the *Lamprolaima rhami* complex by comparing

98   individuals from the five regions where it is distributed, 2) describe the phylogenetic

99   relationships within *L. rhami* using a multilocus dataset (nuclear and mitochondrial DNA),

100   and 3) propose a hypothesis of its evolutionary history. Based on the characteristics of

101   cloud forest and the site fidelity of this hummingbird species, we expect to find high levels

102   of genetic structure supported by congruence in morphological variation within the *L.*

103   *rhami* complex. Thus, phylogenetic discontinuities and spatial disjunction are expected

104   rather than phylogenetic continuity and lack of spatial disjunction.

105

106   **Methods**

107   *Taxon sampling and sequencing.*

108   We obtained tissues from 54 individuals of *L. rhami* from 14 localities across most of

109   its geographic range (Table S1, Fig. 1, using field collecting permit from Instituto Nacional

110   de Ecología, SEMARNAT: FAUT-0169). We defined five groups *a priori* to evaluate genetic

111   and morphological variation among the five allopatric regions where the species occurs: 1)

112   the Sierra Madre Oriental (SMO), 2) the highlands of Guerrero (GRO), 3) the Sierra of

113   Miahuatlan in Oaxaca (MIA), 4) the highlands of Chiapas and Guatemala (CHIS), and 5) the

114   region in Central America comprising the highlands of Honduras and El Salvador (CA).

115   Tissue samples were obtained for four geographic groups (excluding the CA group) from

116   the following collections: "Museo de Zoología Alfonso L. Herrera" (Universidad Nacional

117   Autónoma de México), Museum of Natural Science (Lousiana State University), and

118   Museum of Vertebrate Zoology (University of California, Berkeley).

119   DNA was extracted using the DNAeasy™ kit (Qiagen Inc., Valencia, CA, USA),

120   following the manufacturer's protocols. To evaluate genetic variation in the complex, two

121   mitochondrial markers were obtained from the 54 samples (Control Region, *CR*; and

122   subunits 6 and 8 from ATPase gene, *ATPase 6 & 8*). To evaluate phylogenetic relationships

123   and for species delimitation analyses between groups, two additional mitochondrial

124   markers and four nuclear regions were surveyed in a subsample of 31 individuals (NADH

125   dehydrogenase subunit 2, *ND2*; NADH dehydrogenase subunit 4, *ND4*; the 7th intron of the

126   beta fibrinogen gene, *BFib*, the regions between exons 4 and 5 of the Muscle Skeletal

127   Receptor Tyrosine Kinase gene, *MUSK*; a segment comprising the end of exon 6 and the

128   beginning of exon 8 of the Ornithine Decarboxylase gene, *ODC,* and intron 5 of adenylate

129 kinase gene, *AK1*). We included sequences from the same molecular markers, available in

130 GenBank for *Eugenes fulgens, E. spectabilis*, and *Tilmatura dupontii*, to serve as outgroups

131 (McGuire et al., 2007; Zamudio-Beltrán & Hernández-Baños, 2015).

132  We amplified these molecular markers via polymerase chain reaction (PCR) using

133 specific primers and protocols (Table S3). Reactions contained 10X buffer (1.25 µL), 10mM

134 dNTP (0.19 µL), 50 mM MgCl$_2$ (0.38 µL), 10 µM of each primer (0.25 µL), 0.1 µL of *Taq*

135 (INVITROGEN), and 0.5 µL of genomic DNA (12.5 µL total volume). PCR products were

136 visualized on a 1% agarose gel, and DNA sequencing was performed by the High-

137 Throughput Genomics Unit Service of the University of Washington. We edited and aligned

138 chromatograms with Sequencher v4.8 (GeneCodes Corporation, Ann Arbor, MI). All

139 sequences were deposited in GenBank under accession numbers ####. Multilocus

140 alignment can be found in FigShare (https://figshare.com/s/14d2a747c825fba5f35d).

141

142 *Population structure.*

143  To evaluate the number of haplotypes and their relationships, a statistical

144 parsimony haplotype network was constructed for the concatenated dataset of

145 mitochondrial markers (CR and ATPase 6 & 8), using the program TCS v1.21 (Clement et

146 al., 2000).

147  To analyze genetic diversity and genetic structure, we obtained values of haplotype

148 diversity, nucleotide diversity, mean number of pairwise differences, and population F_{ST}

149 values. These analyses were performed with 1000 replicates, using the program Arlequin

150 v3.11 (Excoffier et al., 2005). Using the same program, we conducted an analysis of

151 molecular variance (AMOVA; Excoffier et al., 1992) to detect structure between populations

152   based on comparisons between geographically defined groups. According to our results,

153   regions on both sides of the Isthmus of Tehuantepec were also evaluated (IT, east and

154   west).

155   To evaluate the isolation by distance among geographic regions, we performed a

156   Mantel Test with 1000 iterations, comparing matrices of genetic and geographic distances,

157   using the program zt v1.1 (Bonnet & de Peer, 2002). Statistical analyses were not

158   performed in the MIA group because of limited number of samples (n=2), but were

159   considered in haplotype networks and phylogenetic analyses.

160

161   *Demographic analyses.*

162   To evaluate demography and population stability, we obtained Tajima's D and Fu's

163   *Fs* values, in Arlequin v2.11 (Excoffier et al., 2005), with 1000 replicates (mtDNA database).

164   Using the same program, parameters, and database, we further evaluated the historical

165   demography of each group under an expansion model with a MISMATCH distribution test

166   and estimated its significance with the raggedness index (Slatkin & Hudson, 1991; Rogers &

167   Harpending, 1992; Harpending, 1994). To analyze variation in effective population size

168   over time, we used Bayesian skyline plots (BSP; Drummond et al., 2005) performed in

169   BEAST v1.6.0 (Drummond & Rambaut, 2007), with 10 million steps for mtDNA, using a

170   mean rate of 0.023 substitutions per site per lineage per million years (s/s/l/My), under

171   Control Region and ATPase estimates (Lerner et al., 2011).

172

173   *Evolutionary Models and Phylogenetic analyses.*

174   For each molecular marker (mtDNA and nuclear DNA), we calculated the

175   evolutionary model that best fit the data based on the Akaike Information Criterion AIC

176   (Akaike, 1987) using jModelTest 0.1.1 (Posada, 2008). We performed a phylogenetic

177   reconstruction using the Bayesian Inference (BI) approach in Mr. Bayes v3.0 (Huelsenbeck

178   & Ronquist, 2002). We assigned different evolutionary models to each gene partition. We

179   ran four simultaneous chains for each Monte Carlo Markov Chain analysis for 50 million

180   generations, sampling every 1000 generations. The results were visualized to ensure ESS

181   (effective sample sizes) values higher than 200, and the burn-in value was determined

182   using Tracer v1.6.0 (Rambaut et al., 2013). The initial 20% of generations were eliminated.

183   The remaining trees were used to construct a majority rule consensus tree with posterior

184   probability distributions, which was visualized using the program FigTree v1.2.3

185   (http//tree.bio.ed.ac.uk/software/figtree/).

