Geographic variation in the advertisement calls of *Hyla eximia* and its possible explanations

Populations of species occupying large geographic ranges are often phenotypically diverse as a consequence of variation in selective pressures and drift. This applies to attributes involved in mate choice, particularly when both geographic range and breeding biology overlap between related species. This condition may lead to interference of mating signals, which would in turn promote reproductive character displacement (RCD). We investigated whether variation in the advertisement call of the mountain treefrog (*Hyla eximia*) is linked to geographic distribution with respect to major Mexican river basins (Panuco, Lerma, Balsas and Magdalena), or to coexistence with its sister (the canyon treefrog, *Hyla arenicolor*) or another related species (the dwarf treefrog, *Tlalocohyla smithii*). We also evaluated whether call divergence across the main river basins could be linked to genetic structure. We found that the multidimensional acoustic space of calls from two basins where *H. eximia* currently interacts with *T. smithii*, was different from the acoustic space of calls from *H. eximia* elsewhere. Individuals from these two basins were also distinguishable from the rest by both the phylogeny inferred from mitochondrial sequences, and the genetic structure inferred from nuclear markers. The discordant divergence of *H. eximia* advertisement calls in the two separate basins where its geographic range overlaps that of *T. smithii* can be interpreted as the result of two independent events of RCD, presumably as a consequence of acoustic interference in the breeding choruses, although more data are required to evaluate this possibility.

Ruth E. RODRÍGUEZ-TEJEDA[1], María Guadalupe MÉNDEZ-CÁRDENAS[2], Valentina ISLAS-VILLANUEVA[3], & Constantino MACÍAS GARCIA[1§].

[1]Instituto de Ecología, [2]Instituto de Investigaciones Antropológicas and [3]Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México.

México City, México.

§Corresponding author: Constantino Macías Garcia, Instituto de Ecología, UNAM, A.P. 70-275, C.P. 04510, México DF., México. Phone: +52 55 56229044. e-mail: maciasg@unam.mx.

Key words: advertisement calls; *Hyla eximia*; geographic variation; acoustic interference; genetic and geographic distances.

Introduction

Populations of species occupying large geographic ranges are likely to experience different selective pressures (West-Eberhard, 1983; Panhuis *et al.*, 2001; Coyne & Orr, 2004) which, together with drift (Wiens, 2004), may result in phenotypic and genotypic differences between populations (e.g. Avise, 2000; Laugen *et al.*, 2003; Amézquita *et al.*, 2009). Local differences in ecology, such as prey availability (e.g. Arnold, 1980; Bonansea & Vaira, 2007) or habitat structure (e.g. Relyea, 2002; Skelly, 2004) can lead to differential adaptation between populations (Newman, 1992), but in species with generalized diets and habitat requirements such variation would have a limited effect on differentiation (e.g. Virgós, 1999). On the other hand, environmental variation in the attributes that determine the transmission and reception of signals used in social (Wells, 1977; Sullivan &Wagner, 1988; Wagner & Wagner, 1989) or sexual contexts (Searcy & Andersson, 1986) may lead to rapid population differentiation (Jennions & Petrie, 1997; Seehausen *et al.*, 1997; Lougheed *et al.*, 2006; Boul *et al.*, 2007). This is because signals used by animals in a breeding context may convey information about the species, sex, breeding status, and even the condition of the sender (Gerhardt, 1992; Wilczynski & Chu, 2001), all of which may be relevant to conspecifics searching for mating partners (Butlin & Ritchie, 1994; Emerson, 2001; Forsman & Hagman, 2006). Geographic variation in mating signals has been widely reported in studies of character displacement, where specific traits (e.g. morphological, behavioral, ecological or physiological traits) differ among sympatric and allopatric populations due to the risk of maladaptive hybridization with related species (Brown & Wilson, 1956; Grant, 1972). Reproductive character displacement (RCD) is often studied in systems where sister species meet at typically narrow hybrid or tension zones (Butlin, 1987; Howard 1993; Butlin & Ritchie, 1994). However an approach involving the study of signal variation across wide geographic areas, coupled with genetic and geographic data, could be helpful in identifying evolutionary patterns of signal evolution and tracing the links between micro and macro evolution of mating signals (Avise *et al.*, 1987).

Amphibians are good models for studying signal evolution and reproductive isolation since their dispersal is restricted by habitat, and they thus are highly philopatric, facilitating the study of genetic divergence amongst populations (c.f. Gamble *et al.*, 2007). Amphibian philopatry results in populations being genetically structured over short geographic distances, which facilitates inferences about the historic events that caused their current distribution (Zeisset & Beebee, 2008). Although other modalities (e. g. visual; Hartmann *et al.*, 2005; Reynolds &

43 Fitzpatrick, 2007; Taylor *et al*., 2008) are sometimes involved, it is common in anurans that

44 species recognition and female preference depend on a single acoustic signal; the advertisement

45 call (Wells, 1977; Cocroft & Ryan, 1995; Wells & Schwartz, 2007). This is produced by males,

46 typically during the mating season. Several attributes of the calls vary within a species as a

47 function of male morphology and condition. For instance, the frequency or pitch (Hertz) of the

48 call is inversely correlated with body size (Zug, 1993; Gerhardt & Huber, 2002). Calls are also

49 affected by temperature, which influences the length of the call as well as the number of pulses

50 that compose the call, and the pulse rate (Gerhardt & Davis, 1988; Wilczynski & Chu, 2001;

51 Gerhardt & Huber, 2002; Yamaguchi *et al*., 2008). Additionally, males at a chorus often adjust the

52 timing of their calls to avoid masking by other vocalizations, and when choruses are formed by

53 more than one species, acoustic interference can select for the use of different acoustic space

54 (Brush & Narins, 1989; Schwartz *et al*., 2002; Check *et al*., 2003; Hoskin & Higgie, 2010).

55 Acoustic space is a multidimensional space defined by acoustic properties of the signals, such as

56 frequency, duration, amplitude, temporal structure, etc.; see for instance Ryan & Rand, (2003). In

57 spite of variation, anuran songs are sufficiently stereotyped to permit females to identify and

58 assess males (e.g. the *Hylidae*; Gerhardt 1991, 1992), so much so that *Hyla* spp. have often been

59 used as models to study pre mating reproductive isolation amongst sister species (e.g. Littlejohn,

60 1965; Ball & Jameson, 1966; Littlejohn, 1970; Duellman, 1973; Gerhardt, 1994; Höbel &

61 Gerhardt, 2003; Gerhardt, 2005).

62   *Hyla eximia* is a relatively small (ca. 3 cm) tree-frog endemic to the geologically complex

63 Trans-Mexican Volcanic Belt (TMVB; de Cserna, 1989; Ferrari *et al*., 2011) and adjacent

64 Mexican High Plateau (Duellman, 1970, 2001). It gives its name to the *H. eximia* species group

65 (Smith *et al*., 2007), which includes eleven other species (Faivovich *et al*., 2005). The very

66 similar *H. wrightorum* replaces *H. eximia* to the north (through Arizona), and *H. euphorbiacea* is

67 found to the south of the TMVB (allopatric with *H. eximia*). *H. plicata* is endemic to the TMVB

68 and is partially sympatric with *H. eximia* (Smith *et al.,* 2007), and *H. arenicolor* is sympatric with

69 *H. eximia* in parts of the Mexican Plateau. Like *H. eximia*, *H. arenicolor* has a wide geographic

70 range; in the Mexican Plateau it is mainly found in the mountain zones of Balsas Basin, at

71 elevations of 300-3000 meters (Duellman, 2001). *Tlalocohyla smithii* (formerly *Hyla smithii*,

72 Faivovich *et al*., 2005) is distributed in the Pacific lowlands of Mexico up to elevations of about

73 1000 meters, from central Sinaloa to southern Oaxaca, and inland within the Balsas Basin

74 (Duellman, 2001). *Tlalocohyla smithii*, is not part of the *H. eximia* group, but occupies the same

75 matings pools.

76   Previous work has shown evidence of geographic variation in *H. eximia* calls. Indeed,

77 variation in pulse rate, call duration and the dominant frequency among recordings from several

78 populations (Blair, 1960; Duellman, 1970; Sullivan, 1986; Duellman, 2001) was so great that

79 Duellman (1970, 2001) concluded that the species lacks a typical call, though sample sizes per

80 population were small. It has also been suggested that some variation in advertisement calls of *H.*

81 *eximia* may be linked to RCD. Based on phonetic data Cortés-Soto (2003) suggested that *H.*

82 *eximia* and *H. plicata* have evolved different advertisement calls in the 500 m altitudinal band

83 (2400-2900 m asl) where their ranges overlap. There, the calls of *H. eximia* are shorter and

84 contain fewer pulses than when the species are found in allopatry, suggesting that RCD has

85 occurred. Here we describe the variation of the advertisement calls of *H. eximia* across a

86 substantial part of its geographic range, and explore whether this variation is linked to genetic

87 structure (based on mitochondrial and nuclear DNA sequences), geography (i.e. hydrographic

88 basins) and/or range overlaps with its sister species the Canyon treefrog (*H. arenicolor*) and a

89 related species the Mexican dwarf treefrog (*Tlalocohyla smithii*).

