Genetic and phenotypic divergence in the Garnet-Throated Hummingbird *Lamprolaima rhami* (Aves: Trochilidae) (#25606)

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

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Background. Cloud forests are one of the most endangered ecosystems in the Americas, but also one of the richest in biological diversity in the world. The species inhabiting these forests are susceptible to changes in these environments and characterized by high levels of geographic structure. The Garnet-Throated Hummingbird *Lamprolaima rhami* mainly inhabits cloud forests, but can also be found in different habitats at elevations between 1200 and 3000 m, such as forest edges, pine-oak forests, upper tropical forests and scrub. Five disjunct regions have been delimited as the current geoprafic distribution for the species from Mexico to Honduras. According to variation in size and color, three taxa have been described: *L. r. rhami* (subspecies) restricted to the Mexican highlands and Guatemala, *L. r. occidentalis* (race) distributed in Guerrero (Mexico), and *L. r. saturatior* (subspecies), distributed in the highlands from Honduras and El Salvador.

Methods. Here, we analyzed if *L. rhami* presents high levels of geographic structure and describe the importance of this study at the population level for a species with a highly restricted distribution in Mesoamerica. We used mitochondrial DNA (subunits 6 and 8 of ATPase, and Control Region) to analyze the genetic variation of 54 individuals. We also evaluated morphological variation in 213 specimens. In addition we analized demographic history (mtDNA), estimated divergence times (nuclear and mtDNA), and produce a multilocus phylogeny (31 *L. rhami* individuals, using four mitochondrial and four nuclear genes) to evaluate phylogenetic relationships within the species.

Results. We found high levels of genetic differentiation in three groups (SMO&SMSn, SMS, and EITn), and significant variation in morphological traits in four groups (SMO&SMS, SMS, EITn, EITs) that corresponded with the disjunct geographic distribution for the species. In general, *L. rhami* presents population stability with the highest genetic variation explained among populations, confirmed by isolation by distance test between groups. Divergence time estimates suggest that *L. rhami* split from its sister group around 10.5 million years ago, and the diversification of the complex was dated ca. 0.61 Mya, during Pleistocene.

Discussion. This study provides evidence of genetic and morphological differentiation between populations in *L. rhami* complex. This differentiation is consistent with geographic areas where the species occurs. Three groups are supported by genetic and morphological data to be consider separate evolutionary lineages: SMO & SMSn, SMS (belonging to *occidentalis* subspecies), and EITn. The Isthmus of Tehuantepec (as geographic barrier), isolation by distance factor (fragmented habitat), and the climatic conditions during Pleistocene represent the main promoters into the geographic structure found in *L. rhami* complex.

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Abstract

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Keywords: *Lamprolaima rhami*, Trochilidae, Mesoamerica, Cloud Forest, Hummingbirds

Introduction

Cloud forests are one of the mostly threatened and biodiverse habitats in the world (Hamilton, 1995; Mulligan, 2010). In Mesoamerica, that biologically represents the transition zone between the Nearctic and Neotropical regions (Ríos-Muñoz, 2013; Morrone, 2014). The cloud forests are restricted to a highland particular sector between 600 and 3000 m above sea level (Foster, 2001). Several studies have tried to describe the evolutionary proceses that have shaped the enormous diversity of cloud forests, concluding

that species show high levels of isolation and population differentiation when compared to those geographically interconnected habitats (Ataroff & Rada, 2000; de Barcellos & Voltolini, 1995; Ornelas et al., 2013). Lack on population differentiation studies could result in underestimating biodiversity as discussed elsewhere (Arbeláez-Cortés & Navarro-Sigüenza, 2013; Bonaccorso et al., 2008; McCormack et al., 2008).

Recently, different studies have focused in describing historical patterns and recognizing new species (Cortés-Rodríguez et al., 2008; González et al., 2011; González-Rodríguez et al., 2004; Ornelas et al., 2010). However, the number of species that inhabit these forests is far from been fully studied, while the pace at which these forests are disappearing due to anthropogenic causes is pushing researches to study more species before the damage would be irrevocable (Martínez-Morales, 2005; Olander et al., 1998).

The Trochilidae family is a well known reference in biological studies containning interesting species models for evolutionary studies (Bleiweiss, 1998a; McGuire et al., 2007), although only a few of them focus on species inhabiting cloud forests (Bleiweiss, 1998b; Chaves et al., 2007; Chaves & Smith, 2011; Cortés-Rodríguez et al., 2008). The Garnet-Throated Hummingbird, *Lamprolaima rhami* (Lesson, 1839), is one of such taxa. It has a restricted Mesoamerican distribution inhabiting upper tropical forest, pine-oak forest, scrub, but primarily occupies cloud forest habitats, comprising an altitudinal range between 1200 and 3000 m (Howell & Webb, 1995; Schuchmann & Boesman, 2018). It is considered a sedentary species, with seasonal movements to higher elevations during breeding season above 1500 m (Schuchmann & Boesman, 2018).

L. rhami has a discontinuos distribution with five distinguishable geographic areas: 1) Sierra Madre Oriental (from Puebla to Veracruz) and northern highlands in Oaxaca

(Mexico), 2) Sierra Madre del Sur, Guerrero (Mexico), 3) southern highlands in Oaxaca (Mexico), 4) highlands of Chiapas (Mexico) and Guatemala, and 5) highlands of Honduras and El Salvador. According to differences on size and color, three taxa have been recognized, and only one presents geographic correspondence with one of the five regions described above (i. e. *L. r. occidentalis*). *L. r. rhami* (Lesson, 1839) is restricted to the highlands of central and southern Mexico (in the states of Puebla, Veracruz, Oaxaca and Chiapas) and Guatemala highlands (Peters, 1945; Schuchmann & Boesman, 2018). *L. r. occidentalis* (Phillips, 1966) has been described as a race, and corresponds to populations found in a restricted patch in south western Mexico, Guerrero (Schuchmann & Boesman, 2018). *L. r. saturatior* (Griscom, 1932) is found in the highlands of Honduras and El Salvador (Peters, 1945; Schuchmann & Boesman, 2018). Contrasting, some authors do not recognize proposed taxa, and consider that *L. rhami* has no distinguishable geographical groups (AOS, 2018; Gill & Donsker, 2018; Howell & Webb, 1995; Ridgway, 1911).

Even when *L. rhami* can be found in different habitats than cloud forest (e. g. pine-oak forests), the vulnerability of these habitats impacts on the stability of the species that move to those sites during year. According to its restricted distribution (isolated areas), and being a resident of mainly high fragmented forests with unique bioclimatic characteristics, *L. rhami* represents an interesting model to assess evolutionary hypotheses about geographic structure and populations dynamics that should be considered in conservation plans. Hence, the main objectives of this paper were: 1) to evaluate the genetic and morphological variation of the *Lamprolaima rhami* complex comparing individuals from the five regions where it is distributed, 2) to describe the phylogenetic relationships within *L. rhami* using a multilocus dataset (nuclear and

mitochondrial DNA), and 3) to propose an hypothesis on its evolutionary history. Based on cloud forest characteristics and site fidelity of this hummingbird species, we would expect to find high levels of genetic structure supported by congruence in morphological variation within *L. rhami* complex. Thus, and under patterns described on phylogeography (Avise et al., 1987), phylogenetic discontinuities and spatial disjunction are expected rather than phylogenetic continuity and lack of spatial disjunction.

Methods

Taxon sampling and sequencing.

We obtained tissues from 54 individuals of *L. rhami* from 14 localities across most of its geographic range (Table S1, Fig. S2). We defined five groups *a priori* to evaluate genetic and morphological variation between the five regions where the species occurs: 1) the Sierra Madre Oriental and the northern portion of the Sierra Madre del Sur (SMO&nSMS), 2) the Sierra Madre del Sur, Guerrero (SMS), 3) the Sierra Sur de Oaxaca (SSO), 4) the highlands of Chiapas and Guatemala (EITn), and 5) the highlands of Honduras and El Salvador (EITs). Tissue samples were obtained for four geographic groups excepting for EITs group, and provided by different biological collections, including the "Museo de Zoología Alfonso L. Herrera" (Universidad Nacional Autónoma de México, Secretaría del Medio Ambiente y Recursos Naturales: scientific collecting permit FAUT-0169), the Museum of Natural Science (Lousiana State University), and the Museum of Vertebrate Zoology (University of California, Berkeley).