186

187   *Morphological variation.*

188   To examine morphological variation between groups of *L. rhami*, we took five

189   measurements from 208 voucher specimens (Table S2) corresponding to four of the five

190   geographic groups defined *a priori* (SMO, GRO, CHIS, CA). These specimens were available

191   from the following collections: Museo de Zoología "Alfonso L. Herrera" (MZFC, UNAM),

192   Museum of Comparative Zoology (MCZ), American Museum of Natural History (AMNH),

193   Donald R. Dickey Bird and Mammal Collection (BMC), and the Moore Lab of Zoology (MLZ).

194   Measurements for bill length (from the base to the tip of the upper mandible), bill width

195   (width at the nostrils), bill depth (from the upper mandible to the base of the bill at the

196   nostrils), and wing chord (distance from the carpal joint to the tip of the longest primary)

197   were taken with a dial calliper with a precision of 0.1 mm, while tail length (distance from

198   the uropigial gland to the tip of the longest rectrix) was determined with a milimetric ruler.

199   A single observer took all measurements. Subsets of individuals were measured twice to

200   confirm consistency between measurements using correlation coefficients obtained in

201   STATISTICA v7 (StatSoft, 2004). When variation among measurements was low or null, all

202   voucher specimens were measured once and these values were used in further analyses. To

203   test the normality of our data, we performed a Lilliefors (Kolmogorov-Smirnov) test using

204   the R package Nortest v1.0-4 (Gross & Liges, 2015). This test was conducted with raw and

205   log-transformed data. Since the data were not normally distributed, a Wilcoxon/Mann-

206   Whitney test (Bauer, 1972) was performed to evaluate differences between males and

207   females. To evaluate differences among groups, and after confirming differences among

208   sexes, we conducted two sets of Kruskall-Wallis tests (Hollander & Wolfe, 1973) to

209   compare: 1) geographic groups (SMO, GRO, CHIS, CA), and 2) groups separated by the

210   Isthmus of Tehuantepec (east, west). All tests were conducted for each variable, treating

211   males and females separately. Statistical analyses were performed using RStudio v.1.1.447

212   (RStudio Team, 2016).

213

214   *Species delimitation and divergence times*

215   According to the results, we assess the limits between different groups based on: 1)

216   disjunct populations (groups by geographic region: SMO, GRO, MIA, CHIS), 2) phylogroups

217   (SMO/MIA, GRO, CHIS), and 3) groups separated by the Isthmus of Tehuantepec (east:

218   SMO/MIA/GRO, and west: CHIS). We used the command line of coalescent approach

219   implemented in Bayesian Phylogenetics and Phylogeography software (BP&P v3.4, Rannala

220   & Yang, 2003; Yang & Rannala, 2010). This method uses the multispecies coalescent model

221   (MSC) to compare different models of species delimitation (Yang and Rannala, 2010; Rannala

222   and Yang, 2013) and species phylogeny (Yang and Rannala, 2014; Rannala and Yang, 2017) in a

223   Bayesian framework, accounting for incomplete lineage sorting due to ancestral polymorphism

224   and gene tree-species tree discordance. We used the concatenated data set of eight molecular

225   markers (mt DNA and nuclear DNA), but mitochondrial markers were treated as one locus,

226   so the total number of this parameter was set to nloci=5. To confirm consistency between

227   runs, we performed multiple analyses using algorithms 0 and 1 (0: species tree given as

228   fixed, 1: species tree given treated as the guide tree), selecting different seed number

229   between runs and changing finetune parameters (ε, algorithm prior), as suggested by Yang

230   and Rannala (2010). After confirming consistency between runs, the subsequent analyses

231   were performed according to species delimitation using a user-specified guide tree

232   (speciesdelimitation =1, speciestree = 0), with values of ε=5. The analyses were conducted

233   using parameter finetune=1, which allows the program to make automatic adjustments to

234   prior parameters. Because different values of θ (ancestral population size, the product of

235   effective population size N and mutation rate μ per site) can result in different posterior

236   probabilities for the same guide tree (Leaché & Fujita, 2010; Yang, 2015), we used three

237   different values: 1) low θ priors (0.0001, IG: 3, 0.0002), 2) medium θ priors (0.001, IG: 3,

238   0.002), and 3) high θ priors (0.01, IG: 3, 0.02). The inverse gamma prior to τ (species

239   divergence times) was set to tauprior=3, 0.03, 1.5% of sequence divergence. Each analysis

240   was run with the reversible-jump Markov chain Monte Carlo algorithm (rjMCMC) for 100

241   thousand generations, sampling every 5, and discarding 30 thousand generations as burn-

242   in.

243   To estimate divergence times, we decided to evaluate the hypothesis of three

244   independent groups based on full evidence (phylogroups: SMO/MIA, n=12; GRO, n=9; CHIS,

245   n=10). We used the concatenated data set (mt DNA and nuclear DNA) that included data

246   from *Eugenes fulgens, E. spectabilis*, and *Tilmatura dupontii* as outgroups. For each

247   partition, we assigned the previous selected evolutionary model. This analysis was

248   performed using StarBeast (*Beast; Heled & Drummond, 2010). We employed an

249   uncorrelated lognormal relaxed clock and a Yule process speciation model to model the

250   tree prior. We assigned a calibration node based on a secondary calibration obtained for

251   the split between the "Mountain Gems" clade (*L. rhami, E. fulgens*, and *E. spectabilis*) and

252   "Bees" clade (*T. dupontii*; 12.5 Mya; McGuire et al., 2014). We incorporated mean

253   substitution rates reported previously (ATPase 6 and 8, ND2, ND4: Pacheco et al., 2011; CR:

254   Lerner et al., 2011; AK1, BFib, MUSK, ODC: McGuire et al., 2014). Three independent

255   analyses were run for 30 million generations, sampling every 1000. The log and tree files

256   from each analysis were combined in LogCombiner, and visualized in Tracer to confirm

257   convergence and ensure acceptable ESS values (ESS>200). We discarded the first 15% of

258   trees as burn-in. We used TreeAnnotator v1.8.2 (Rambaut & Drummond, 2007) to

259   summarize trees as a maximum clade credibility tree, and to obtain mean divergence times

260   with 95% highest posterior density intervals. The resulting tree was visualized in FigTree

261   v.1.2.3.

262

263   **Results**

264   *Genetic diversity and population structure.*

265   We obtained a concatenated dataset of 1402 bp for 54 individuals (527 bp of CR and

266   875 bp of ATPase 6 & 8). The complementary dataset of five molecular markers for 31

267   individuals included 875 bp of ATPase 6 and 8, 527 bp of CR, 918 bp of ND2, 521 bp of ND4,

268   758 bp of BFib, 559 bp of MUSK, 495 bp of ODC, and 416 bp of AK1. The initial dataset

269   included 33 haplotypes (24 found with CR and 15 with ATPase 6 & 8). Estimates of

270   haplotype and nucleotide diversity are presented in Table 1. Overall, high haplotype

271   diversity and low nucleotide diversity were observed within groups (SMO, GRO, CHIS).

272   The mtDNA network revealed significant population structure within the *L. rhami*

273   complex (Fig. 1). There was a clear separation between populations on either side of the IT,

274   which were separated by twelve mutational steps, while individuals of GRO were closely

275   linked to those of the SMO. In general, the most frequent haplotype was present in

276   populations from the Sierra Madre Oriental group (SMO). Haplotypes from GRO tended to

277   separate from the main haplotype. In Table 2, F_{ST} values confirm high levels of geographic

278   structure between regions. This further translates into a significant correlation between

279   the genetic distance and the geographic distance matrices, according to the Mantel test,

280   thus suggesting isolation by distance between groups (r = 0.87, p < 0.005).

281   AMOVA results indicated that the highest genetic variation was observed among

282   rather than within populations, with similar percentages when grouping populations

283   according to geographic groups or on either side of the IT: 76.41% and 78.74%

284   respectively (*P* <0.0001, Table 3).