90 **Materials and methods**

91 **Song description**

92 Based on information from public databases we surveyed 51 locations where *H. eximia* has been

93 previously collected. We found *H. eximia* in nine of these locations. Surveys were carried out

94 during a single rainy season to avoid bias due to inter-seasonal call variation and to ensure the

95 presence of the species in the breeding pools. *Hyla eximia* was alone (allopatric) in four of the

96 nine locations that were sampled. In two locations, *H. eximia* was sympatric and formed breeding

97 choruses with *H. arenicolor*, and in the remaining three with *T. smithii* (Table 1, Fig.1). Here we

98 considered "sympatric" the populations in which two species were found to simultaneously

99 occupy the same microhabitat during the breeding season (form breeding choruses); this

100 preliminary classification that will have to be confirmed with repeated sampling.

101   During the summer of 2011 we recorded advertisement calls from males of the three

102 species. Calls were recorded in natural breeding aggregations with a directional microphone

103 (Sennheiser™ ME66) connected to a digital recorder (Marantz™ PMD660). The microphone

104 was held 1 m in front of the calling male, and the recording volume was adjusted to avoid

105   saturation (0-6 dB). Each male was recorded for 1-1.5 min to ensure that adequate call series

106   were obtained. Then we measured the body temperature of the frog by holding it from a hind

107   limb and pointing an infrared thermometer (Extech™ 42529; ± 0.05 °C) at its body, thus

108   preventing heat transfer from the observer. Finally, we measured the frog's snout-vent length with

109   a digital calliper (± 0.05 mm) and collected a toe-clip from the distal phalanx of the fourth right

110   front-leg digit for subsequent DNA extraction (see Gonser & Collura, 1996).

111   We used only recordings containing at least 10 advertisement calls. In order to maximize

112   the opportunity to detect differences in the songs of *H. eximia* between populations, we measured

113   a large number of variables from our recordings. Using Avisoft-SASLab Pro™ we quantified the

114   following attributes (definition, abbreviation; units): call duration (length of the call, continuous

115   trace in the sonogram, CD; s), inter-call duration (silent period between consecutive calls, IC; s),

116   call period (sum of CD + IC, CP; s), number of pulses (number of short signals that compose the

117   call, NP; count), pulse duration (length from the beginning to the end of the pulse, PD; s), inter-

118   pulse duration (length of the silent period between the end of one pulse and the beginning of the

119   next, ID; s), pulse amplitude (peak intensity of the pulse, PA; V), pulse peak frequency (highest

120   energy within the pulse, PPF; kHz), pulse period (the sum of PD + IP, PP; s), pulse rate (pulses

121   per second measured from the start of the first pulse to the start of the last pulse, PR; Hz),

122   dominant frequency (frequency with the highest energy within the call, DF; kHz) and

123   fundamental frequency (the lowest, or reference frequency of series of frequencies -called

124   harmonics- that are its integer multiples, FF; kHz). Temporal variables were measured directly on

125   the spectrogram, all the pulse variables were extracted with the *Pulse Train Analysis* option, and

126   the frequency variables were obtained with *Power spectrum logarithmic* function (Fig. S1).

127   Individual frogs were represented in the analyses by the average value of each call attribute.

128   To detect the call variation in *H. eximia* we defined three chorus compositions: 1)

129   allopatric, composed of *H. eximia* males only, 2) *H. eximia* in mixed choruses with *H. arenicolor*,

130   and 3) *H. eximia* in mixed choruses with *T. smithii*. We also analyzed the calls of 4) *H. arenicolor*

131   males in mixed choruses with *H. eximia*, and 5) *T. smithii* males in mixed choruses with *H.*

132   *eximia*.

Call variation

134   To reduce the number of variables included in multivariate analyses in an objective way, we

135   calculated for each chorus composition the correlation matrix of the twelve variables and

136 discarded one attribute of any pair that had a correlation coefficient of ≥ 0.7. Since temperature is

137 known to affect several amphibian call attributes (Blair, 1958; Zweifel, 1959; Zweifel, 1968;

138 Gerhardt & Mudry, 1980; Gayou, 1984; Gerhardt & Huber, 2002) we performed an analysis of

139 covariance (ANCOVA) for each species and variable to assess the potential effect of temperature

140 on call attributes from different chorus compositions. Since ANCOVAs showed a significant

141 effect of temperature on call variables, we used ANCOVA residuals for all subsequent analyses.

142   Once the number of variables had been reduced and the effect of temperature removed,

143 we compared the attributes of the calls of *H. eximia* from the three chorus compositions using a

144 multivariate analysis of variance (MANOVA) to determine whether and to what extent call

145 variation was determined by locality and/or chorus composition. The absence of a significant

146 effect of locality on call attributes might suggest that song variation occurs at a larger

147 geographical scale, such as by basin, thus when there was no locality effect we repeated the

148 analysis replacing locality with major river basins (Panuco, Lerma, Magdalena and Balsas). All

149 analyses were performed using R (v. 2.15.1 development core team 2011). The above analyses

150 were designed to evaluate whether the vocalizations of *H. eximia* produced at different chorus

151 compositions were different in some -or in a combination of- attributes. To visualize such

152 differences we performed a canonical discriminant analysis on the calls of *H. eximia,* and used

153 the resulting discriminant function to plot the distribution of the calls of *H. arenicolor* and *T.*

154 *smithii* in the same canonical space. This allowed the visualization of any significant differences

155 revealed by the MANOVA as displacements away the calls of sympatric species in a canonical

156 multivariate space. Finally we ran an analysis of variance to compare the first two canonical

157 scores of *H. eximia* amongst basins -a major geographic source of variation. Analyses in this

158 section were conducted with NCSS™ (2007, Kaysville, UT).

159 **Genetic variation**

160 Using mitochondrial and nuclear genes we analyzed phylogenetic patterns to explore the extent to

161 which geographic variation of songs across populations is related to genetic variation. Total

162 genomic DNA of 30 individuals of *Hyla eximia* from 12 localities was extracted from EtOH-

163 preserved toe clippings using the DNeasy Tissue Kit (Qiagen™ Inc., Valencia, CA, USA).

164 Standard PCRs were carried out in 25µl reaction mixes with final concentrations of 1µM of each

165 primer, 1.5mM of MgCl2, 0.2mM of each dNTP, 1xNH4 reaction buffer (50mM Tris-HCl pH

166 8.8; 16mM [NH4]2 SO4), 1.25 units of Taq DNA polymerase, and 1-4µl of DNA. Successful

167 amplifications were performed using the following protocol: initial denaturing for 5 min at 95°C,

168   denaturing for 45 s at 94°C, annealing for 45 s at 48°C (for Cyt b) and 57°C (for ATP), extension

169   for 1 min at 72°C, final extension min for 7 min at 72°C; denaturing, annealing and the first

170   extension stage were cycled 35-40 times.

171   We amplified two mitochondrial gene regions; cytochrome-*b* (754 bp) with the primers

172   MVZ16-H AAATAGGAARTATCAYTCTGGTTTRAT and MVZ15-L

173   GAACTAATGGCCCACACWWTACGNAA (Moritz *et al*., 1992; Stöck *et al*., 2008, 2011), and

174   ATPase subunits 8 and 6 (778 bp) with the primers LysAF CAACCACCCTTGATGAATGCC

175   and C3FR GGGCTGGGGGTTKACTATGTG (Stöck *et al*., 2008; Bryson *et al*., 2010). These

176   mitochondrial gene regions have been found to be informative at different levels of variation

177   within hylid frogs (references given above). We also amplified two nuclear gene regions using

178   the same protocol as above, with a slight modification (see below). The Pro-opiomelanocortin

179   gene region (POMC; 447 pb) with the primers POMC_DRV_F1

180   ATATGTCATGASCCAYTTYCGCTGGAA and POMC_DRV_R1

181   GGCRTTYTTGAAWAGAGTCATTAGWGG (annealing temperature = 58°C; modified from

182   Wiens *et al*., 2005; Bryson *et al*., 2010), and the Rhodopsin (Rho) gen region (291 pb) with the

183   primers Rho1A ACCATGAACGGAACAGAAGGYCC and Rho1C CCAAGGGTAGCG

184   AAGAARCCTTC (Bossuyt & Milinkovitch, 2000; Faivovich *et al*., 2005; Klymus *et al*., 2010).

185   PCR products were sent for sequencing to the University of Washington High-Throughput

186   Genomics Unit, Department of Genome Sciences, to the Service of DNA Sanger Sequencing.

187   Sequences were aligned with Clustal X (Jeanmougin *et al*., 1998). Some regions were difficult to

188   align, and were therefore adjusted manually using BioEdit (Hall, 1999). DNAsp (Librado &

189   Rozas, 2009) was used to determine the number of unique haplotypes and invariable and

190   polymorphic sites.

191   Phylogenetic analyses were performed for each gene using the maximum likelihood

192   algorithm (ML; Felsenstein, 1981). We used the Hasegawa-Kishino-Yano model (HKY) -G+I

193   (gamma distribution with invariable sites) for mitochondrial genes and the GTR +I+G for nuclear

194   genes. In both analyses the nearest neighbor interchange (NNI) was used. The best model was

195   identified with the program Modeltest 3.7 and MrModeltest modified version of Modeltest

196   version 3.6 (Posada, 2008) using the Akaike information criterion (AIC).