DNA was extracted using the DNAeasy™ kit (Qiagen Inc., Valencia, CA, USA), and following manufacturer's protocols. For evaluating the general genetic variation of the

130 complex, two mitochondrial markers were obtained for the 54 samples (Control Region,
131 *CR*; and subunits 6 and 8 from ATPase gene, *ATPase 6 & 8*), while for evaluating the
132 phylogenetic relationships between groups, two additional mitochondrial markers and four
133 nuclear regions were surveyed for a subsample of 31 individuals (NADH dehydrogenase
134 subunit 2, *ND2*; NADH dehydrogenase subunit 4, *ND4*; the 7th intron of the beta fibrinogen
135 gene, *BFib*, the regions between exons 4 and 5 of the Muscle Skeletal Receptor Tyrosine
136 Kinase gene, *MUSK*; a segment comprising the end of exon 6 and the beginning of exon 8 of
137 the Ornithine Decarboxylase gene, *ODC,* and intron 5 of adenylate kinase gene, *AK1*). These
138 31 individuals were chosen to analyze phylogenetic relationships by increasing loci
139 number. Also, we included sequences from these molecular markers available in GenBank
140 for *Eugenes fulgens, E. spectabilis, Heliomaster longirostris*, *Atthis heloisa*, *Doricha eliza*, and
141 *Tilmatura dupontii* as sister groups and outgroups (McGuire et al., 2007; Zamudio-Beltrán
142 & Hernández-Baños, 2015).
143 We amplified these molecular markers via the polymerase chain reaction (PCR)
144 using specific primers and protocols (Table S3). Reactions contained 10X buffer (1.25 µL),
145 10mM dNTP (0.19 µL), 50 mM $MgCl_2$ (0.38 µL), 10 µM of each primer (0.25 µL), 0.1 µL of
146 *Taq* (INVITROGEN), and 0.5 µL of genomic DNA (12.5 µL total volume). PCR products were
147 visualized on a 1% agarose gel, and DNA sequencing was performed in the High-
148 Throughput Genomics Unit Service of the University of Washington. We edited and aligned
149 chromatograms with Sequencher v4.8 (GeneCodes Corporation, Ann Arbor, MI). All
150 sequences were deposited in FigShare (https://figshare.com/s/dac62b5c84ac760d8613).
151
152 *Population structure.*

153 To evaluate the number of haplotypes and their relationships, statistical parsimony
154 haplotype networks were constructed for each mitochondrial marker (CR and ATPase 6 &
155 8) and for their concatenated dataset using the program TCS v1.21 (Clement et al., 2000).
156 To analyze genetic diversity and genetic structure, we obtained values of haplotype
157 diversity, nucleotide diversity, mean number of pairwise differences, and population F_{ST}
158 values. These analyses were performed with 1000 replicates, using the program Arlequin
159 v3.11 (Excoffier et al., 2005). Using the same program, we conducted an analysis of
160 molecular variance (AMOVA; Excoffier et al., 1992) to detect structure between
161 populations, based on comparisons between groups defined geographically, and according
162 to our results, we also evaluated regions at both sides of the Isthmus of Tehuantepec (IT,
163 east and west).
164 To evaluate the isolation by distance among geographic regions, we performed a
165 Mantel Test with 1000 iterations, comparing matrices of genetic and geographic distances,
166 using the program zt v1.1 (Bonnet & de Peer, 2002). Statistical analyses were not
167 performed in SSO group because of limited number of samples (n=2), but were considered
168 in haplotype networks and phylogenetic analyses.
169
170 *Demographic analyses.*
171 To evaluate demography and population stability, we obtained Tajima's *D* and Fu's
172 *Fs* values, in Arlequin v2.11 (Excoffier et al., 2005), with 1000 replicates (mtDNA database).
173 Using the same program, parameters, and database we further evaluated the historical
174 demography of each group under an expansion model with a MISMATCH distribution test
175 and estimated its significance with the raggedness index (Harpending, 1994; Rogers &

Harpending, 1992; Slatkin & Hudson, 1991). To analyze variation in effective population size through time, we used Bayesian skyline plots (BSP; Drummond et al., 2005) performed in BEAST v1.6.0 (Drummond & Rambaut, 2007), with 10 million steps for mtDNA, using a mean rate of 0.023 substitutions per site per lineage per million years (s/s/l/My), under Control Region and ATPase estimates (Lerner et al., 2011).

Evolutionary Models and Phylogenetic analyses.

For each molecular marker (mtDNA and nuclear DNA), we calculated the evolutionary model that better fits the data using jModelTest 0.1.1. (Posada, 2008), based on the Akaike Information Criterion AIC (Akaike, 1987). We performed a phylogenetic reconstruction with the Bayesian Inference (BI) approach available in Mr. Bayes v3.0 (Huelsenbeck & Ronquist, 2002). We assigned different evolutionary models to each gene partition. We ran four simultaneous chains for each Monte Carlo Markov Chain analysis for 5 million generations, and sampling every 250 generations. We determined the burn-in value using Tracer v1.6.0 (Rambaut et al., 2013), and eliminated the initial 20% of generations. The remaining trees were used to construct a majority rule consensus tree with posterior probability distributions, which was visualized using the program FigTree v1.2.3 (http//tree.bio.ed.ac.uk/software/figtree/).

We performed a species tree estimate using StarBeast (*Beast, Heled & Drummond, 2010) to test the uncertainty associated with each group and the limits between our three populations with multilocus sequence data corresponding to SMO&SMSn, SMS, and EITn (mtDNA and nuclear DNA). We ran 100 million generations, sampling every 10 thousand,

198 discarding the first 20% as burn-in. Convergence was visualized in the program Tracer
199 v1.6.0., and we used TreeAnnotator v1.8.2 to summarize trees.
200
201 *Divergence times.*
202 Divergence time estimates were obtained using BEAST v1.6.0 (Drummond &
203 Rambaut, 2007). We used the concatenated data set (mt DNA and nuclear DNA) that
204 included data from *Eugenes fulgens, E. spectabilis*, *Heliomaster longirostris*, *Atthis heloisa*,
205 *Doricha eliza*, and *Tilmatura dupontii* as outgroups. For each partition, we assigned
206 previous selected evolutionary model. We employed a uncorrelated lognormal relaxed
207 clock, and a Birth-Death process speciation model to model the tree prior. We assigned a
208 calibration node based on a secondary calibration obtained for the split between the
209 "Mountain Gems" clade (*L. rhami*, *E. fulgens*, *E. spectabilis*, and *H. longirostris*) and "Bees"
210 clade (*A. heloisa*, *D. eliza*, and *T. dupontii*; 12.5 Mya; McGuire et al., 2014). We incorporated
211 mean substitution rates reported previously (ATPase 6 and 8, ND2, ND4: Pacheco et al.,
212 2011; CR: Lerner et al., 2011; AK1, BFib, MUSK, ODC: McGuire et al., 2014). This analysis
213 was run for 50 million generations, sampling every 2000 generations, with a burnin period
214 of 20%. We used TreeAnnotator v1.8.2 (Rambaut & Drummond, 2007) to summarize the
215 sampled trees as a maximum clade credibility tree, and to obtain mean divergence times
216 with 95% highest posterior density intervals.
217
218 *Morphological variation.*
219 To examine morphological variation between groups of *L. rhami*, we took five
220 measures from 213 voucher specimens corresponding to four or the five geographic

groups defined *a priori* (SMO&SMSn, SMS, EITn, EITs). These specimens were available from different biological collections, including the Museo de Zoología Alfonso L. Herrera (UNAM), the Museum of Comparative Zoology (MCZ), the American Museum of Natural History (AMNH), the Bird and Mammal Collection (UCLA), and the Moore Lab of Zoology (MLZ).

Measures for bill length (from the upper base of the bill to the tip of the upper mandible), bill width (width by the location of the nostrils), bill depth (from the upper mandible to the base of the bill by the location of the nostrils), and wing chord (distance from the carpal joint to the tip of the longest primary) were taken with a dial calliper with a precision of 0.1 mm , while tail length (distance from the uropigial gland to the tip of the longest rectrix) was determined with a milimetric ruler. All measurements were taken by a single observer. We performed a statistical analysis (*t*-student test) to detect significative differences between males and females using the statistical software STATISTICA v7 (StatSoft, 2004). Subsequently usign the same program, we performed an analysis of variance (ANOVA) comparing four groups defined *a priori* for each variable, treating males and females separately, and performed a post-hoc analysis (Fisher's Least Significant Difference Test, LSD; Williams & Abdi, 2010) to detect significant differences between groups.