285

286   *Demographic analyses*

287   Differing conclusions among the methods used to evaluate demographic history led

288   to ambiguous results. The occurrence of historical population expansion was supported by

289   negative and significant values of neutrality tests (Tajima's *D* and Fu's *Fs*), except for

290   Tajima's *D* statistic in SMO and GRO groups (Table 1). A mismatch distribution unimodal

291   curve was recovered for CHIS population, but no significant values of the raggedness index

292   indicated possible demographic expansion in all populations, as curves under the

293   expansion model did not deviate from a unimodal distribution. BSP estimates revealed that

294   effective population size was flat across time for GRO. This pattern was also found for CHIS,

295   however, the higher posterior density low interval suggested a growing demographic

296   tendency, and a subtle demographic expansion was recovered in SMO population (Fig. 2).

297

298   *Evolutionary models and Phylogenetic analyses.*

299   We obtained a concatenated dataset of 5069 bp. The best-fit models for each

300   molecular marker were as follows: HKY (MUSK), HKY+I (ATPase 6 and 8, ND4), HKY+G

301   (AK1), HKY+I+G (CR), TNR+G (ND2), TPM3uf (ODC), and TPM2uf+I (BFib). Phylogenetic

302   relationships using the multilocus dataset resulted in one main monophyletic group

303   corresponding to individuals from west of the IT (PP > 0.95, Fig. 3: Bayesian Inference).

304   Most individuals from east of the IT were grouped into two well-supported separate clades,

305   but no resolution was recovered for three individuals from this region. Moreover, one well-

306   supported clade included most individuals from GRO group from west of the IT, with the

307   rest of individuals merged in a polytomy with individuals from SMO region.

308

309   *Morphological variation.*

310   Normality of our data was rejected and there was dimorphism between males and

311 females in all variables (Table S4). General comparisons between geographic groups

312 resulted in significant differences in all variables for both males and females, while

313 comparisons between groups separated by the Isthmus of Tehuantepec showed significant

314 differences in bill width (χ^2 = 6.3669, p = 0.01163) and wing chord (χ^2 = 8.0642, p =

315 0.004515) for males; in females all traits were statistically different except for bill length

316 (Table 4). Paired differences between geographic groups (comparing each group against

317 the others), revealed that CA and GRO present significant differences in several traits

318 (Table S4).

319

320 *Species delimitation and divergence times*

321   Three different species hypotheses were assessed: A) species delimited by disjunct

322 populations (groups by geographic region: SMO, GRO, MIA and CHIS), B) phylogroups

323 (SMO/MIA, GRO and CHIS), and C) groups separated by the Isthmus of Tehuantepec (east:

324 SMO/MIA/GRO, and west: CHIS), using different values of θ priors (population size

325 parameters) to test the sensitivity of the species delimitation results. BP&P analyses testing

326 the first hypothesis (that allopatric populations are different species) resulted in low

327 statistical support for the split of groups SMO and MIA, and suggested the split of GRO

328 group as a valid species only when using low θ priors. The high probabilities for an

329 ancestral node suggest splitting into multiple species, as well as the validity of CHIS as an

330 independent group (Fig. 4A). Analysis of the three lineages corresponding to phylogroups

331 resulted in high speciation probabilities in all cases, except when using high θ priors (0.01,

332 IG: 3, 0.02) for the splitting of GRO and SMO/MIA groups (Fig. 4B). Analysis of the two-

333   lineage hypothesis (i.e. two groups separated by the Isthmus of Tehuantepec) suggested

334   that these groups are separate species (Fig. 4C). Varying the θ priors affected the resulting

335   speciation probabilities; higher θ priors resulted in lower probabilities than lower θ priors.

336   Our divergence time estimates (Fig. 4D) showed that the split between *L. rhami* complex

337   and its sister group (genus *Eugenes*) was around 10.55 Mya (8.28-13.14 Mya). The estimate

338   for the first split within the *L. rhami* complex was dated ca. 0.207 Mya (0.091-0.317 Mya),

339   corresponding to the divergence between populations on either side of the IT. The split

340   between the groups from west of the IT (SMO/MIA and GRO) was dated at ~0.087 Mya

341   (0.035-0.146 Mya). The independence of groups was supported by high values of posterior

342   probability.

343

344   **Discussion**

345   Our study provides evidence of high levels of genetic and morphometric

346   differentiation among most of the disjunct populations comprising the *L. rhami* complex.

347   Our results support the existence of four separate evolutionary lineages: SMO/MIA (*rhami*),

348   GRO (*occidentalis*), CHIS (new suggested taxon: *tacanensis*), and CA (*saturatior*). The

349   AMOVA and F_{ST} values also indicate the presence of strong population structure between

350   these same geographic regions (e.g. 76.41% variation among populations). We found

351   significant morphological differences between the southern populations (Honduras and El

352   Salvador), which we expect to be reflected in genetic differentiation, though we were

353   unable to test this hypothesis here due to a lack of tissue samples.

354   Morphometric variation in the 208 specimens of *L. rhami* also showed geographic

355   structure among the compared areas (four of the five regions were defined *a priori*).

356   Although the group sampled in the highlands of Chiapas and Guatemala (CHIS) was the

357   most genetically differentiated, the populations of Guerrero (GRO) and Central America

358   (CA) were the most different in morphometric traits. We had no access to genetic samples

359   from the CA region (Honduras and El Salvador), so we cannot confirm if this morphological

360   variation is consistent at the genetic level. Also, we did not have access to enough voucher

361   specimens from MIA to conduct a reasonable morphological statistical analysis. A larger

362   sampling effort in the southern highlands of Oaxaca (Miahuatlan, Mexico) and in Central

363   America (Honduras and El Salvador) will allow evaluation of species limits in these regions.

364   The genetic variation found in the *L. rhami* complex corresponds to a phylogenetic

365   discontinuity and a spatial vicariance pattern (Avise et al., 1987) resulting from long-term

366   isolation and/or restricted gene flow among groups, probably promoted by geographic

367   barriers. This pattern of high genetic differentiation is congruent with other studies of

368   Mesoamerican vertebrate species (Barber, 1999; Zarza et al., 2008; Bonaccorso, 2009;

369   Bryson et al., 2011; Smith et al., 2011; Arbeláez-Cortés et al., 2014; Castañeda-Rico et al.,

370   2014). In Trochilidae, high levels of geographic structure have been previously reported,

371   related to differences in current or historical ecological conditions (*Adelomyia*

372   *melanogenys*: Chaves et al., 2007; *Lampornis amethystinus*: Cortés-Rodríguez et al., 2008;

373   Ornelas et al., 2016). Also, moderate levels of differentiation have been found in

374   hummingbird species co-distributed in Mesoamerican cloud forests (*Campylopterus*

375   *curvipennis*: González et al., 2011; *Amazilia cyanocephala*: Rodríguez-Gómez et al., 2013).

376   As expected, the levels of genetic variation were correlated with a pattern of isolation by

377   distance associated with the patchy distribution of cloud forests, where particular

378   environmental characteristics have been reported as drivers of differentiation between

379   populations (Ramírez-Barahona & Eguiarte, 2014). In the case of populations from west of

380   the Isthmus of Tehuantepec, geographic structure could be explained by limited gene flow

381   between regions (SMO, MIA and GRO) promoted by isolation by distance. In contrast, the

382   genetic separation between populations on either side of the Isthmus of Tehuantepec is

383   almost certainly influenced by this geographic barrier in addition to distance.