197   We also constructed a haplotype network using TCS 2.1.1 for preliminary exploration of

198   the phylogeographic relationships of the obtained haplotypes (Clement *et al*., 2000). Genetic

199   diversity was calculated as nucleotide (π) and haplotype (h) diversity for each of the sub-regions

200   of the basins (northern, central and southern) using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010),

201   for both pairs of concatenated genes (mitochondrial and nuclear). We also calculated the

202   population differentiation index F_{ST} and its significance levels, between pairs of basins, using

203   Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). To visualize the differences in nuclear genes

204   between basins, the genetic structure was investigated using Structure 2.3 (Pritchard *et al*., 2000)

205   with the Admixture model and correlated frequencies. A 500 000 burnin was employed and 5 000

206   000 generations of K 1 to 10 were performed with 10 iterations for each gene. We used the

207   Evanno method to determine the true value of K (Evanno *et al*., 2005) with the program Structure

208   Harvester (Earl & vonHoldt, 2012). This analysis was employed only for nuclear genes, as it is

209   not designed to find structure in linked or haploid genes.

210   **Phenotypic, genetic and geographical distance**

211   We performed Mantel tests and partial Mantel tests with 1000 resamplings using R v. 2.15.1 (R

212   development core team 2011) to evaluate the correlation between acoustic (phenotypic), genetic

213   and geographic distances. To calculate the acoustic matrix we employed the distances between

214   canonical variables for each basin obtained from a discriminant analysis. The geographic distance

215   was constructed with the geo- reference points taken at each locality; as the basin could include

216   several localities, an average geo-reference was employed. The genetic distance matrix was built

217   using the D_{xy} values.

218   The project was reviewed and approved by the Consejo Técnico de la Investigación Científica

219   (CTIC) of UNAM.

220   **Results**

221   **Song description**

222   We obtained 169 good quality recordings of male advertisement calls. These came from nine

223   *Hyla arenicolor*, 20 *Tlalocohyla smithii* and 140 *Hyla eximia* from both sympatric and allopatric

224   localities. A detailed description of the calls of *H. eximia* based on our complete sample is

225   available in the supplemental information (see song description S1) along with descriptions of

226   those of *H. arenicolor* and *T. smithii* which are also summarized in Tables 2 and 3. Briefly, the

227   calls of *H. eximia* consist of a single note that lasts less than a quarter of a second, has a dominant

228   frequency of about 2.6 kHz, and is composed of ca. 18 pulses. The call of *Hyla arenicolor* (Table

229   3) is roughly three times longer, (0.88 s), although it is composed of only a few more (about 20

230 pulses, but these pulses are longer and louder than the calls of *H. eximia*. Calls of *H. arenicolor*

231 are lower-pitched than those of *H. eximia* (dominant frequency = 1.26 kHz). The structure of the

232 call of male *T. smithii* is very different from those of *H. eximia* and *H. arenicolor*. Often the

233 males emit calls with two elements; a long note followed by an extremely short one. Most

234 individuals, however, produce more long than short notes, and in several recordings short notes

235 are absent. Some males produced a third note with different structure and duration than the

236 previous two, but it is not clear whether this is also part of the advertisement call. For our

237 analyses we considered just the first note, as it is the most constant element in our recordings, and

238 it is the one more likely to interfere with the advertisement calls of *H. eximia* calls. Spectrograms

239 of typical advertisement calls of the three species are shown in Fig. 2.

240 **Call variation**

241 The following variables were highly correlated in the calls of *Hyla eximia* in allopatry: pulse

242 amplitude and interval between calls (0.997), and call period and inter-call duration (0.993). In

243 sympatry with *H. arenicolor*, the correlated variables of calls of *H. eximia* were: call period and

244 inter-call duration (0.991), pulse rate and inter-pulse duration (-0.984), pulse period and inter-

245 pulse duration (0.954), and pulse rate and pulse period (-0.953). Correlated variables in the

246 contact zone between *H. eximia* and *T. smithii* were: call period and inter-call duration (0.999),

247 pulse period and pulse duration (0.996), pulse rate and inter-pulse duration (-0.966), dominant

248 frequency and pulse peak frequency (0.943), and pulse rate and number of pulses (0.920).

249 The following attributes of the calls of *H. arenicolor* were intercorrelated: pulse rate and

250 inter-pulse duration (-0.985), pulse period and inter-pulse duration (0.965), pulse rate and pulse

251 period (-0.941), and call period and inter-call duration (0.917). In the case of *T. smithii* the

252 highest correlations amongst variables were: call period and inter-call duration (0.998), pulse

253 period and inter-pulse duration (0.990), pulse rate and inter-pulse duration (-0.979) and pulse rate

254 and pulse period (-0.967).

255 Considering that correlations ≥ 0.7 indicate redundant variables, we dropped from the

256 analyses the following variables: call period (CP), pulse period (PP), pulse rate (PR), inter-pulse

257 duration (ID) and pulse peak frequency (PPF). Consequently, analyses were performed using

258 seven variables: call duration (CD), inter-call duration (IC), number of pulses (NP), pulse

259 duration (PD), pulse amplitude (PA), dominant frequency (DF) and fundamental frequency (FF).

260   Covariance analyses revealed that temperature, chorus composition and the interaction

261   between them affect call attributes of each species in different ways (Table S1). To control for the

262   effect of temperature, in cases where the interaction between temperature and chorus composition

263   was significant we used the residuals from the ANCOVA, rather than the original variable, in all

264   subsequent analyses.

265   MANOVA results show that call variability in *H. eximia* is due to the locality ($F_{(6,42)}$

266    $=3.91$, $P = 1.66e^{-14}$), whereas the fact that individuals call in allopatry or sympatry does not

267   contribute significantly to call variation. However, when populations are grouped according to

268   watershed (Panuco, Lerma, Magdalena y Balsas), it is amongst basins, rather than localities, that

269   calls diverge significantly ($F_{(3,21)} = 7.7241$, $P < 2e^{-16}$).   The seven call variables entered in the

270   MANOVA analyses allow for the discrimination between the four basins with a 34% reduction in

271   classification error, classifying correctly 51% of the calls according to the basin of origin. The

272   first two canonical functions explain 99.8% of the variance and define a multivariate acoustic

273   space where the calls of *H. eximia* from Balsas and Magdalena are distinct from those from the

274   other basins (Figs. 2 and 3). Subsequent ANOVAs and Tukey tests confirm that these differences

275   are significant (function 1, $F_{3,134} = 87.67$, $P < 0.0001$; function 2, $F_{3,134} = 6.84$, $P = 0.0002$; Fig.

276   4). Discriminant function 1 distinguishes amongst calls from different basins based on the

277   interval between calls (r= -0.809), call duration (r= -0.274) and number of pulses (r= 0.254),

278   while the discriminant function 2 discriminates by dominant frequency (r= 0.974). Applying the

279   functions that discriminate between calls of *H. eximia* from different basins to the call attributes

280   (or the ANCOVA residuals) of *H. arenicolor* and *T. smithii* reveals no overlap of canonical

281   acoustic space between the calls of *H. eximia* from the Balsas basin and those of co-occurring *T.*

282   *smithii*, and a partial overlap between the calls of *H. eximia* at Magdalena those of the local *T.*

283   *smithii* (Fig. 3). There is complete overlap between the canonical acoustic space of calls of *H.*

284   *eximia* from the several populations in the Panuco basin, whether they are sympatric with *H.*

285   *arenicolor* or not, and between those and the calls of *H. eximia* in the Lerma basin (Fig. 3).

286   **Genetic variation**We found a total of 31 different haplotypes of concatenated mitochondrial

287   genes (Table 4). Nucleotide diversity, number of analyzed samples and total haplotypes per basin

288   for each concatenated gene pair are shown in Table 4. Mitochondrial nucleotide diversity was

289   generally low, and the high values from Magdalena ($\pi = 0.462$) and Balsas ($\pi = 0.269$) are

290   probably not reliable since standard deviations are high due to small sample size (Table 4).

291   Mitochondrial haplotype diversity was very high in all regions (Table 4). Since in most cases the

292 number of haplotypes is identical to the sample size, it does not represent the populations'

293 haplotype diversity.

294   The phylogenetic reconstruction based on mitochondrial genes recovers four major

295 lineages of *H. eximia* loosely grouped by basin, with some admixture (Fig. 5). The basal clade

296 (A) includes frogs from the southern reaches of the Panuco drainage, at the edge of the ancient

297 closed basin of Apan, as well as frogs from the Balsas and one from Magdalena. This is the best

298 -supported clade with bootstrap values of 56-96 %. The next mitochondrial clade to diverge (B)

299 includes frogs from central Lerma, Magdalena and Balsas basins. The most recent, sister clades

300 include only Lerma (C) frogs, or frogs from north and central Panuco and central Lerma (D).

301 Bootstrap values are higher in mitochondrial genes than in nuclear genes, and for the basal

302 groups more than for the recent groups. As expected from the above reconstruction, the

303 populations comprising clade A (southern Panuco, Balsas and Magdalena) are the most

304 differentiated; they differ mostly (and significantly) from populations in clade D; central and

305 northern Panuco, as well as from central Lerma as shown by the F_{ST} values (see Table 5).