A category classification was performed with a discriminant analysis using the five morphological measurements as independent variables, and the geographic groups defined as grouping variable. In a second discriminant analysis we used as grouping variable the populations east and west of the Isthmus of Tehuantepec (IT). The results of discriminant

243 analysis were plotted in R statistical software (Ripley, 2001), using the package ggplot2
244 (Wickham, 2009).
245
246 **Results**
247 *Genetic diversity and population structure*.
248 We obtained a concatenated dataset of 1402 bp for 54 individuals (527 bp of the CR
249 and 875 bp of ATPase 6 & 8). The complementary dataset of ~~five~~ molecular markers for 31
250 individuals included 875 bp of ATPase 6 and 8, 527 bp of CR, 918 bp of ND2, 521 bp of ND4,
251 758 bp of BFib, 559 bp of MUSK, 495 bp of ODC, and 416 bp of AK1. The initial dataset
252 included 33 haplotypes (24 found with CR and 15 with ATPase 6 & 8). Estimates of
253 haplotype and nucleotide diversity can be found in Table 1. Overall, high values of
254 haplotype diversity, and low levels of nucleotide diversity were observed within groups
255 (SMO&SMSn, SMS, EITn).
256 ~~Haplotype~~ networks revealed a significant population structure within the *L. rhami*
257 complex (Fig. 1). There was a clear separation between populations at both sides of the IT,
258 which were separated by three to twelve mutation steps depending on the dataset, while
259 the localities of the SMS were closely linked to those of the SMO&SMSn and SSO. In general,
260 the most frequent haplotype was present in populations from the Sierra Madre Oriental
261 and the northern portion of the Sierra Madre del Sur group (SMO&SMSn). Haplotypes from
262 SMS showed a tendendy of separation from the main haplotype. In Table 2, we can observe
263 that F_{ST} values confirm high levels of geographic structure between regions, moreover all
264 values are significant. This further translated into a significant correlation between the

265 genetic distance and the geographic distance matrices, according to the Mantel test, thus
266 suggesting isolation by distance between groups (r=0.87, p<0.005).
267 AMOVA results indicated that the highest genetic variation was observed among and
268 not within populations, with similar percentages when grouping populations according to
269 geographic region or at both sides of the IT, 76.41% and 78.74% respectively (*P*<0.0001,
270 Table 3).
271
272 *Demographic analyses*
273 The different methods used to evaluate demographic history resulted in ambiguous
274 results. The occurence of historical population expansion was supported by negative and
275 significant values of neutrality tests (Tajima's *D* and Fu's *Fs*), except that for Tajima's *D*
276 statistic in SMO&SMSn and SMS populations (Table 1). Mismatch distribution unimodal
277 curve was recovered only for EIT population, but no significant values of raggedness index
278 indicated possible demographic expansion in all populations as curves under the expansion
279 model did not deviate from a unimodal distribution. BSP estimates revealed that effective
280 population size was flat across time for SMS. This pattern was also found for EIT, however,
281 higher posterior density low interval presented a growing demographic tendency, and
282 subtle demographic expansion is recovered in SMO&SMS population (Fig. 2).
283
284 *Evolutionary models and Phylogenetic analyses.*
285 We obtained a concatenated dataset of 5069 bp. The best-fit models for each
286 molecular marker were as follows: HKY (MUSK), HKY+I (ATPase 6 and 8, ND4), HKY+G
287 (AK1), HKY+I+G (CR), TNR+G (ND2), TPM3uf (ODC), and TPM2uf+I (BFib). Phylogenetic

relationships using multilocus dataset resulted in one main monophyletic group corresponding to individuals at west from the IT (PP>0.95, Fig. 3: Bayesian Inference). Most individuals at east from the IT were grouped into two well-supported separated clades, but no resolution was recovered for three individuals from this region. Moreover, one well-supported clade includes most individuals from SMS group at west from the IT, the rest of individuals are merged in a politomy with individuals from SMO&SMSn region.

We tested the evolutionary independency of individuals grouped in three regions: SMO&SMSn, SMS, and EITn (groups defined *a priori* as independent ones), and obtained high values of posterior probability in the species tree estimate in all cases (Fig. 3, *BEAST).

Divergence times.

Our divergence time estimates (Fig. 4) showed that the split between *L. rhami* complex and its sister group (genus *Eugenes*) was dated around 10.50 Mya (8.34-12.83 Mya). Time estimate for *L. rhami* complex was dated ca. 0.61 Mya (0.41-0.84 Mya), that corresponded to the divergence between populations at both sides of the IT. Group at west from the IT (SMO&SMSn and SMS) was dated at ~0.24 Mya (0.15-0.34 Mya), and within this group the clade corresponded to SMS region was dated around 0.14 Mya (0.07-0.21 Mya). Finally east group (EITn) was dated around 0.24 Mya (0.15-0.35 Mya).

Morphological variation.

Dimorphism tests between males and females revealed significant differences for all variables. Females showed differences for all variables excepting for bill lenght ($F=2.35$, $p=0.07$), while in the males all variables showed significant statistically differences

311 between groups (Fig. S4). In all cases, except in female bill lenght comparison, statistical
312 differences between groups were detected when LSD test was performed. Such differences
313 were further observed in the discriminant analyses using only the first two cannonical
314 roots (Fig. 5). For both sexes the most informative variables were bill depth and bill lenght.
315 Comparisons between groups were significant in all cases except between group A
316 (SMO&SMSn) and D (EITn).
317
318 **Discussion**
319 Our study provides evidence of high levels of genetic and morphological
320 differentiation with geographic correspondence among most of the disjunct areas where *L.*
321 *rhami* complex occurs. Three groups are supported by genetic and morphological data to
322 be consider separate evolutionary lineages: SMO & SMSn, SMS (belonging to *occidentalis*
323 subspecies), and EITn. AMOVA and F_{ST} values also indicate presence of a strong population
324 structure between these regions (e.g. 76.41% variation among populations). Even though
325 we genetically analized most of the five areas where *L. rhami* inhabits, further work is
326 needed, including a larger sampling effort in the southern highlands of Oaxaca (Mexico),
327 and Central American highlands (Hounduras and El Salvador) to properly evaluate species
328 limits. According to morphological traits, the southern populations (Honduras and El
329 Salvador) showed significant differences that must be supported by genetic information
330 lacking in our analyses.
331 Results of phenotypic variation for 213 voucher specimens of *L. rhami* showed
332 geographic structure between analyzed areas (four of the five regions defined *a priori*).
333 Despite that the group sampled east of the Isthmus of Tehuantepec (EITn) was the most

334 genetically differentiated, the morphological results showed that groups at Sierra Madre
335 del Sur (SMS), and the southern group east of the Isthmus of Tehuantepec (EITs,
336 corresponding to *saturatior* subspecies), were the most differentiated under phenotypic
337 traits. Unfortunately, we had no access to samples from EITs region (Honduras and El
338 Salvador), so we could not confirm if this morphological variation is consistent at genetic
339 level. Also, we neither had access to enough voucher specimens from SSO to conduct a
340 reasonable morphological statistical analysis.

341 Under the original descriptions of phylogeographic patterns, the genetic variation
342 found in *L. rhami* corresponds to a phylogenetic discontinuity and spatial vicariance
343 pattern (Avise et al., 1987), which represents the result of long-term isolation, and/or
344 restricted gene flow among groups, probably promoted by geographic barriers. This
345 pattern of high genetic differentiation is congruent with some others found in many
346 Mesoamerican species of vertebrates (Arbeláez-Cortés et al., 2014; Barber, 1999;
347 Bonaccorso, 2009; Bryson et al., 2011; Castañeda-Rico et al., 2014; Smith et al., 2011; Zarza
348 et al., 2008). In Trochilidae high levels of geographic structure have been reported before,
349 related to differences on present or historical ecological conditions (*Adelomyia*
350 *melanogenys*: Chaves et al., 2007; *Lampornis amethystinus*: Cortés-Rodríguez et al., 2008;
351 Ornelas et al., 2016). Also, moderated levels of differentiation have been found on
352 hummingbird species codistributed in Mesoamerican cloud forests (*Campylopterus*
353 *curvipennis*: González et al., 2011; *Amazilia cyanocephala*: Rodríguez-Gómez et al., 2013).
354 As expected, levels of genetic variation were correlated with a pattern of isolation by
355 distance associated with discontinuous distribution of cloud forests, where particular
356 environmental characteristics have been reported as drivers of differentiation between

357 populations (Ramírez-Barahona & Eguiarte, 2014). In the case of populations west of the
358 Isthmus of Tehuantepec, geographic structure could be explained by limited gene flow
359 between regions (SMO&SMSn, and SMS) promoted by isolation by distance. In contrast, the
360 genetic separation between populations at both sides of the Isthmus of Tehuantepec is
361 certainly influenced by this geographic barrier plus the distance variable.
362 Many phylogeography studies have shown the influence of the Isthmus of
363 Tehuantepec as driver of isolation in Mesoamerican species. This valley in southeastern
364 Mexico, locates near of three plates: North American, Cocos and Caribbean, resulting from
365 different tectonic episodes that took place since the Late Miocene (Barrier, et al. 1998).
366 Two main diversification events across the Isthmus of Tehuantepec were detected in
367 regional fauna placing both events within the Pleistocene (Barber & Klicka, 2010).
368 Divergence time estimates provided evidence of a recent Pleistocene origin of *L.*
369 *rhami* complex that coincides with the split of populations across the Isthmus of
370 Tehuantepec (0.61 Mya, 0.41-0.84 Mya), followed by a subsequent separation of the
371 youngest group found in the Sierra Madre del Sur (0.14 Mya, 0.07-0.21 Mya). According to
372 these estimates, population differentiation may have ocurred from south to north. This
373 process took place during latest Pleistocene, a well known period where highland
374 distributed species expanded and contracted their ranges, promoting allopatric
375 differentiation due to climatic fluctuations (Still et al., 1999). Recent estimates support the
376 hypothesis of differentiation promoted by these climatic oscilations rather than conditions
377 generated during older events such as uplift mountains. Demographic history was
378 evaluated under different methods (neutrality tests, mismatch distributions and BSP),
379 showing ambiguous patterns of populations dynamics. Range expansion is revealed in