384   Many phylogeography studies have shown the influence of the Isthmus of

385   Tehuantepec as a driver of isolation in Mesoamerican species. This valley in southeastern

386   Mexico is located near three tectonic plates -North American, Cocos and Caribbean-

387   resulting from different tectonic episodes that took place in the Late Miocene (Barrier, et al.

388   1998). Two main diversification events across the Isthmus of Tehuantepec were detected

389   in the regional bird fauna, placing both events within the Pleistocene (Barber & Klicka,

390   2010). It is clear that ecological conditions in this area act as a geographic barrier limiting

391   gene flow of *L. rhami* populations across the Isthmus, and the lack of shared haplotypes

392   across this area demostrates that the Isthmus of Tehuantepec is better considered a hard

393   rather than soft barrier as has been proposed in co-distributed species (e. g. *Amazilia*

394   *cyanocephala*; Rodríguez-Gómez et al., 2013).

395   Divergence time estimates show that the split between putative lineages was very

396   recent. The lack of reciprocal monophyly in the multilocus phylogenetic reconstruction

397   (Bayesian inference) may be influenced by low nuclear marker signal due to the temporal

398   scale, resulting in a failure to reconstruct discrete clades in recently evolved species

399   (Knowles & Carstens, 2007). This signal of ancestral polymorphism and/or incomplete

400   lineage sorting is taken into account in BP&P estimates, where information of the chosen

401   molecular markers is fully used in closely related species (Yang, 2015). Results of species

402 delimitation were sensitive to different θ priors, favoring lumping with higher values, as

403 has been previously reported (McKay et al., 2013). However, splitting of phylogroups was

404 consistently supported in most cases. These findings reveal the potential for recognizing at

405 least three distinct cryptic species, also supported by other criteria evaluated here

406 (morphometric differences, population structure and geographic isolation).

407 Our evaluations of demographic history used different methods (neutrality tests,

408 mismatch distributions and BSP), showing ambiguous patterns of populations dynamics.

409 Range expansion was revealed in the basal group CHIS (Tajima's D and Fu's Fs, mismatch),

410 and subtle population size changes over time were detected by the BSP approach.

411 Additionally, expansion signal was not fully supported in the SMO group, and population

412 stability was found in the youngest clade, GRO. Our divergence time estimates provide

413 evidence of recent Pleistocene diversification of the *L. rhami* complex, with the first

414 population split occurring in the Isthmus of Tehuantepec (0.207 Mya, 0.091-0.317 Mya),

415 followed by subsequent separation of groups west of the Isthmus, resulting in the splitting

416 of the SMO/MIA and GRO groups (0.087 Mya, 0.07-0.21 Mya). These processes took place

417 during the Pleistocene, when climatic fluctuations resulted in the expansion and

418 contraction of the ranges of highland species, promoting allopatric differentiation (Still et

419 al., 1999). Our estimates of recent splitting support the hypothesis of differentiation

420 promoted by climatic oscillations rather than by older events related to the complex

421 volcanic history of Mexican highlands, such as mountain uplift.

422 Two demographic scenarios for cloud forests species during Pleistocene have been

423 proposed, and they corresponded to the dry refugia and the moist forests hypotheses

424 (Ramírez-Barahona & Eguiarte, 2013). The dry hypothesis (Haffer, 1969) suggests that

425   during glacial cycles, climatic oscillations in cloud forests displaced them downslope into

426   refugia, forcing populations to contract their ranges, which subsequently expanded and

427   recolonized them during interglacial cycles. The moist forest hypothesis suggests that

428   unchanging precipitation conditions did not reduce cloud forests into refugia but favored

429   downslope altitudinal migration, where adapted species expanded their ranges during

430   glacial cycles (connectivity), and fragmented them into higher altitudes during interglacial

431   periods. Historical signals of demographic expansion and high levels of structure are more

432   related to dry refugia, while low levels of structure are consistent with moist forest model

433   due to processes of recurrent population connectivity. Therefore, the high levels of genetic

434   structure found in *L. rhami* are consistent with the dry refugia model. Population dynamics

435   in CHIS were led by down-slope fragmented ranges (glacial cycles) and expanding up-slope

436   (interglacials) revealed by the unimodal distribution of allele differences. In contrast, the

437   SMO and GRO populations show no clear evidence of expansion, and seem to have been

438   maintained *in situ*, a hyphothesis first supported by the shrub *Moussonia deppeana*, a

439   Mesoamerican cloud-forest adapted species (Ornelas & González, 2014).

440   Despite the well-known movement abilities of Trochilidae species, some studies

441   have found that geographical barriers are crucial in promoting high levels of differentiation

442   and the diversification of independent evolutionary lineages in various regions, such as the

443   Andes region (e.g. *Adelomyia melanogenys*, Chaves & Smith, 2011), Mesoamerica (Ornelas

444   et al., 2016), the Motagua fault region (Rodríguez-Gómez & Ornelas, 2014), and the Isthmus

445   of Tehuantepec (Cortés-Rodríguez et al., 2008; González et al., 2011). In contrast, in

446   lowland Neotropical birds, high levels of intraspecific diversification are better explained

447   by the hypothesis of limited dispersal ability (Burney & Brumfield, 2009). *L. rhami* exhibits

448   some altitudinal movements related to the presence of resources available along elevation

449   gradients (Schuchmann & Boesman, 2018), but long-distance dispersal has not been

450   reported for this species, so both geographic barriers and limited longitudinal and

451   latitudinal dispersal movements could be influencing the geographic separation we

452   observed.

453   Earlier taxonomic studies described different subspecies for this complex: *L. r.*

454   *rhami* (Lesson, 1839; Peters, 1945), *L. r. occidentalis* (Phillips, 1966), and *L. r. saturatior*

455   (Griscom, 1932; Peters, 1945). Our study supports the taxonomic validity of the

456   *occidentalis* (based on genetic and morphometric data) and *saturatior* (based on

457   morphometric data) groups. Thus, the suggestion of considering *occidentalis* and *saturatior*

458   taxa as races (Schuchmann & Boesman, 2018) should be reevaluated.

459   The original description of *L. r. rhami* comprises populations in the highlands of

460   central and southern Mexico, including populations in the state of Guerrero, and

461   populations in Chiapas and Guatemala, which, in agreement with our study, belong to

462   different lineages. Therefore, *rhami* will just include populations from the Sierra Madre

463   Oriental and the highlands of Oaxaca, while *occidentalis* belongs to populations distributed

464   in the highlands of Guerrero. Finally, populations in Chiapas and Guatemala belong to a

465   new suggested taxon. The Honduras and El Salvador populations belong to *saturatior,* for

466   which the differentiation is supported by our morphometric results. Supported by our

467   multilocus phylogenetic approach, by the species delimitation estimates, and by the

468   differences in morphometric traits, we found that *L. rhami* is a complex formed by four

469   groups that correspond to separate evolutionary lineages and that should be treated as full

470   species. The importance of delimitation of taxonomic units increases given the level of

471   threat that is reported for the cloud forests of Mesoamerica (resulting from the growth of

472   agricultural and urban areas), and the restricted geographic distribution of *L. rhami*. The

473   problem of an incorrect placement of subspecies is that this could promote

474   underestimation of biodiversity, and therefore mismanagement in conservation efforts

475   (Zink, 2004).