306   The mitochondrial haplotype network seems to reflect the distribution of individuals into

307 the major basins (Fig. 6). Again, haplotypes from southern Panuco are not connected to any other

308 basin. Two of the three Balsas haplotypes are also isolated from those from other basins, except

309 for one haplotype from Magdalena (the other Balsas haplotype is linked to haplotypes from

310 central Lerma). By and large, the haplotypes from central and northern Panuco are closely linked,

311 and relatively closely linked also to those from the three Lerma sub basins (Fig. 6).

312   We found 25 different haplotypes of concatenated nuclear genes (Table 4). The

313 greatest nucleotide diversity of nuclear genes was found in central Panuco ($\pi = 0.200$) central (π

314 $= 0.190$) and southern Lerma (0.157), but the latter has a high standard deviation (Table 4). Just

315 as with mitochondrial genes, nuclear haplotype diversities were often $\simeq 1$ and are certainly

316 overestimations.

317   The reconstruction based on nuclear genes is much less clear, with very shallow branches,

318 although it also suggests a link between Lerma and both Balsas and Panuco (Fig. 5). The degree

319 of nuclear genetic differentiation of *H. eximia* between regions is roughly consistent with that

320 shown by mitochondrial genes: populations from southern Lerma (mitochondrial clade A) are

321 significantly differentiated from those in northern and central Panuco, as well as central Lerma

322 (mitochondrial clade D), yet this latter clade is formed by frogs from regions that are significantly

323 differentiated by nuclear genes. Frogs from Magdalena (mitochondrial clade B) are significantly

324 differentiated from those from northern Panuco (mitochondrial clade D; Table 5).

325   The Evanno method obtained a true value of K=3 which is, however, not too sharply

326 delimited. Both genes show different patterns among the populations analyzed and little

327 correspondence to the patterns inferred from mitochondrial genes. This could be due to

328 recombination or to the fact that Structure fails to find differentiation when the population

329 structure is too subtle (Latch *et al*., 2006), which could be our case as the reduced sample size

330 failed to produce significant values of population differentiation.

331 **Phenotypic, genetic and geographical distance**

332 There was no significant correlation between call variation and geographic distance (Mantel test

333 r= 0.125, P= 0.306) even when controlling for mitochondrial (Mantel partial test r= 0.126, P=

334 0.218) or nuclear (r= 0.185, P= 0.234) D_{xy}. Nuclear D_{xy} was not correlated with geographic

335 distance (r= 0.097, P= 0.311). This pattern remains when controlling for call variation (r= 0.167,

336 P= 0.293). We did find a significant correlation between geographic distance and mitochondrial

337 D_{xy} (r= 0.372, P= 0.035), and this association remained when controlling for call variation (r=

338 0.372, P= 0.042). Call variation was not correlated with either mitochondrial (r= 0.022, P= 0.409)

339 or nuclear (r= 0.426, P= 0.108) D_{xy}, even when controlling for geographic distance

340 (mitochondrial D_{xy}, r= 0.026, P= 0.419; nuclear D_{xy}, r= 0.443, P= 0.128).

341 **Discussion**

342 **Song description**

343 Intraspecific variation in the attributes of the advertisement calls of *H. arenicolor* and of the *H.*

344 *eximia* species group is common (e.g. Blair, 1960; Klymus *et al.,* 2010), but its origin and

345 biological significance are not fully understood. A comprehensive study of call variation across

346 the whole geographic range of the Canyon treefrog revealed that such differences are not large

347 enough to promote speciation, although a degree of assortative mating preferences was evident

348 over large geographic scales (Klymus *et al.,* 2012), and at least some of that variation may be

349 linked to introgression with species in the *H. eximia* group (see Klymus *et al*., 2010). Three

350 populations of *Hyla wrightorum*, a member of the latter group, were found to produce calls of

351 different dominant frequency (and two differed in the duration of their calls; Gergus *et al*., 2004).

352 Pulse rate, an attribute often involved in mate recognition in these treefrogs (e.g. Klymus *et al*.,

353 2012), was not different between populations, thus the authors suggested that call variation in *H.*

354 *wrightorum* is unlikely to promote mating isolation (although we note that differences in

355 dominant frequency may be due to local adaptation to facilitate transmission [Littlejohn 1971]

356 and could thus lead to assortative mating).

357   Variation in the calls of *H. eximia* has been less comprehensively studied. An early report

358 by Blair (1960) classified some populations as either producing "slow" (PR ~ 50 Hz) or "fast"

359 (PR ~100 Hz) calls. That study was not concerned with exploring the possible origin of such

360 variation, but we now know that two populations then categorized as producing slow calls

361 correspond to chorus where *H. eximia* is sympatric with *T. smithii*; in these we recorded song

362 with low PR and relatively high PPF and DF. In fact, the calls of frogs recorded in sympatry with

363 *T. smithii* were more variable (mean CV = 29%) than those recorded elsewhere (see Table 2 and

364 Fig. 2). This is unlikely to be an artifact of a small sample size, as this estimate is based on a

365 larger sample than that used to assess variation in the attributes of calls produced in sympatry

366 with *H. arenicolor*. In mixed choruses, calls of *H. eximia* may be masked by the long and/or the

367 short notes of *T. smithii* calls. This may mean that males of *H. eximia* confront unpredictable note

368 periodicity, since calls of *T. smithii* are made of different notes, both of variable length, and each

369 with a different and also variable period (Table 3). This would promote high variation of inter-

370 call interval in *H. eximia*, which may explain the high coefficient of variation of IC in those

371 sympatric localities (Table 2).

372   We could not discern a link between type of variable (temporal, frequency, structural) and

373 the amount of variation it harbors (Table 2; Fig. 2), but pulse peak frequency, dominant

374 frequency, call duration and pulse duration were the least variable elements of *H. eximia* calls,

375 and may thus be useful elements for species recognition.

376 **Call variation**

377 In his comprehensive work, Duellman (1979; 2001) suggested that *H. eximia* lacks a typical call.

378 This was puzzling since anuran advertisement calls are often stereotypical, which is why they

379 often constitute pre-mating barriers between closely-related species. We did find a substantial

380 degree of variation in calls, but mostly restricted to two geographic regions: Balsas and

381 Magdalena (Fig. 3). Calls of *H. eximia* from these two basins were significantly different from

382 calls elsewhere, mostly in structure/length (IC, CD, NP; discriminant function 1). Number of

383 pulses (NP) was highly correlated with pulse rate (PR; e. g. *H. eximia* in contact with *T. smithii*:

384 0.9204), which together with dominant frequency plays an important role in species recognition

385   (Littlejohn, 1965; Gerhardt & Davis, 1988; Gerhardt, 1994) and mate discrimination in other

386   hylids (e. g. *Hyla ebraccata*, Wollerman, 1998). There are no published studies of mate choice in

387   *H. eximia*, but in the related *H. arenicolor* female mate choice is influenced by call PR (Klymus

388   *et al.*, 2012), which is a key element for maintaining isolation between lineages of *H. arenicolor*

389   and might also lead, through its correlation with NP, to reproductive isolation between

390   populations of *H. eximia*. Call (CD) and inter-call duration (IC) reflect calling effort, and the

391   energy required to increase them depends on body size (Gerhardt, 1994). We found no differences

392   between populations or basins in body size, thus it is possible that variation of call structure in the

393   Balsas localities is adaptive, enabling *H. eximia* to avoid interference from the calls of *T. smithii*

394   (see below).

395   Calls of *H. eximia* in Balsas and Magdalena were also different in frequency (DF;

396   function 2) from calls elsewhere. This a static trait (Gerhardt, 1991) that, like other such

397   attributes of treefrog calls, is determined by morphology (several body structures, muscle size,

398   larynx structure, among others; Dubley & Rand, 1991; McClelland, 1996; Wells, 2001; Gerhardt

399   & Huber, 2002), resulting in structural similarity of notes and seemingly simple calls (compared

400   to the more complex bird song). A change in this attribute would be energetically costly, and it is

401   therefore likely that the observed variation in DF is the result of selection to improve

402   communication efficiency, which may include avoiding interference from heterospecific calls.

403   In the Balsas and Magdalena basins, *H. eximia* shares choruses with *T. smithii*. Calls of

404   *H. eximia* from Balsas are very variable, and their canonical acoustic space (as defined by

405   discriminant functions 1 and 2; Fig. 3) is different from that of the local *T. smithii*, which seems

406   to indicate reproductive character displacement, a possibility that requires corroboration from

407   more intense sampling and phonotaxis studies. Interestingly, the distribution in the same

408   canonical space of the calls of *H. eximia* from Magdalena shows that they are shifted in the

409   opposite canonical direction than in Balsas. This can be caused by random differences being

410   selected in different populations, as long as they reduce overlap with *T. smithii*. However, we note

411   that in Magdalena *H. eximia* also faces interference with another frog species; *Gastrophryne sp*.

412   Probably because this is a larger frog, its calls are much longer and lower-pitched (Duellman,

413   2001) than to those of *H. eximia*. This results in a more limited acoustic space available for *H.*

414   *eximia* to accommodate its call to avoid masking, being simultaneously limited by the temporal

415   and spectral attributes of the calls of *T. smithii* and of *Gastrophryne sp*.