380 basal group EIT (Tajima's *D* and Fu's *Fs*, mismatch), and also sublte population size changes
381 through time were detected by the BSP approach. Additionally, expansion signal was not
382 fully supported in SMO&SMSn group, and population stability was found in the youngest
383 clade SMS. Populations west of the Isthmus seem to have mantained population stability
384 through Pleistocene conditions demonstrating more tolerance to climatic changes, in
385 contrast to the southern population belonging to the most genetically differentiated group
386 (EIT).

387 Despite the well-known movement abilities of Trochilidae species, some studies
388 have found that geographical barriers are crucial in promoting high levels of differentiation
389 and in the diversification of independent evolutionary lineages in various regions, such as
390 the Andes region (e.g. *Adelomyia melanogenys*, Chaves & Smith, 2011), Mesoamerica
391 (Ornelas et al., 2016), the Motagua fault region (Rodríguez-Gómez & Ornelas, 2014), and
392 the Isthmus of Tehuantepec (Cortés-Rodríguez et al., 2008; González et al., 2011). By
393 contrast, there is a hypothesis suggesting that the high levels of intraspecific diversification
394 found mostly on lowland Neotropical birds, are related to limited dispersal ability (Burney
395 & Brumfield, 2009). *L. rhami* exhibits some altitudinal movements related with the
396 presence of resources available along elevational gradients (Schuchmann & Boesman,
397 2018), but long dispersal movements have not been reported for this species, so both
398 factors could be influencing the geographic separation observed herein (geographic
399 barriers and limited longitudinal and latitudinal dispersal movements).

400 Supported by our multilocus phylogenetic approach, by the species tree estimate
401 and the topology recovered from the divergence times, we found that *L. rhami* is a complex
402 conformed by three groups that correspond to separate evolutionary lineages. According to

the most recent taxonomy, no taxa representing geographic variation is recognize for *L. rhami* (AOS, 2018; Gill & Donsker, 2018). However, earlier taxonomic studies described and confirm the existence of different subspecies for this complex: *L. r. rhami* (Lesson, 1839; Peters, 1945; Schuchmann & Boesman, 2018), *L. r. occidentalis* (Phillips, 1966; Schuchmann & Boesman, 2018), and *L. r. saturatior* (Griscom, 1932; Peters, 1945; Schuchmann & Boesman, 2018). Our study provides the existence of *occidentalis* (based on genetic and phenotypic data), and *saturatior* (based on phenotypic differentiation) groups. Original description of *rhami* comprises populations in the highlands of central and southern Mexico, including populations in the state of Guerrero (corresponding to *occidentalis*), which have shown independence according to this study. Within this subspecies (*rhami*), the populations distributed in the highlands of Chiapas and Guatemala are included, but our results indicate that this group represents a different and isolated group. Ultimately, *saturatior* corresponds to populations from Central America (Honduras and El Salvador), which is phenotypically differentiated from the others. The problem of an incorrect placement of subspecies is that this could promote mismanagement in conservation efforts (Zink, 2004). The importance of taxonomic delimitation units increases, taking into account the level of threat that is reported in cloud forests in Mesoamerica (growth of agricultural systems and urban areas), and the reduced geographic distribution of this species.

Conclusions

This study shows evidence of morphological and genetic differentiation between populations within *Lamprolaima rhami*. Pleistocene historical events, the influence of the

Isthmus of Tehuantepec as a geographical barrier, and the effects of isolation by distance have been shaped the geographical structure found in *L. rhami* complex. Also, contemporary fragmentation of habitat and unique bioclimatic characteristics of cloud forests are probably still influencing this pattern of isolation between populations.

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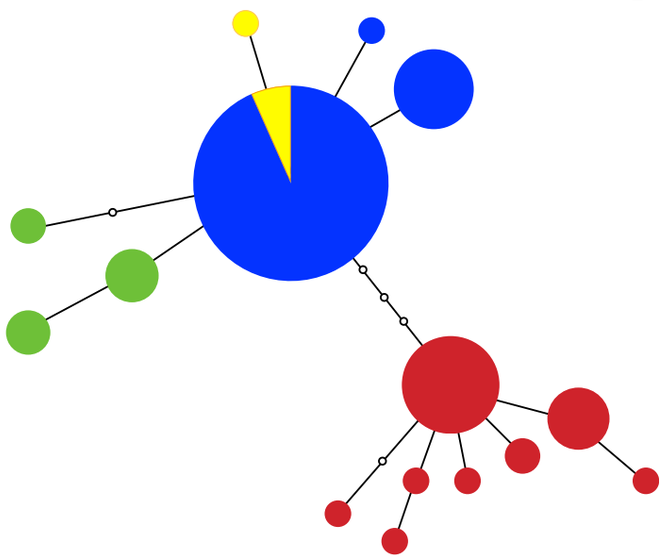
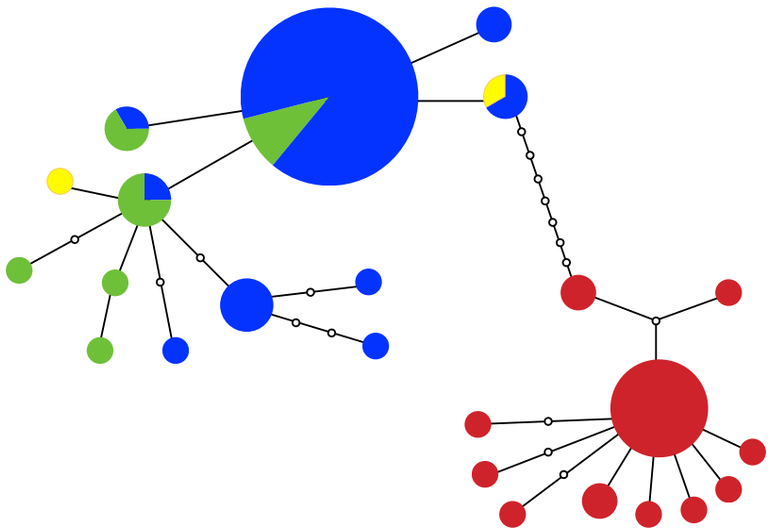
Figure 1(on next page)

Statistical parsimony haplotype networks

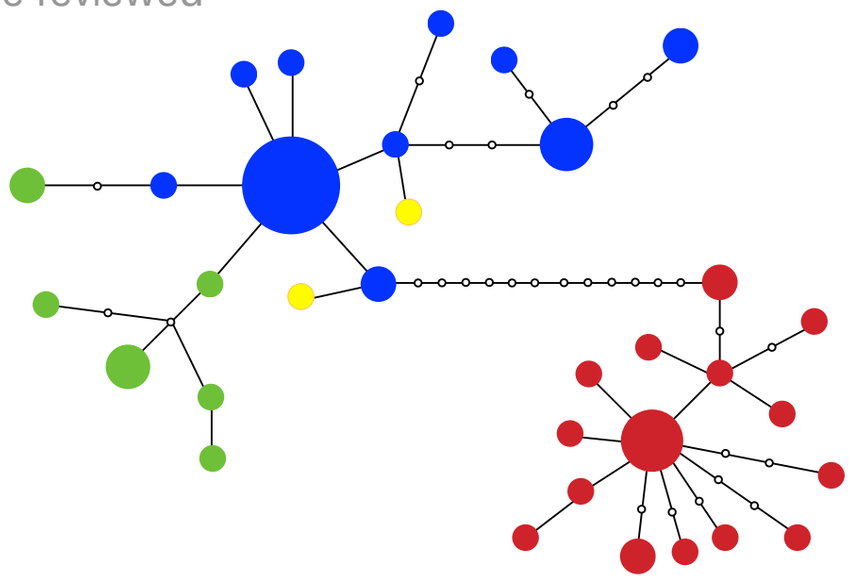
Statistical parsimony haplotype networks for 54 individuals of *L. rhami*, constructed with three different databases: ATPase 6 and 8, CR and concatenated mtDNA markers. Different colors in networks correspond to the different geographic groups on the map. Size of each circle is proportional to the number of individuals carrying each haplotypes