476

477   **Conclusions**

478   This study presents clear evidence of morphometric and genetic differentiation

479   between populations of the hummingbird *Lamprolaima rhami*. Pleistocene historical

480   events, the influence of the Isthmus of Tehuantepec as a geographical barrier, and the

481   effects of isolation by distance have shaped the geographical structure found in the *L. rhami*

482   complex. Contemporary habitat fragmentation and the unique bioclimatic characteristics of

483   cloud forests are probably still influencing this pattern of isolation between populations. In

484   general, we found that species diversity within the *L. rhami* complex is currently

485   underestimated, and four taxa should be recognized: 1) populations from the Sierra Madre

486   Oriental and highlands in Oaxaca (*rhami*), 2) populations from highlands in Guerrero

487   (*occidentalis*), 3) populations from highlands in Chiapas and Guatemala (new suggested

488   taxon: *tacanensis*), and 4) populations from highlands in Honduras and El Salvador

489   (*saturatior*). This study emphasizes the importance of evaluating multiple characters in

490   species complexes that presumably diverged recently or are in an incipient process of

491   speciation.

492

493   **Acknowledgements**

494   We thank the Museo de Zoología Alfonso L. Herrera (MZFC, UNAM), Museum of

495   Natural Science (LSU), Museum of Vertebrate Zoology at University of California Berkeley

496   (MVZ), D. Dittman (LSU), C. Cicero (MVZ), and R. Bowie (MVZ) for provided tissues samples

497   and logistical help; The Moore Lab of Zoology at Occidental College (MLZ), Bird and

498   Mammal Collection at the University of California Los Angeles (UCLA), American Museum

499   of Natural History (AMNH), Museum of Comparative Zoology at Harvard University (MCZ),

500   J. McCormack (MLZ), W. Tsai (MLZ), J. Maley (MLZ), K. Molina (UCLA), P. Sweet (AMNH), L.

501   Garetano (AMNH), J. Trimble (MCZ), K. Eldridge (MCZ), for provided assistance and help in

502   carrying out morphological measurements on museum specimens; A. Gordillo, Isabel

503   Vargas, S. Robles, and F. Rebón for technical help; G.F. Calvillo for drawing *L. rhami* (Figure

504   1), and to all the collectors at MZFC. J. J. Morrone, and C. Cordero provided helpful

505   comments that improved the manuscript. L. Kiere reviewed the English.

506

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722

Figure 1

Geographic distribution of the Lamprolaima rhami complex

Geographic distribution of the *Lamprolaima rhami* complex. Hexagons represent sampled localities corresponding to tissues used in this study. Geographic groups defined *a priori* are drawn in different colors. Geographic groups: Sierra Madre Oriental (SMO, *blue*), highlands of Guerrero (GRO, *green*), Sierra of Miahuatlan in Oaxaca (MIA, *yellow*), highlands of Chiapas and Guatemala (CHIS, *red*), highlands of Honduras and El Salvador, Cental America (CA, *purple*). The statistical parsimony haplotype network for 54 individuals of *L. rhami* constructed with concatenated mtDNA dataset (ATPase 6 and 8, and control region) is shown above the map. The size of each circle is proportional to the number of individuals carrying each haplotype. Illustration of *L. rhami* by Giselle Fernanda Calvillo García.

geographic region / subspecies

■ SMO (*L. r. rhami*)
■ MIA (*L. r. rhami*)
■ GRO (*L. r. occidentalis*)
■ CHIS (*L. r. rhami*)
■ CA (*L. r. saturatior*)