416   While at Balsas and Magdalena the calls of *H. eximia* differ significantly from those of

417   their conspecifics elsewhere (and are completely different from those of *T. smithii* in Balsas),

418   calls from localities sympatric with *H. arenicolor* (mostly in the Panuco basin) were similar to

419   those from allopatric populations. This was unexpected since, although both species are

420   morphologically very different (*H. arenicolor* has a rough, granulated skin and is somewhat

421   larger than smooth-skinned *H. eximia*, which is more similar to the smaller *T. smithii*), they

422   belong to the same phylogenetic group, share the same mating ponds, and in the Balsas (where

423   we did not sample mixed choruses of these two species) there is evidence suggesting ancient

424   mitochondrial introgression (Bryson *et al.*, 2010; Klymus & Gerhardt, 2012), thus mating

425   interference between them was expected. It may be that, since the calls of *H. eximia* and *H.*

426   *arenicolor* are substantially different, there is no, or little, interference between their calls to drive

427   character displacement, although it may also be that the interaction of the two species is too

428   recent in northern and central Panuco (see below).

429   **Genetic variation**

430   While call variation between some regions covered in our sample is supported by mitochondrial

431   phylogeny and population structure, it cannot be properly interpreted as a consequence of the

432   phylogeny of *H. eximia*. The mitochondrial reconstruction is generally well supported, although it

433   draws a puzzling phylogenetic history of *H. eximia*. It suggests, very preliminarily, that the

434   populations in our sample originated in the south-east of the current species distribution, and

435   expanded clockwise in their colonization of what are now the basins of rivers Balsas, Magdalena

436   (Ameca), and central (lower) Lerma, from where they may have reached the northern (Santiago)

437   and southern (upper) Lerma, as well as the central and upper Panuco basins (Fig. 1). Published

438   phylogeographic hypotheses for *H. arenicolor* often include a few sequences of *H. eximia* and

439   cover a limited portion of its geographic range, so that no phylogeographic hypotheses for this

440   species can be drawn from them (see Bryson *et al.*, 2010; Klymus & Gerhardt, 2012). Thus we

441   provisionally propose the above phylogenetic scenario of *H. eximia* based on our best -resolved

442   (mitochondrial) reconstruction (Fig. 1).

443   **Phenotypic, genetic and geographical distance**

444   Call variation between the regions covered in our sample may not be random (i.e. due to drift); it

445   was greater in particular basins (Balsas and Magdalena) and chorus compositions (*H. eximia-T.*

446   *smithii*), but did not correlate with genetic or geographic distance. Absence of a correlation

447   between acoustic and genetic distance has been reported in other studies. For instance, in

448   *Oophaga* (*Dendrobates*) *pumilio* (Pröhl *et al*., 2007), the correlation is lost when the analysis

449   controls for geographic distance, and a similar effect occurred in some populations of the

450   Túngara frog *Engystomops* (*Physalaemus*) *pustulosus* (Ryan *et al*., 1996). These findings are

451   perhaps not surprising, since one of the forces that most effectively shape anuran call attributes is

452   female mate choice (Boul *et al*., 2007), and this is most effective when mistakes are penalized as

453   in a hybrid zone. Thus we should in fact expect greater call variation between populations when

454   reproductive character displacement is favored in at least one of them (Pfennig & Pfennig, 2010),

455   than when populations are distant geographically but do not face mating call interference. Indeed,

456   we rule out the possibility that a correlation between genetic and acoustic distance exists but was

457   undetected, since we have adequate call samples, and, as expected, mitochondrial genetic

458   distance was correlated with geographic distance, making it likely that our genetic screening is

459   unbiased.

460   We have argued that call variation in the regions where *H. eximia* and *T. smithii* coexist is

461   consistent with the expected effects of acoustic interference, but it might also be the result of

462   morphological differentiation in attributes that influence call production (e. g. the body size in

463   *Hyla leucophyllata*, Lougheed *et al*., 2006; see also Emerson, 2001; Gerhardt & Huber 2002;

464   Castellano *et al*., 2002; Hoskin *et al*., 2005). However, an exploratory analysis failed to detect

465   significant differences in body size among populations of *H. eximia*, thus it is unlikely that the

466   variation in calls found between basins is due to differences in the body size. Other causes of call

467   differentiation include local differences in call assemblage (Wollerman & Wiley, 2002), in signal

468   transmission efficiency (related to the type of habitat), or in sexual selection (acting through

469   reproductive isolation mechanisms). The first is compatible with our explanation of call

470   differentiation to avoid masking by the calls of *T. smithii*, whereas the reported lack of

471   differentiation amongst the populations not sympatric with *T. smithii*, which cover a much larger

472   and presumably environmentally variable geographic area, argues against the other two. We thus

473   provisionally propose that the geographic variation of advertisement calls of *H. eximia* in the

474   Balsas and Magdalena basins populations is due to reproductive character displacement produced

475   by sharing mating choruses with the related and morphologically similar *T. smithii* and with the

476   distantly related *Gastrophryne sp*.

477   **Acknowledgments**

478 Filed work was conducted under SEMARNAT license SGPA/DGVS/03580/09, and was made

479 possible by the help of J. Ávila, A. Archundia, A. Briones, J. Carillo, C. Chávez, M. A. Martínez,

480 M. Nuñez, Tomás, M. J. Godínez, B. Peña, I. Barbosa, C. Montes, N. Lifshitz, E. Quiróz, N.

481 Miranda, M. Suárez, M. Méndez, E. Bermúdez, R. Beamonte, F. Mendieta, A. Freyermuth, C.

482 Ríos, A. García, E. Vázquez and E. Ávila-Luna, who also provided logistical support. G. Cortés-

483 Soto and A. Ríos- Chelén helped with the initial sound analyses, S. Benitez-Vieyra and a former

484 referee provided useful statistical advice and L. Kiere helped improving the English. This project

485 was founded with a grant from CONACyT (83779) to CMG, and a scholarship to RERT also

486 from CONACyT. This paper constitutes a partial fulfilment of the Graduate Program in

487 Biological Sciences of the National Autonomous University of Mexico (UNAM). We declare that

488 the authors have no conflict of interests, and that we adhered to the local legislation and to ethical

489 guidelines for the use of animals in research.

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Populations sampled

Populations where advertisement calls of *Hyla eximia*, *Hyla arenicolor* and *Tlalocohyla smithii* were recorded

Population	Symbol map	Latitude	Longitude	Chorus composition	N
El Realejo, San Luis Potosí.	R	22° 40' 23.98'' N	-100° 25' 5.45'' W	*H. eximia* allopatric	26
Laguna de Gerardo, San Luis Potosí.	L	22° 38' 27.36'' N	-100° 26' 20.94'' W	*H. eximia* allopatric	29
Sierra de Álvarez, San Luis Potosí.	SA	22° 01' 52.56'' N	-100° 36' 49.86'' W	*H. eximia* allopatric	15
Playita de San Rafael, Jalisco.	SR	20° 02' 52.98'' N	-103° 09' 46.26'' W	*H. eximia* allopatric	25
Rancho Santa Elena, Hidalgo.	SE	20° 08' 7.38'' N	-98° 30' 43.50'' W	*H. eximia* sympatric with *H. arenicolor*	18
Magdalena, Jalisco.	MA	20° 54' 0.41'' N	-103° 59' 36.85'' W	*H. eximia* sympatric with *T. smithii*	20
Presa de Malinaltenango, Edo. de México.	PM	18° 48' 32.01'' N	-99° 43' 26.18'' W	*H. eximia* sympatric with *T. smithii*	7
Rancho Santa Elena, Hidalgo.	SE	20° 08' 7.38'' N	-98° 30' 43.50'' W	*Hyla arenicolor*	2
Km 58, San Luis Potosí.	K58	22° 01' 12.18'' N	-100° 36' 7.26'' W	*Hyla arenicolor*	7
Magdalena, Jalisco.	MA	20° 54' 0.41'' N	-103° 59' 36.85'' W	*Tlalocohyla smithii*	12
Presa de Malinaltenango, Edo. de México.	PM	18° 48' 32.01'' N	-99° 43' 26.18'' W	*Tlalocohyla smithii*	5
Tecomatepec, Edo. de México.	TC	18° 50' 9.06'' N	-99° 41' 40.62'' W	*Tlalocohyla smithii*	3

Descriptive data for *H. eximia*

Descriptive data of morphology, body temperature and attributes of the advertisement calls of