ATPase 6 & 8



CR



CONCATENATED

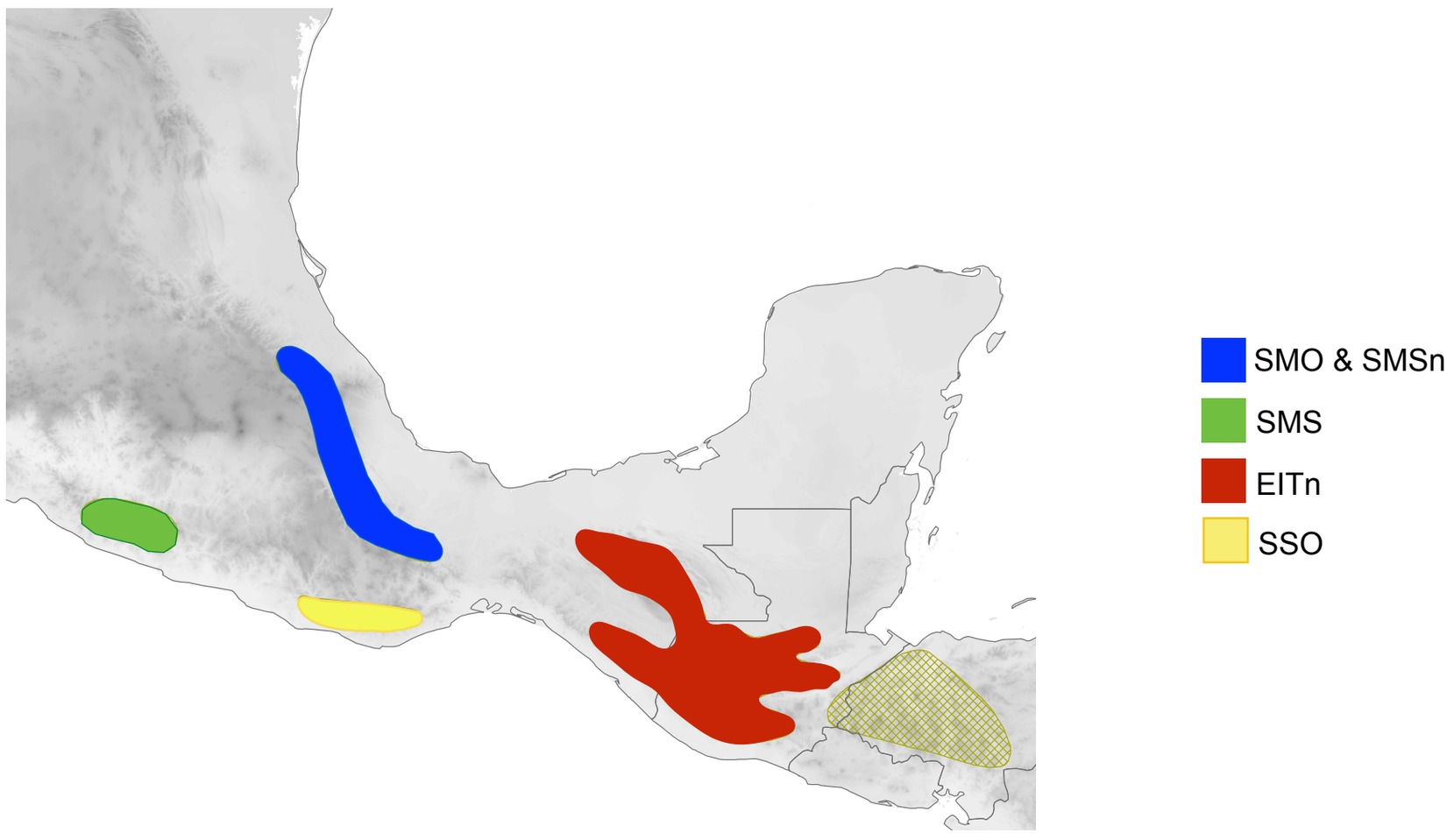
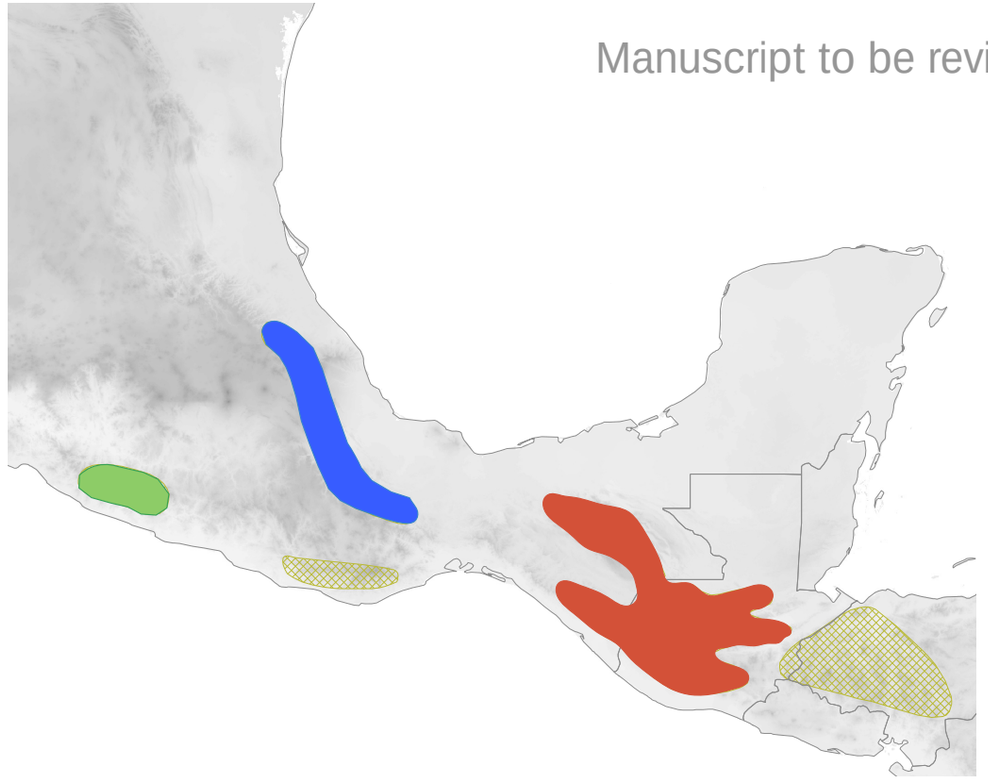
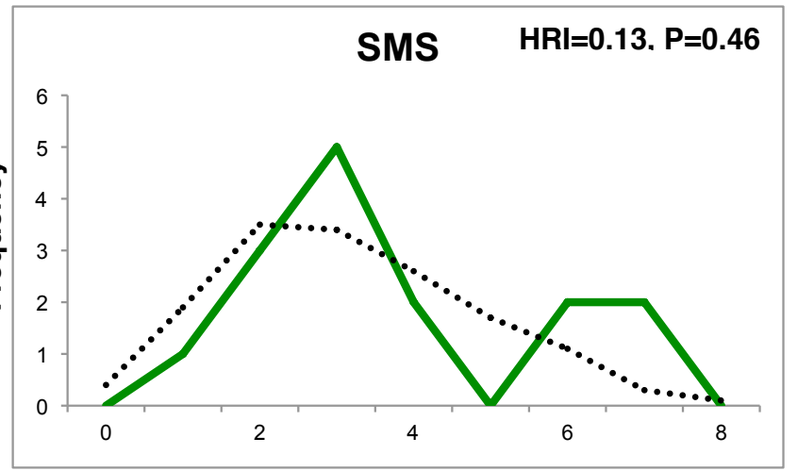


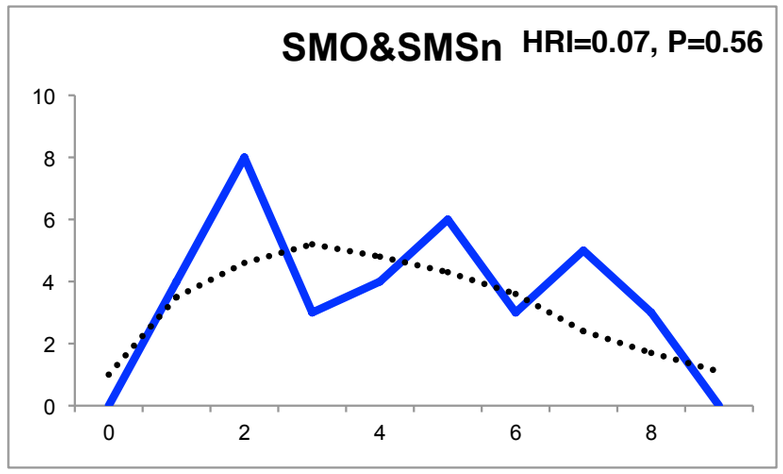
Figure 2(on next page)