Sierra Madre Oriental

Sierra Madre del Sur

Isthmus of Tehuantepec

Chiapas highlands

Guatemala highlands

Honduras

El Salvador

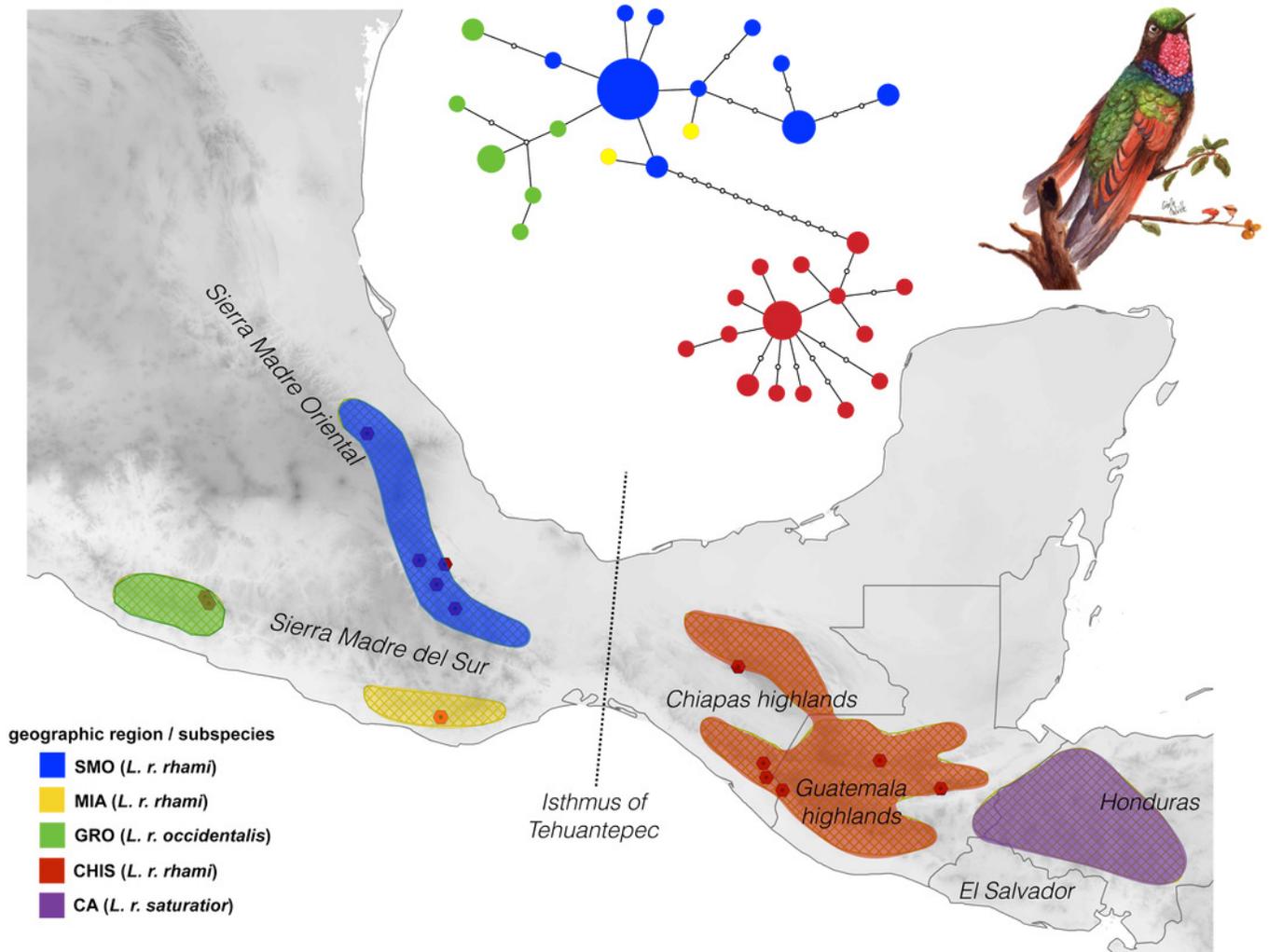
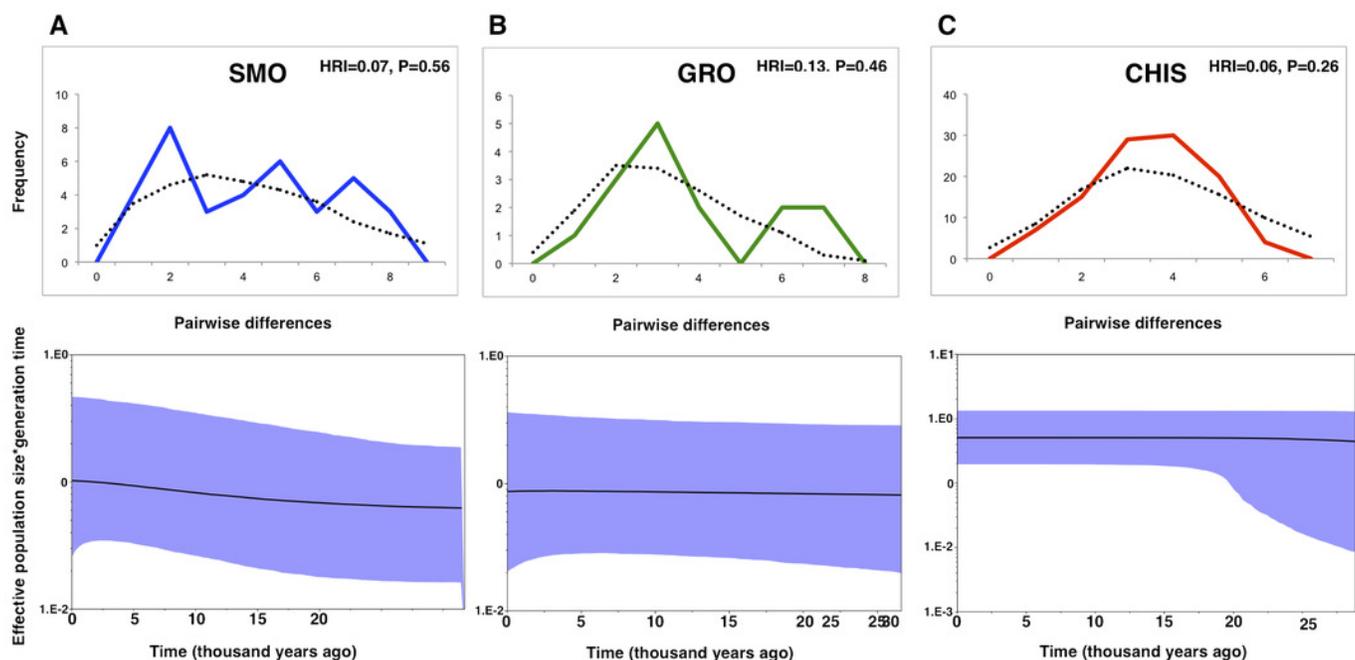
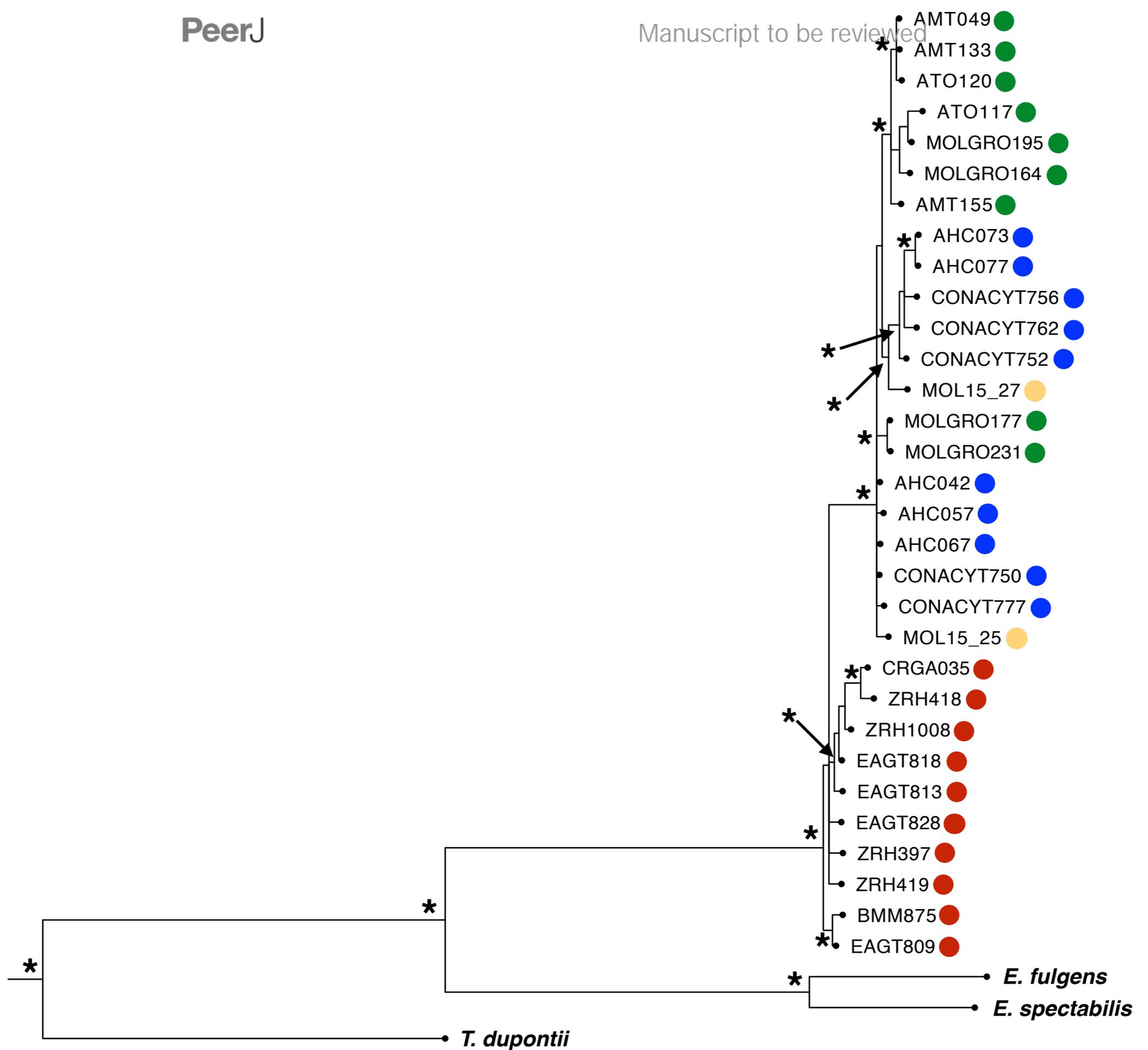