H. eximia

Variable (units)	H. eximia allopatric (n=95)			H. eximia (sympatric with H. arenicolor) (n=18)			H. eximia (sympatric with T. smithii) (n=27)		
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
SLV (mm)	**28.149**	2.426	8.618	**32.252**	1.734	5.376	**31.011**	3.197	10.311
T (°C)	**17.634**	2.041	11.572	**14.478**	1.663	11.489	**19.556**	3.226	16.497
CD (s)	**0.199**	0.015	7.417	**0.267**	0.019	7.018	**0.196**	0.025	12.907
IC (s)	**0.401**	0.124	30.826	**0.548**	0.133	24.344	**0.657**	0.416	63.282
CP (s)	**0.599**	0.123	20.58	**0.815**	0.127	15.563	**0.853**	0.432	50.669
NP	**20.435**	1.791	8.766	**16.133**	1.605	9.947	**12.066**	2.925	24.244
PD (s)	**0.005**	0	9.148	**0.005**	0	4.587	**0.007**	0.002	24.963
ID (s)	**0.009**	0.001	12.086	**0.017**	0.002	9.455	**0.016**	0.005	29.066
PA (V)	**0.184**	0.135	73.674	**0.437**	0.149	34.216	**0.261**	0.111	42.48
PPF (kHz)	**2.380**	2.147	9.023	**2.260**	0.079	3.5	**2.760**	0.484	17.51
PP (s)	**0.014**	0.001	9.381	**0.021**	0.002	7.367	**0.023**	0.006	24.969
PR (Hz)	**98.139**	9.776	9.961	**56.641**	5.196	9.174	**57.241**	15.683	27.398
DF (kHz)	**2.590**	0.207	8.004	**2.410**	0.135	5.61	**3.003**	0.618	20.59
FF (kHz)	**0.649**	0.293	45.19	**0.647**	0.216	33.4	**0.534**	0.222	41.5

Notes: SVL= snout vent length; T = corporal temperature; CD= call duration; IC= inter-call duration; CP= call period; NP= number of pulses; PD= pulse duration; ID= inter-pulse duration; PA= pulse amplitude; PPF= pulse peak frequency; PP= pulse period; PR= pulse rate; DF= dominant frequency; FF= fundamental frequency.

Table 3_(on next page)

Descriptive data for *H. arenicolor* and *T. smithii*

Descriptive data of morphology, body temperature and attributes of the advertisement calls of *H. arenicolor* and *T. smithii.*

variable	*H. arenicolor* (n=9)			*T. smithii* (n=20)		
(units)	Mean	SD	CV	Mean	SD	CV
SLV (mm)	**42.664**	2.999	7.029	**24.523**	1.299	5.297
T (°C)	**19.044**	2.783	14.613	**18.285**	1.446	7.91
CD (s)	**0.884**	0.182	20.623	**0.469**	0.055	11.714
IC (s)	**1.6**	0.473	29.583	**1.18**	0.923	78.266
CP (s)	**2.499**	0.527	21.089	**1.649**	0.902	54.714
NP	**22.613**	4.453	19.693	**23.735**	4.935	20.794
PD (s)	**0.008**	0.003	42.701	**0.005**	0.001	9.823
ID (s)	**0.039**	0.007	16.904	**0.02**	0.004	16.95
PA (V)	**0.368**	0.209	56.86	**0.214**	0.152	71.076
PPF (kHz)	**1.470**	0.66	45.031	**4.320**	0.163	3.8
PP (s)	**0.047**	0.009	19.567	**0.025**	0.003	13.41
PR (Hz)	**24.837**	4.91	19.767	**48.218**	7.661	15.888
DF (kHz)	**1.264**	0.666	52.662	**4.450**	0.158	3.6
FF (kHz)	**-**	-	-	**1.920**	1.515	79.1

Notes: SVL= snout vent length; T = corporal temperature; CD= call duration; IC= inter-call duration; CP= call period; NP= number of pulses; PD= pulse duration; ID= inter-pulse duration; PA= pulse amplitude; PPF= pulse peak frequency; PP= pulse period; PR= pulse rate; DF= dominant frequency; FF= fundamental frequency. The calls of *H. arenicolor* showed no fundamental frequency.

Genetic diversity of populations of *H. eximia*

Haplotype (*h*) and nucleotide (π) diversity of populations of *H. eximia* from each sub-region of the sampled basins. The two mitochondrial genes were concatenated as well as the two the nuclear genes.

Basin	mtDNA N, H	h	π	Nuclear N, H	h	π
Northern Panuco	6, 4	0.800 (±0.172)	0.022 (±0.016)	5, 4	0.900 (±0.161)	0.123 (±0.080)
Central Panuco	7, 5	0.857 (±0.137)	0.023 (±0.016)	6, 5	0.933 (±0.122)	0.200 (±0.121)
Southern Panuco	3, 3	1.000 (±0.272)	0.064 (±0.052)	1, 1	1.000 (±0.000)	0.000 (±0.000)
Northern Lerma	2, 2	1.000 (±0.500)	0.058 (±0.062)		------	------
Central Lerma	9, 9	1.000 (±0.052)	0.077 (±0.045)	8, 8	1.000 (±0.062)	0.190 (±0.109)
Southern Lerma	3, 3	1.000 (±0.272)	0.064 (±0.052)	3, 3	1.000 (±0.272)	0.157 (±0.123)
Magdalena Lerma	2, 2	1.000 (±0.500)	0.461 (±0.466)	3, 3	1.000 (±0.272)	0.118 (±0.094)
Balsas	3, 3	1.000 (±0.272)	0.269 (±0.205)	2, 2	1.000 (±0.500)	0.088 (±0.095)

Notes: Standard deviation in parentheses. N= analyzed samples number, H= number of haplotypes.

Population differentiation of *H. eximia*

Basin	Northern Panuco	Central Panuco	Southern Panuco	Central Lerma	Southern Lerma	Magdalena	Balsas
Northern Panuco	-----	-0.025 (P= 0.513)	0.133 (P= 0.405)	**0.175** (P= **0.009**)	**0.304** (P= **0.036**)	**0.303** (P= **0.045**)	0.300 (P= 0.054)
Central Panuco	-0.088 (P= 0.639)	-----	0.189 (P= 0.117)	0.060 (P= 0.090)	**0.239** (P= **0.000**)	0.089 (P= 0.234)	0.075 (P= 0.225)
Southern Panuco	**0.904** (P= **0.027**)	**0.915** (P= **0.018**)	-----	**0.182** (P= **0.045**)	0.223 (P= 0.486)	0.294 (P= 0.099)	0.363 (P= 0.333)
Central Lerma	0.101 (P= 0.090)	0.077 (P= 0.126)	**0.840** (P= **0.000**)	-----	**0.236** (P= **0.027**)	-0.017 (P= 0.504)	0.098 (P= 0.063)
Southern Lerma	**0.328** (P= **0.027**)	**0.309** (P= **0.009**)	0.863 (P= 0.090)	0.131 (P= 0.063)	-----	0.268 (P= 0.063)	0.299 (P= 0.18)
Magdalena	0.334 (P= 0.081)	0.445 (P= 0.054)	0.565 (P= 0.099)	0.364 (P= 0.387)	0.161 (P= 0.108)	-----	-0.031 (P= 0.396)
Balsas	**0.538** (P= **0.018**)	**0.611** (P= **0.000**)	0.628 (P= 0.153)	**0.560** (P= **0.000**)	0.409 (P= 0.198)	-0.431 (P= 0.495)	-----

Notes: Mitochondrial F_{ST} below, and nuclear F_{ST} above the diagonal.