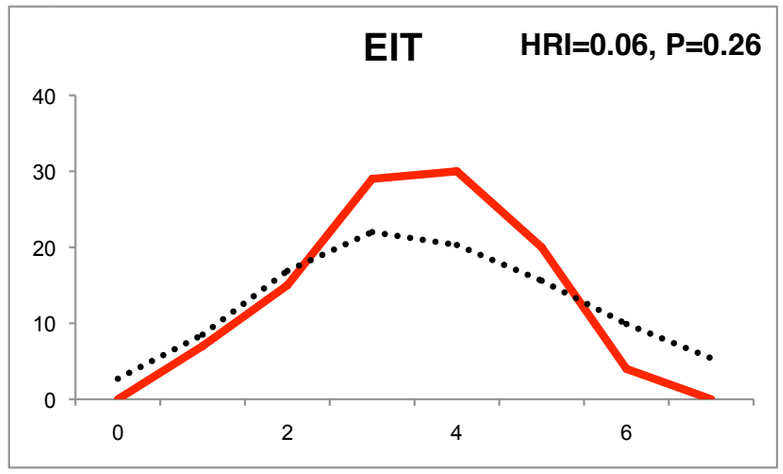
Mismatch distribution and Bayesian skyline plots

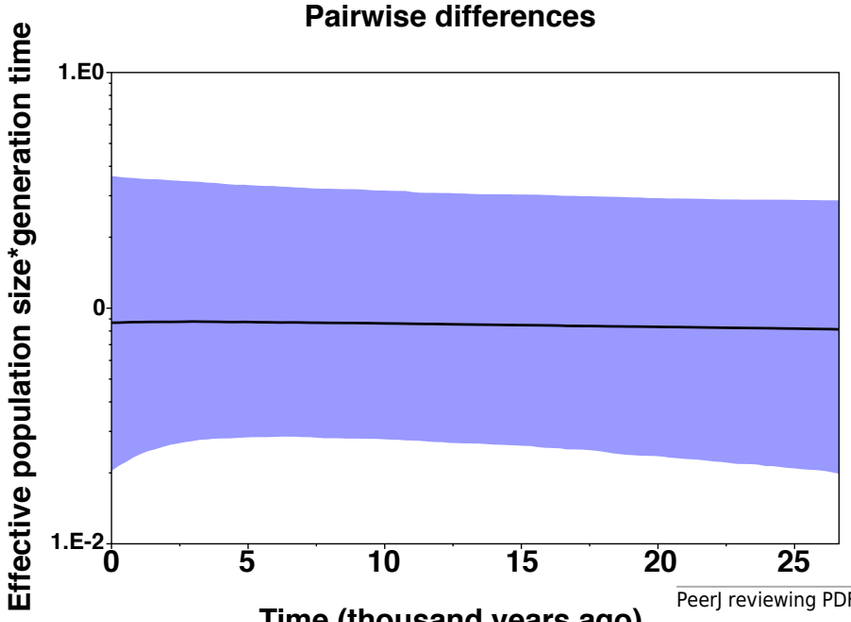
Mismatch distributions and Bayesian skyline plots for each geographic group of *L. rhami* (mtDNA: CR, ATPase 6 and 8). In mismatch distributions, solid lines indicate the observed distributions of pairwise differences, and dotted lines represent simulated distributions under a model of population expansion. In Bayesian skyline plots, solid lines represent median estimates and shaded areas represent 95% confidence intervals. Geographic groups are represented in different colors according to the geographic regions on the map

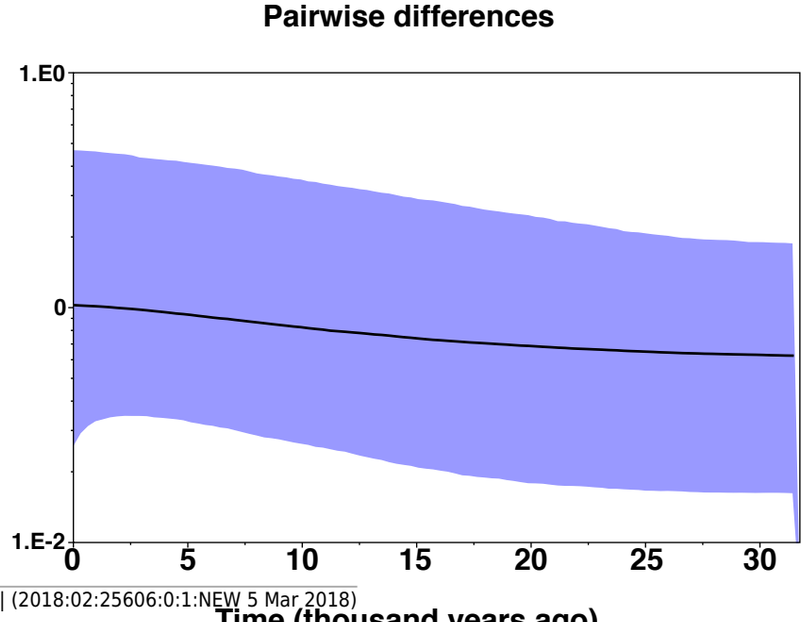



SMS
HRI=0.13. P=0.46
Frequency
0
1
2
3
4
5
6
0
2
4
6
8
Pairwise differences


SMO&SMSn
HRI=0.07, P=0.56
0
2
4
6
8
10
0
2
4
6
8
Pairwise differences


EIT
HRI=0.06, P=0.26
0
10
20
30
40
0
2
4
6
Pairwise differences


Effective population size*generation time
1.E0
0
1.E-2
0
5
10
15
20
25
Time (thousand years ago)


1.E0
0
1.E-2
0
5
10
15
20
25
30
Time (thousand years ago)

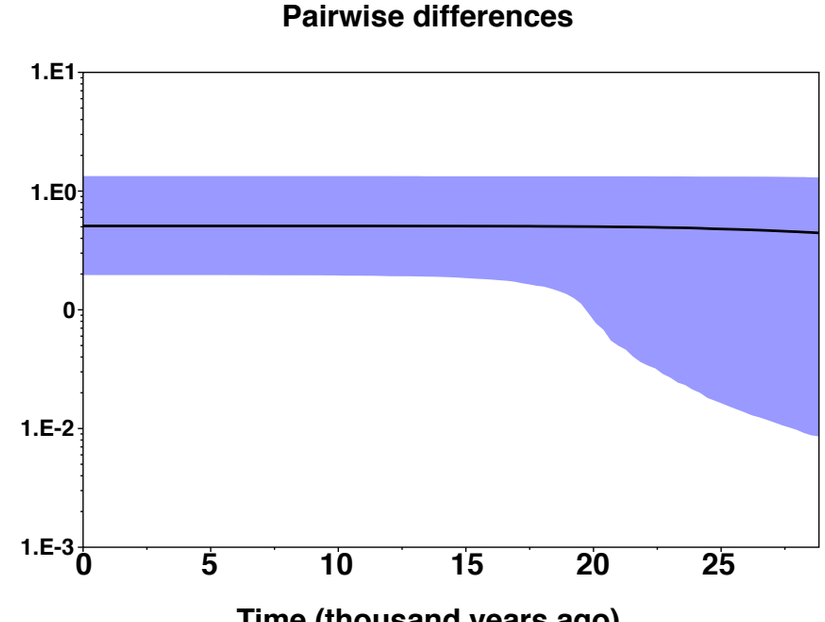
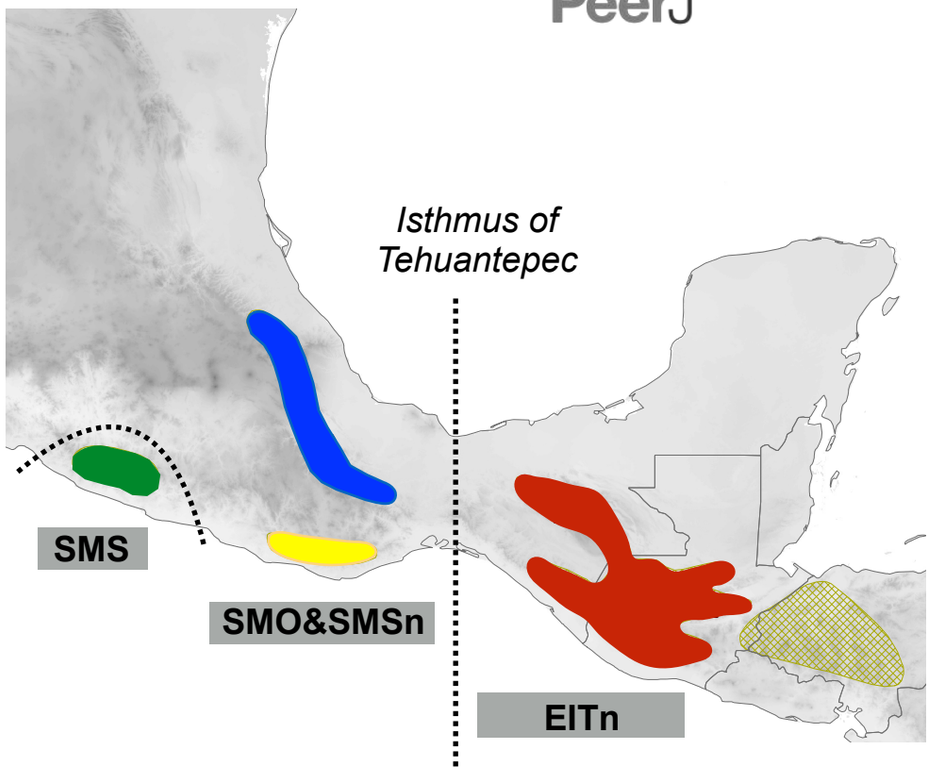
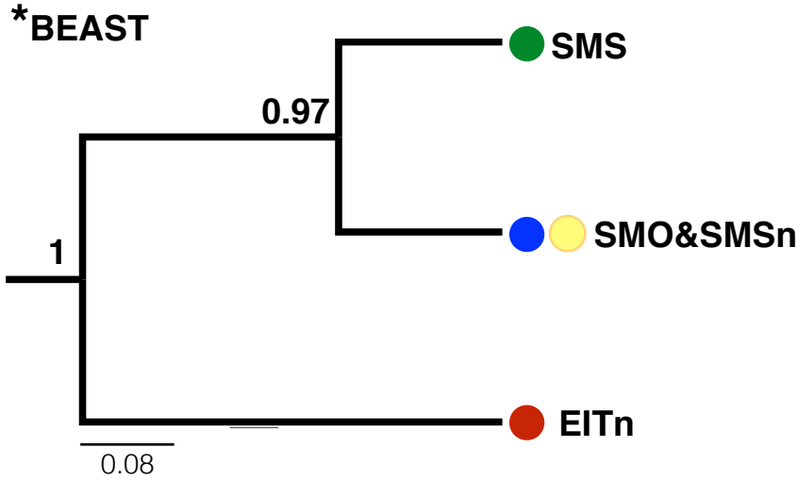