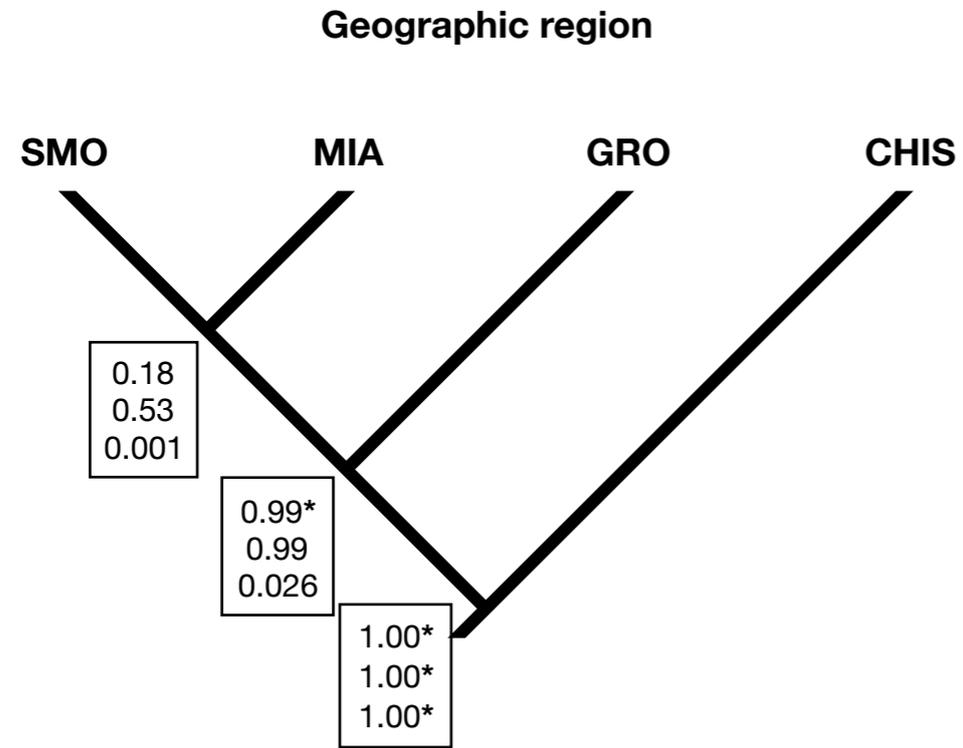
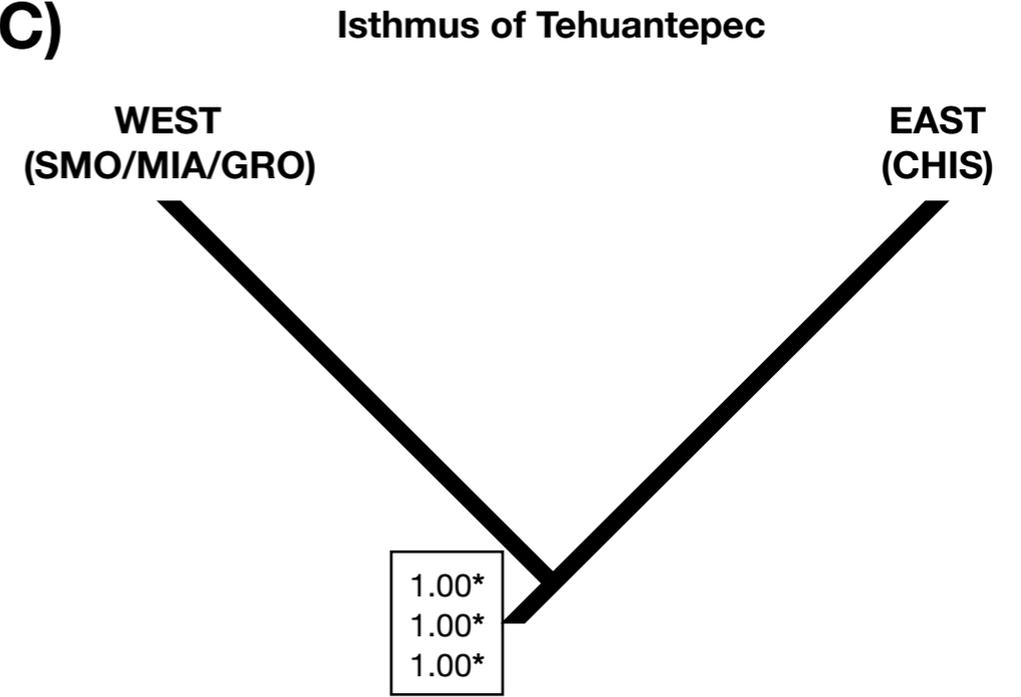
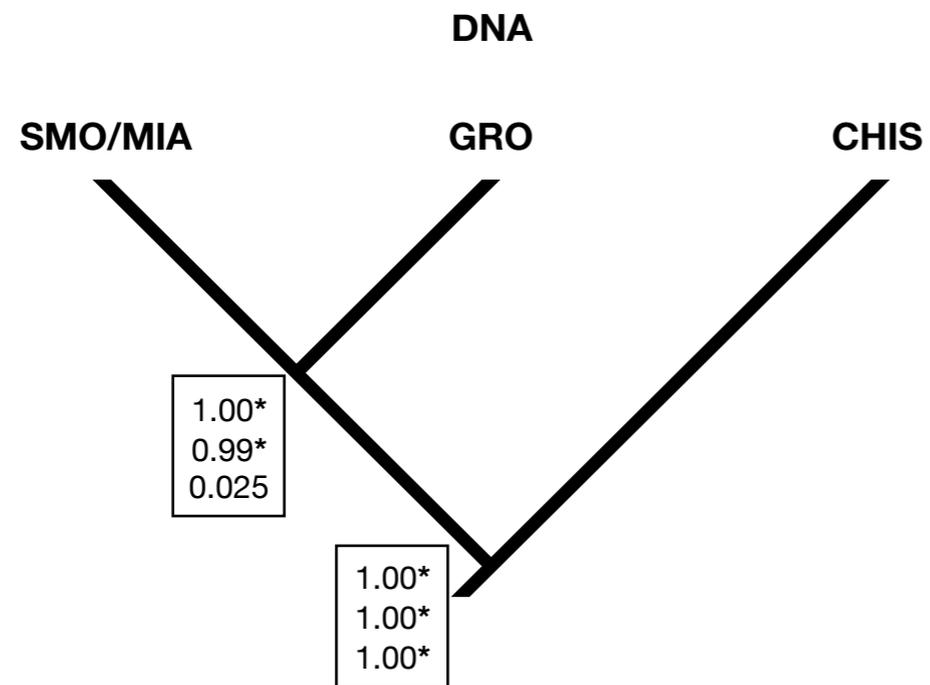
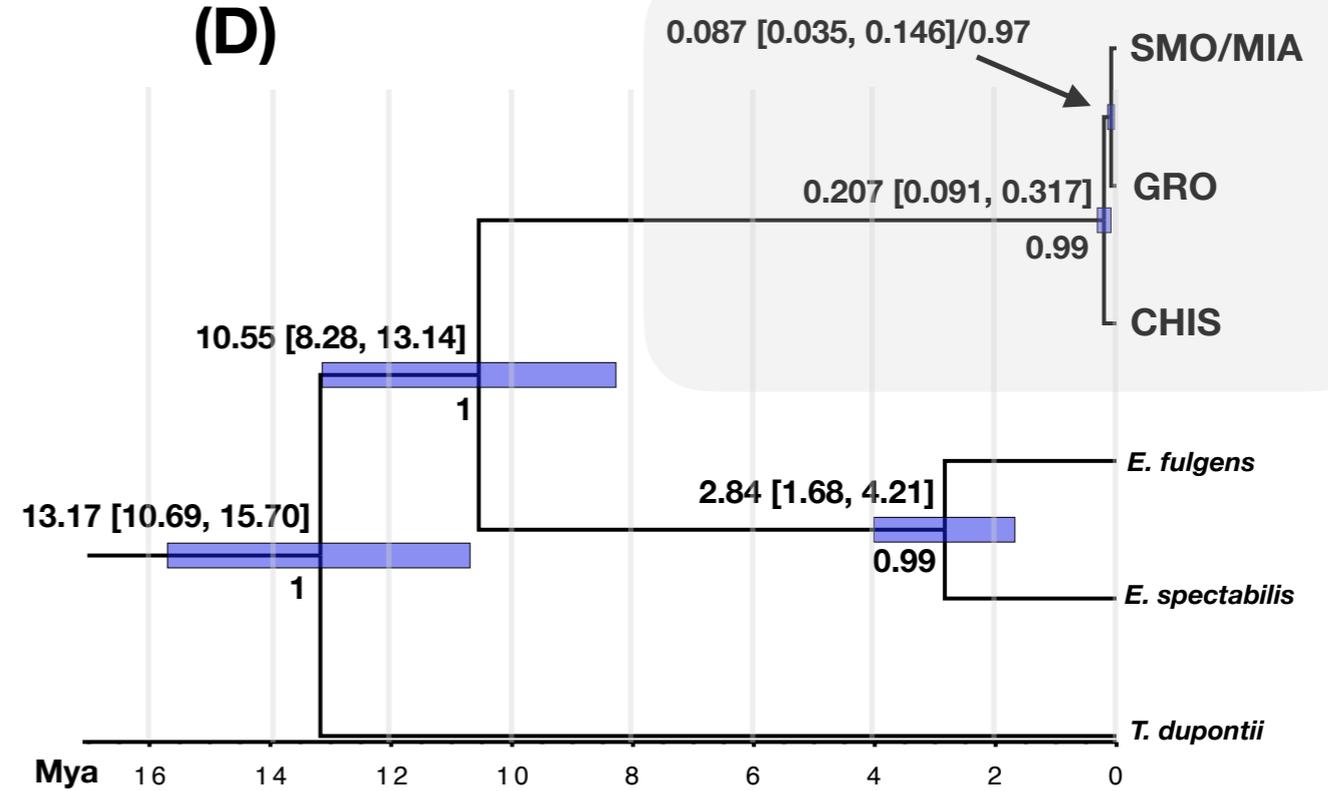
Figure 2