Figure 1

Localities sampled, chorus composition and mitochondrial clades

Hypothesized colonization routes of *H. eximia* in central Mexico. The distribution of frogs belonging to clades A-D from a phylogenetic analysis of two concatenated mitochondrial genes (see text) is indicated with globes of different color (clade A=turquoise blue; clade B= red; clade C= yellow; D= green). Dotted arrows indicate the possible direction of the colonization, and are not intended to show the exact paths followed by the frogs. The occurrence of one individual form Magdalena in clade A is indicated with a dotted turquoise-blue line. Thin grey dotted lines represent boundaries between the major basins (Panuco, Lerma, Balsas and the currently closed Valley of Mexico), whereas rivers are indicated with continuous grey lines. Lerma River flows northwards until it meets the Santiago, which drains in the Pacific Ocean to the west. Balsas drains southwards, also in the Pacific, whereas the Panuco drains to the east, in the Gulf of Mexico. See Table 1 for population codes.
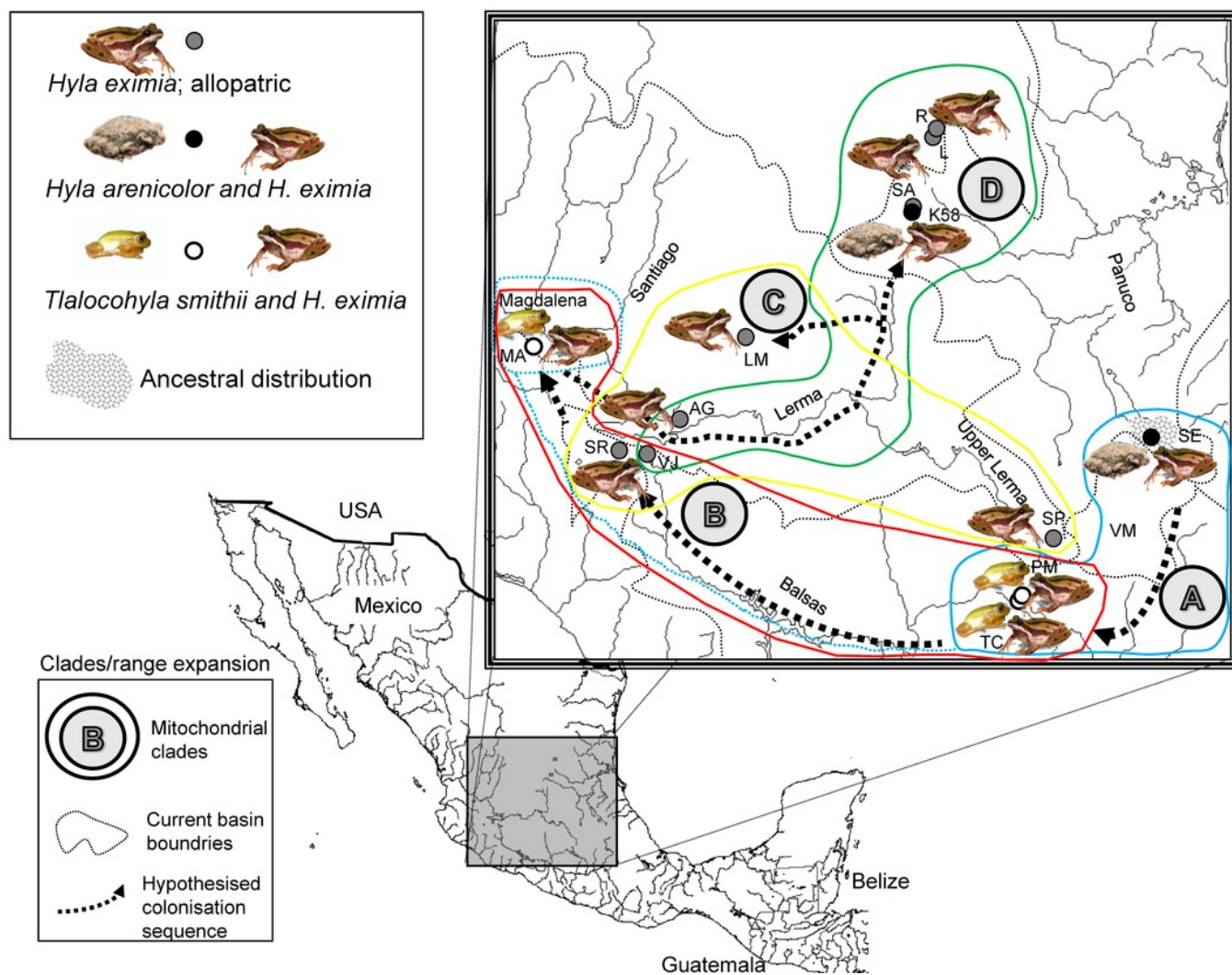
Figure 2

Call spectrograms and averages of call attributes

A) Spectrograms of advertisement calls produced by individuals of each chorus composition (A= allopatric *Hyla eximia*; B= *Hyla arenicolor*; C= *H. eximia* sympatric with *H. arenicolor*; D= *Tlalocohyla smithii*; and E= *H. eximia* sympatric with *T. smithii*). B) From top to bottom, mean temporal, pulse and frequency variables. Categories in the X axis correspond to the chorus composition. Variable identity, indicated next (right) to the solid symbols, apply also to the open symbols (e.g. circles of both types represent the same variable). Solid symbols indicate *H. eximia* populations and open symbols represents the heterospecific species.
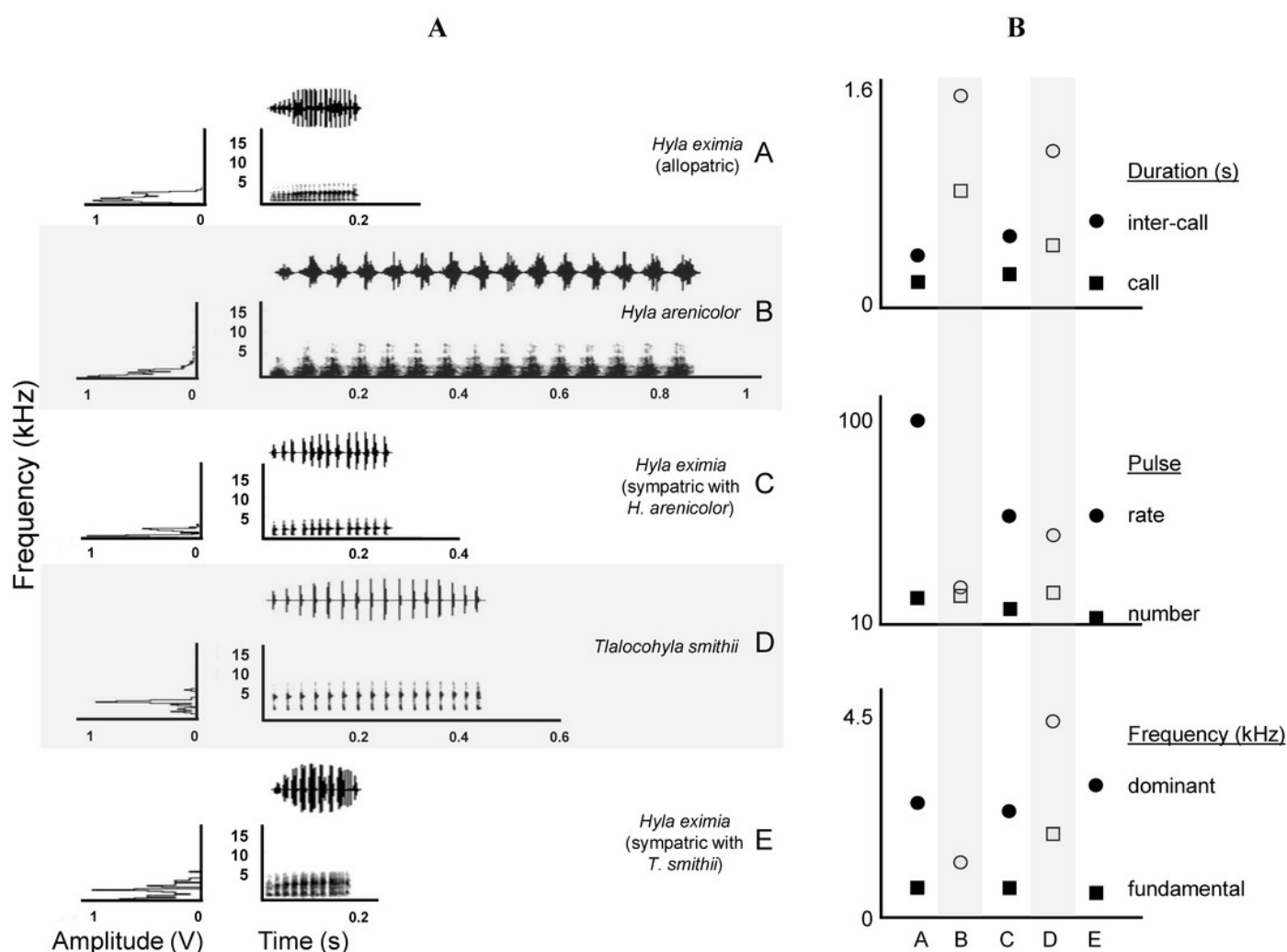
Figure 3