1.E1
1.E0
0
1.E-2
1.E-3
0
5
10
15
20
25
Time (thousand years ago)

Figure 3(on next page)

Bayesian species tree and Phylogenetic Bayesian Inference

Bayesian species tree topology (*BEAST), and Phylogenetic Bayesian Inference reconstruction from 31 individuals of *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 on the map





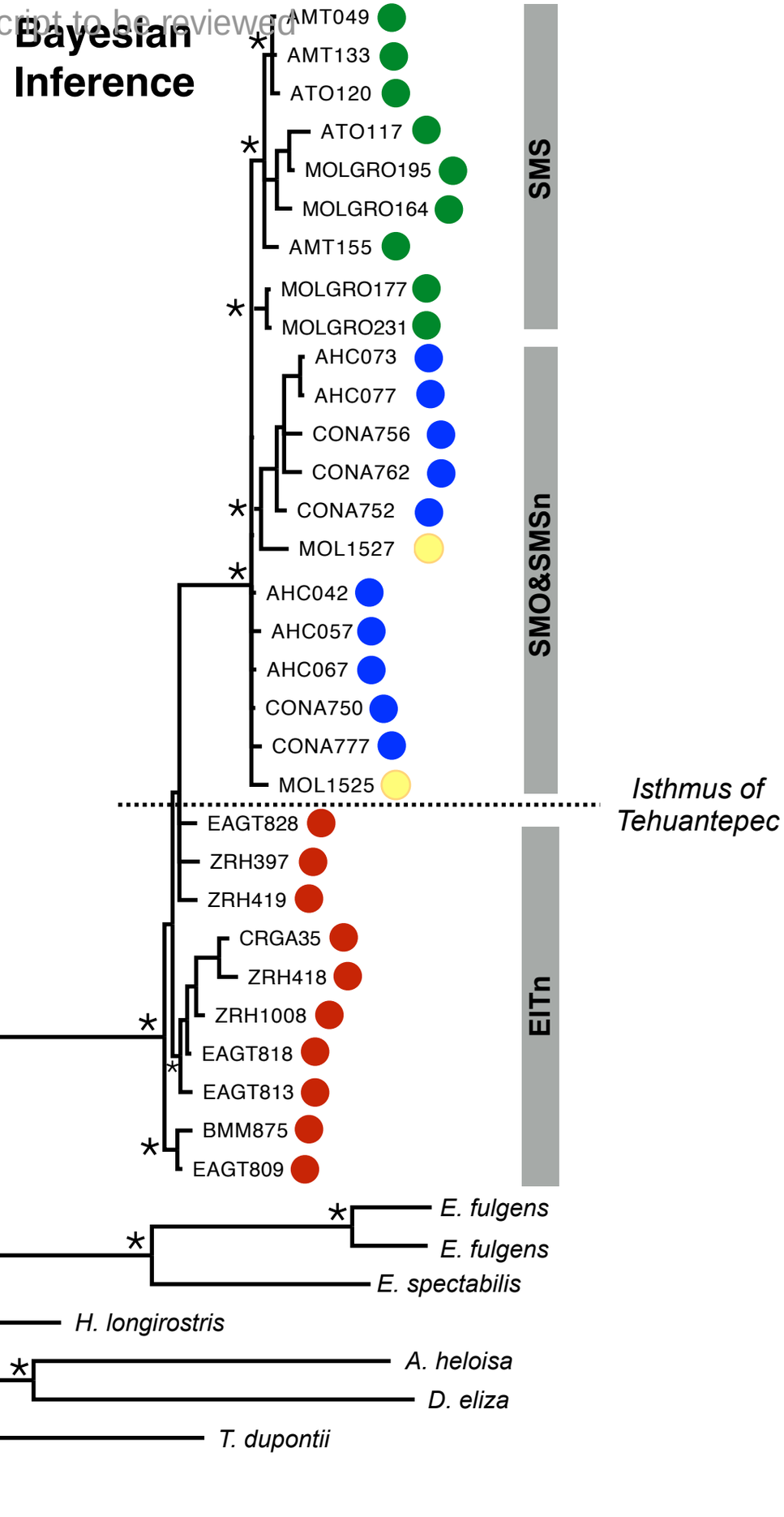


Figure 4(on next page)

Divergence times

Phylogeny illustrating the divergence times for *L. rhami* complex as generated by BEAST, using mitochondrial and nuclear markers (ATPase 6 and 8, CR, ND2, ND4, MUSK, BFib, ODC, and AK1). Bars on each node represent 95% of high posterior densities of divergence times (HPD). Ma (Million years)