Mismatch distribution and Bayesian skyline plots

Mismatch distributions and Bayesian skyline plots for three geographic groups (A. Sierra Madre Oriental, SMO; B. highlands of Guerrero, GRO; C. highlands of Chiapas and Guatemala, CHIS) of *L. rhami* (mtDNA: CR, ATPase 6 and 8). In mismatch distributions, solid lines indicate the observed distributions of pairwise differences, and dotted lines represent simulated distributions under a model of population expansion. In Bayesian skyline plots, solid lines represent median estimates and shaded areas represent 95% confidence intervals.

Phylogenetic Bayesian Inference

Phylogenetic Bayesian Inference reconstruction of 31 individuals from the *L. rhami* complex using mitochondrial and nuclear markers (ATPase 6 and 8, CR, ND2, ND4, MUSK, BFib, ODC, and AK1). Posterior probabilities PP>0.95 are shown (*). Different colors represent different groups according to the geographic regions defined *a priori* (see Figure 1 legend).

0.007

Species delimitation and divergence times

Results from a coalescent-based species delimitation analysis implemented in Bayesian Phylogenetics and Phylogeography (BP&P) of three possible hypotheses based on: (A) disjunct populations (groups by geographic region: SMO, GRO, MIA, CHIS), (B) DNA groups (SMO/MIA, GRO, CHIS), and (C) groups separated by the Isthmus of Tehuantepec (east: SMO/MIA/GRO, and west: CHIS). Speciation probabilities for each node are shown in boxes: top, low θ prior (0.0001); middle, medium q prior (0.001); bottom, high q prior (0.01). * Indicates statistical support for splitting (posterior probabilities of 0.95 or higher). (D) Divergence times and Bayesian species tree topology (*BEAST) for *L. rhami* complex, bars on each node represent 95% of high posterior densities of divergence times (HPD), Mya (Million years ago). Posterior probabilities are shown below node ages.

Statistical parameters

Statistical parameters of genetic diversity, population structure and population demography for mtDNA (CR, ATPase 6 and 8). n: number of sequences used, h: number of haplotypes, Hd: haplotype diversity, π: nucleotide diversity, Pi: mean number of pairwise differences. *p-value<0.05, **p-value<0.01. ***p-value<0.001.

1 Statistical parameters of genetic diversity, population structure and population demography for
2 mtDNA (CR, ATPase 6 and 8). n: number of sequences used, h: number of haplotypes, Hd:
3 haplotype diversity, π: nucleotide diversity, Pi: mean number of pairwise differences. *p-
4 value<0.05*, **p-value<0.01. ***p-value<0.001.

GROUP	n	h	Hd	π	Pi(theta)	Tajima's D	Fu's Fs Test
SMO	22	9	0.81	0.0022	4.22	-0.559	-5.505**
GRO	9	6	0.89	0.0026	3.73	-0.886	-2.77*
CHIS	21	15	0.94	0.0021	3.50	-1.988*	-14.93***

Population pairwise

Population pairwise F_{ST} mtDNA (CR, ATPase 6 and 8). *p-value<0.05.

1   Population pairwise F_{ST} mtDNA (CR, ATPase 6 and 8). *p-value<0.05.

	SMO	GRO	CHIS
SMO	----		
GRO	0.176*	----	
CHIS	0.769*	0.784*	----

AMOVA results

AMOVA results on *Lamprolaima rhami* populations defined according to geographic groups, and groups on either side of the Isthmus of Tehuantepec using mtDNA (CR, ATPase 6 and 8). ***p-value<0.0001.

1 AMOVA results on *Lamprolaima rhami* populations defined according to geographic groups,
2 and groups on either side of the Isthmus of Tehuantepec using mtDNA (CR, ATPase 6 and 8).
3 ***p*-value<0.0001.

	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices
Geographic groups (*a priori*)					
Among populations	2	167.63	5.04	76.41	
Within populations	49	76.23	1.56	23.59	
Total	51	243.87	6.59		F_{ST}=0.76***
Groups on either side of the Isthmus of Tehuantepec					
Among populations	1	159.05	6.28	78.74	
Within populations	50	84.82	1.70	21.26	
Total	51	243.86	7.98		F_{ST}=0.79***

Multiple Kruskall-Wallis tests

Multiple Kruskall-Wallis tests to evaluate differences between groups: a) by geographic region (SMO, GRO, CHIS, CA), and b) by groups on either side of the Isthmus of Tehuantepec (IT, east and west).

1 Multiple Kruskall-Wallis tests to evaluate differences between groups: a) by geographic
2 region (SMO, GRO, CHIS, CA), and b) by groups on either side of the Isthmus of Tehuantepec
3 (IT, east and west).
4

		a) Geographic region			b) IT		
		χ^2	df	p-value	χ^2	df	p-value
MALES	Bill length	7.8661	3	**0.04886**	1.6847	1	0.1943
	Bill width	19.386	3	**0.0002275**	6.3669	1	**0.01163**
	Bill depth	26.425	3	**7.77E-06**	0.91719	1	0.3382
	Wing chord	19.14	3	**0.0002558**	8.0642	1	**0.004515**
	Tail length	16.982	3	**0.0007127**	1.834	1	0.1757
FEMALES	Bill length	9.4421	3	**0.02396**	0.40845	1	0.5228
	Bill width	13.125	3	**0.004374**	6.346	1	**0.01176**
	Bill depth	20.159	3	**0.0001574**	5.3064	1	**0.02125**
	Wing chord	29.698	3	**1.60E-06**	18.547	1	**1.66E-05**
	Tail length	28.092	3	**3.48E-06**	10.168	1	**0.001429**