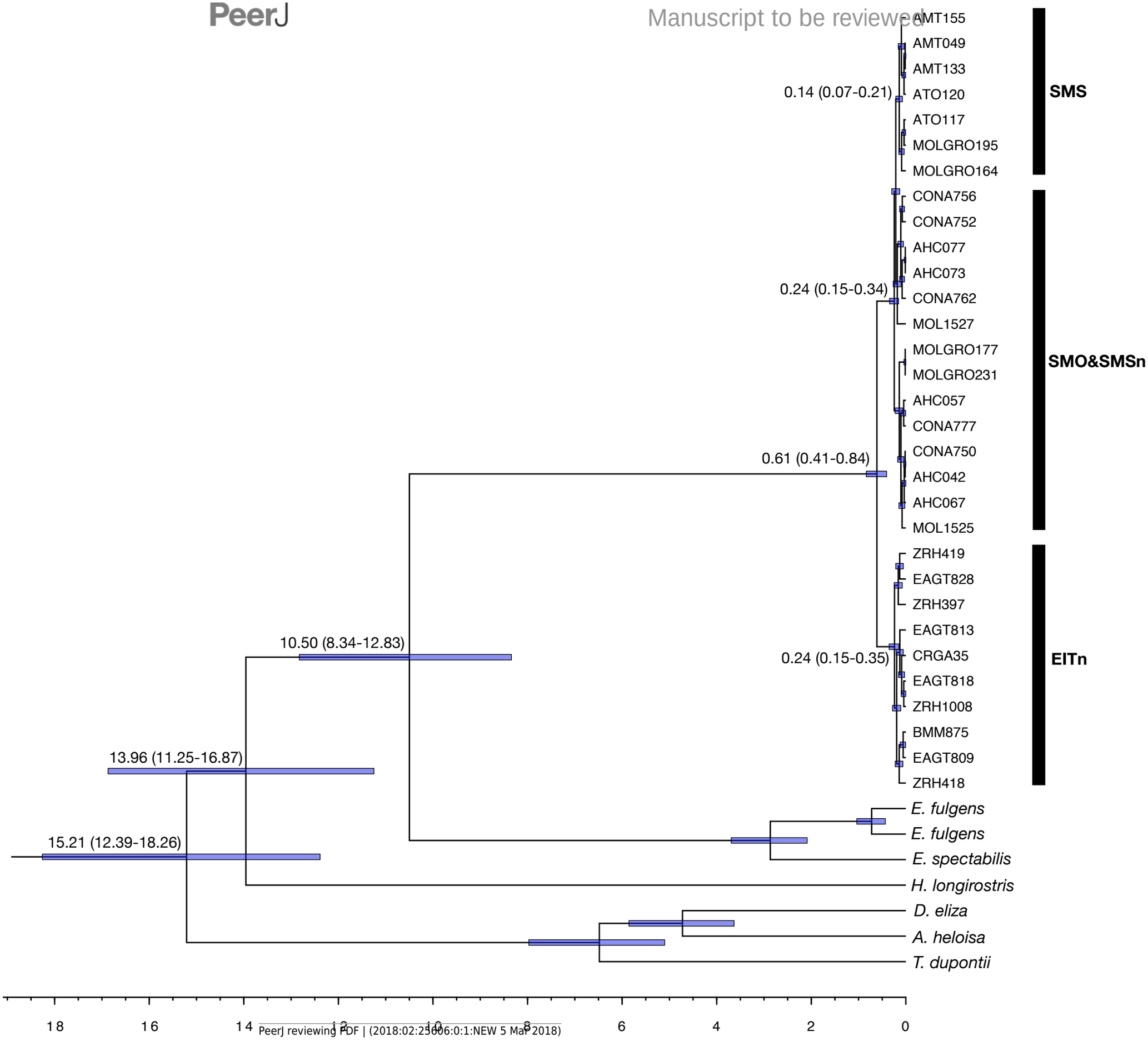
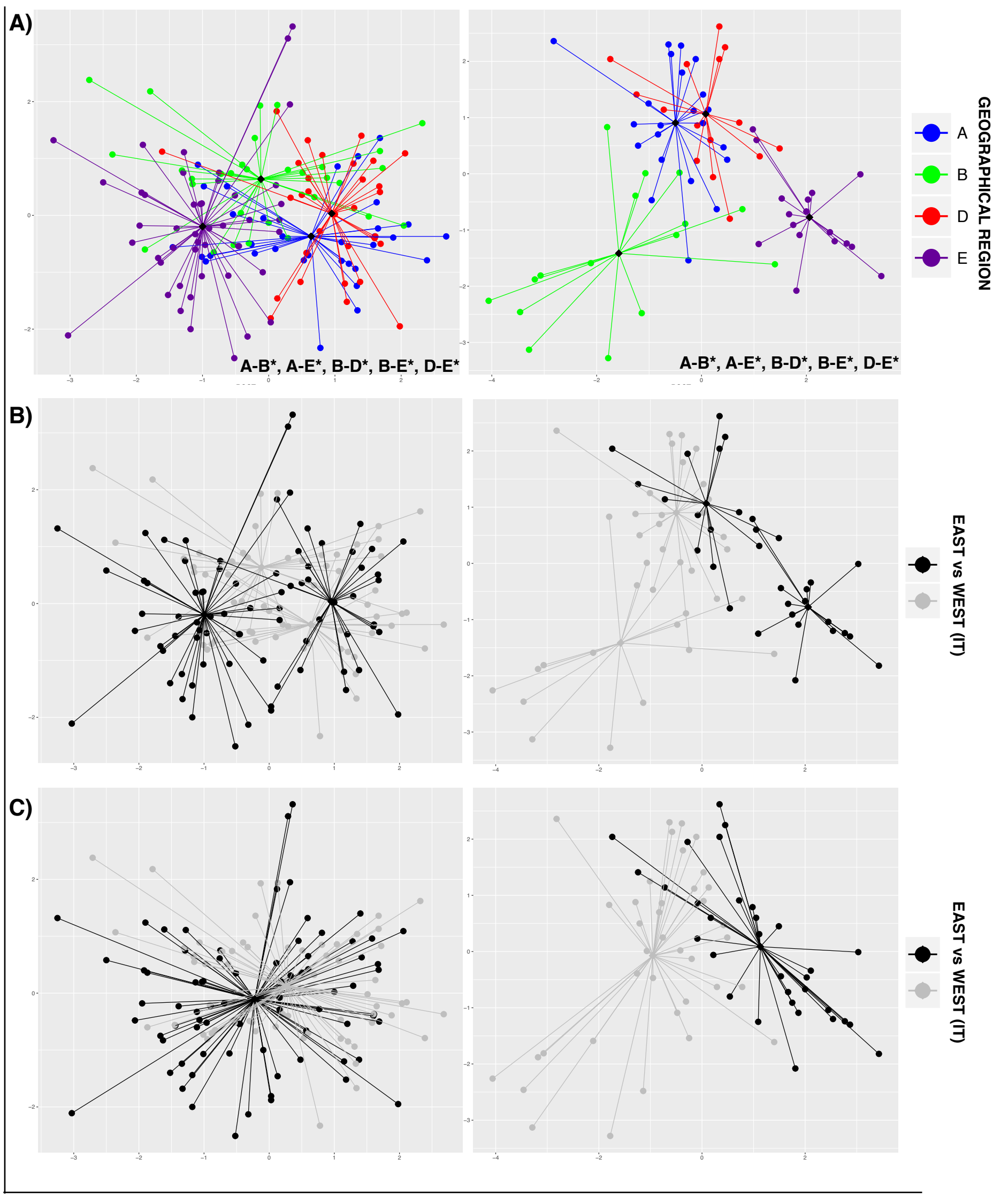

AMT155
AMT049
AMT133
0.14 (0.07-0.21)
ATO120
ATO117
MOLGRO195
MOLGRO164
SMS
CONA756
CONA752
AHC077
AHC073
0.24 (0.15-0.34)
CONA762
MOL1527
MOLGRO177
MOLGRO231
AHC057
CONA777
0.61 (0.41-0.84)
CONA750
AHC042
AHC067
MOL1525
SMO&SMSn
ZRH419
EAGT828
ZRH397
EAGT813
0.24 (0.15-0.35)
CRGA35
EAGT818
ZRH1008
BMM875
EAGT809
ZRH418
EITn
10.50 (8.34-12.83)
13.96 (11.25-16.87)
15.21 (12.39-18.26)
E. fulgens
E. fulgens
E. spectabilis
H. longirostris
D. eliza
A. heloisa
T. dupontii
18
16
14
12
10
8
6
4
2
0

Figure 5(on next page)

Discriminant analysis

Discriminant analysis for males and females of *L. rhami*. A) Plots representing geographic groups in different colors, mean values are represented by a black dot for each group. B) Plots by geographic groups differentiating populations at east and west from the Isthmus of Tehuantepec. C) Plots representing populations at east and west from the Isthmus of Tehuantepec. Statistical differences between groups are represented with an asterisk (*P<0.05)


MALES
FEMALES
A)
A-B*, A-E*, B-D*, B-E*, D-E*
A-B*, A-E*, B-D*, B-E*, D-E*
GEOGRAPHICAL REGION
A
B
D
E
B)
C)
EAST vs WEST (IT)
EAST vs WEST (IT)
ROOT_2
ROOT_1

*P<0.05

Table 1(on next page)

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, p: nucleotide diversity, Pi: mean number of pairwise differences

1

GROUP	n	h	Hd	π	Pi(theta)	Tajima's D	Fu's Fs Test
SMO&SMSn	22	9	0.81	0.0022	4.22	-0.559 (P=0.30)	-5.505 (P=0.003)
SMS	9	6	0.89	0.0026	3.73	-0.886 (P=0.22)	-2.77 (P=0.022)
EIT	21	15	0.94	0.0021	3.50	-1.988 (P=0.012)	-14.93 (P=0.000)

2
3
4

Table 2(on next page)

Population pairwise

Population pairwise F_{ST} mtDNA (CR, ATPase 6 and 8)

1

	SMO&SMSn	SMS	EIT
SMO&SMSn	----		
SMS	0.176*	----	
EIT	0.769*	0.784*	----

2 *P<0.05

3

4

5

Table 3(on next page)

AMOVA results

AMOVA results on *Lamprolaima rhami* populations defined according to biogeographic regions, and grouped into groups separated by the Isthmus of Tehuantepec using mtDNA (CR, ATPase 6 and 8)

1

	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices
Biogeographic region (*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***
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***

2

3