Discriminant plot

A) Discriminant functions of advertisement call attributes of *Hyla eximia*. Discriminant function 1 represents the inter-call duration (ID), call duration (CD) and number of pulses (NP); function 2 is loaded by dominant frequency (DF). B) Discriminant functions of advertisement call attributes of *Hyla eximia* (H. e.), *Hyla arenicolor* (H. a.) and *Tlalocohyla smithii* (T. s.) separated according to basin. Discriminant function 1 is largely loaded by ID, CD and NP, and function 2 by DF. The dashed line encompasses the canonical space occupied by calls of *T. smithii* from Balsas (red) and Magdalena (black) based on the functions generated using the calls of *H. eximia*.
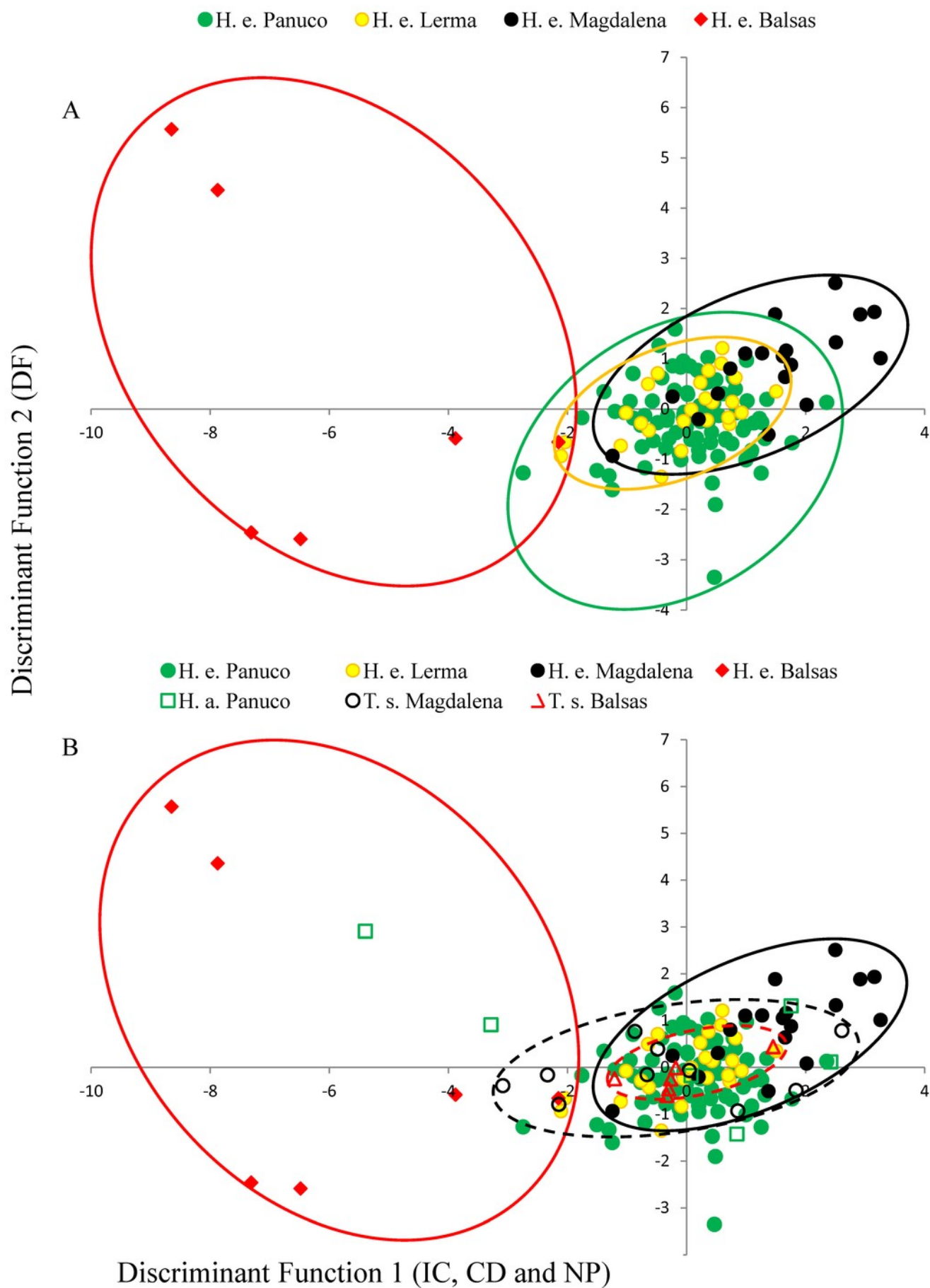
Figure 4

Scores of *Hyla eximia* calls from the first two canonical functions

Post hoc tests reveal significant differences (indicated by different letters above the bars) between the mean values of canonical scores of calls from different basins.
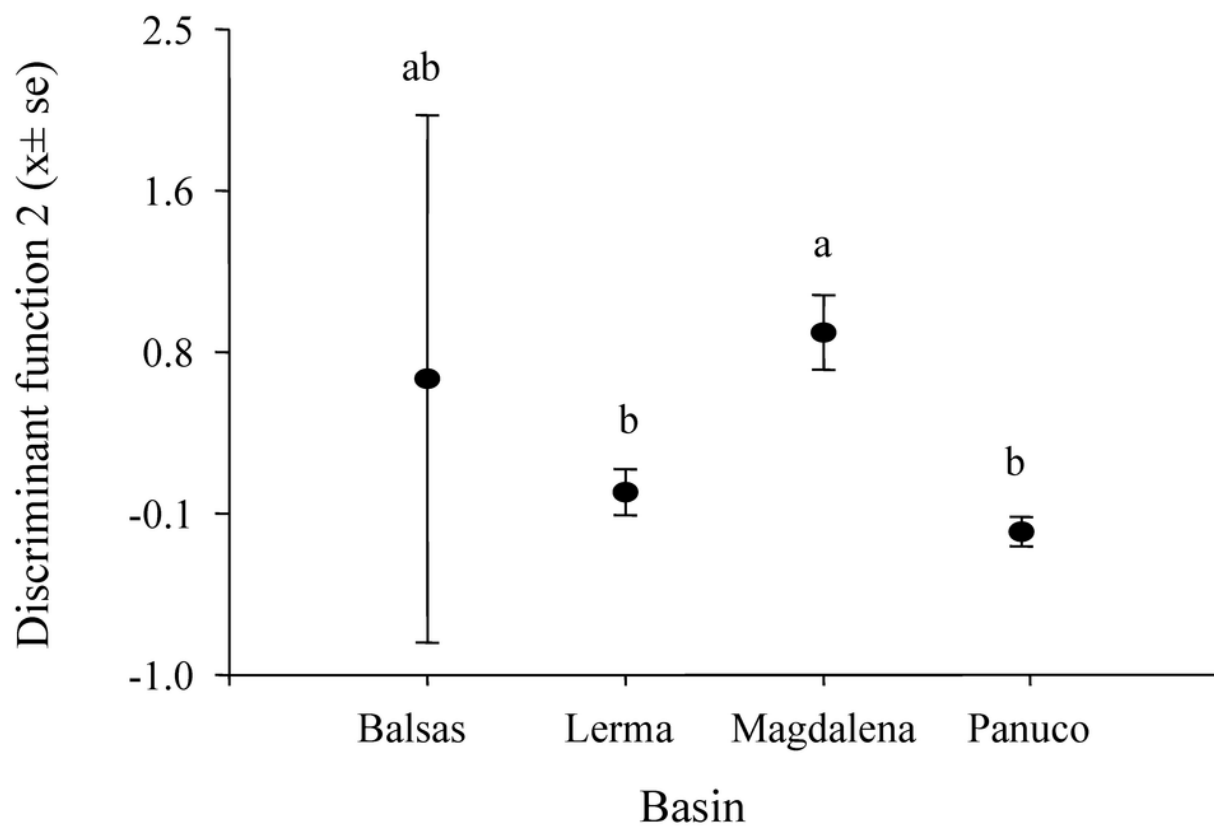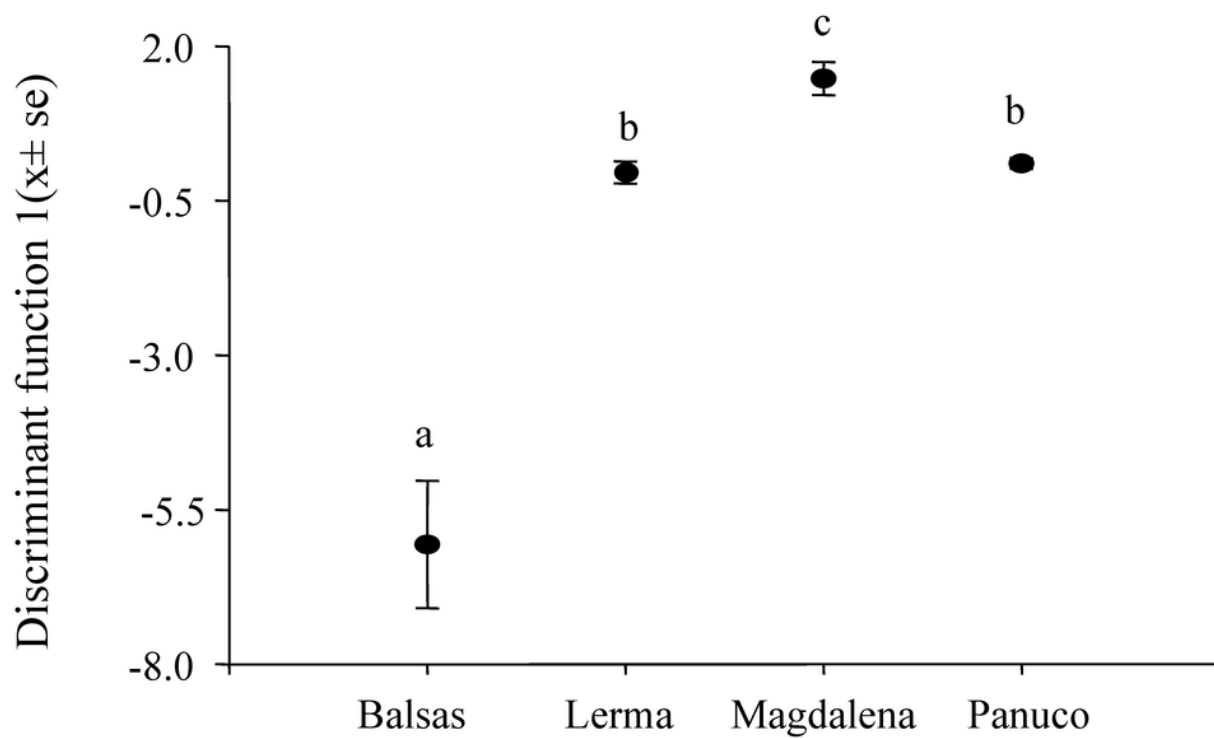
Figure 5

Phylogeny of concatenated mitochondrial (ATPase and cyt-*b*) and nuclear (POMC and Rho) genes

Individuals are separated by basin: northern Panuco (green), central Panuco (brown), southern Panuco (turquoise blue), northern Lerma (blue), central Lerma (yellow), southern Lerma (purple), Magdalena (black), Balsas (red) and out groups (gray). Letters to the left designate proposed clades.
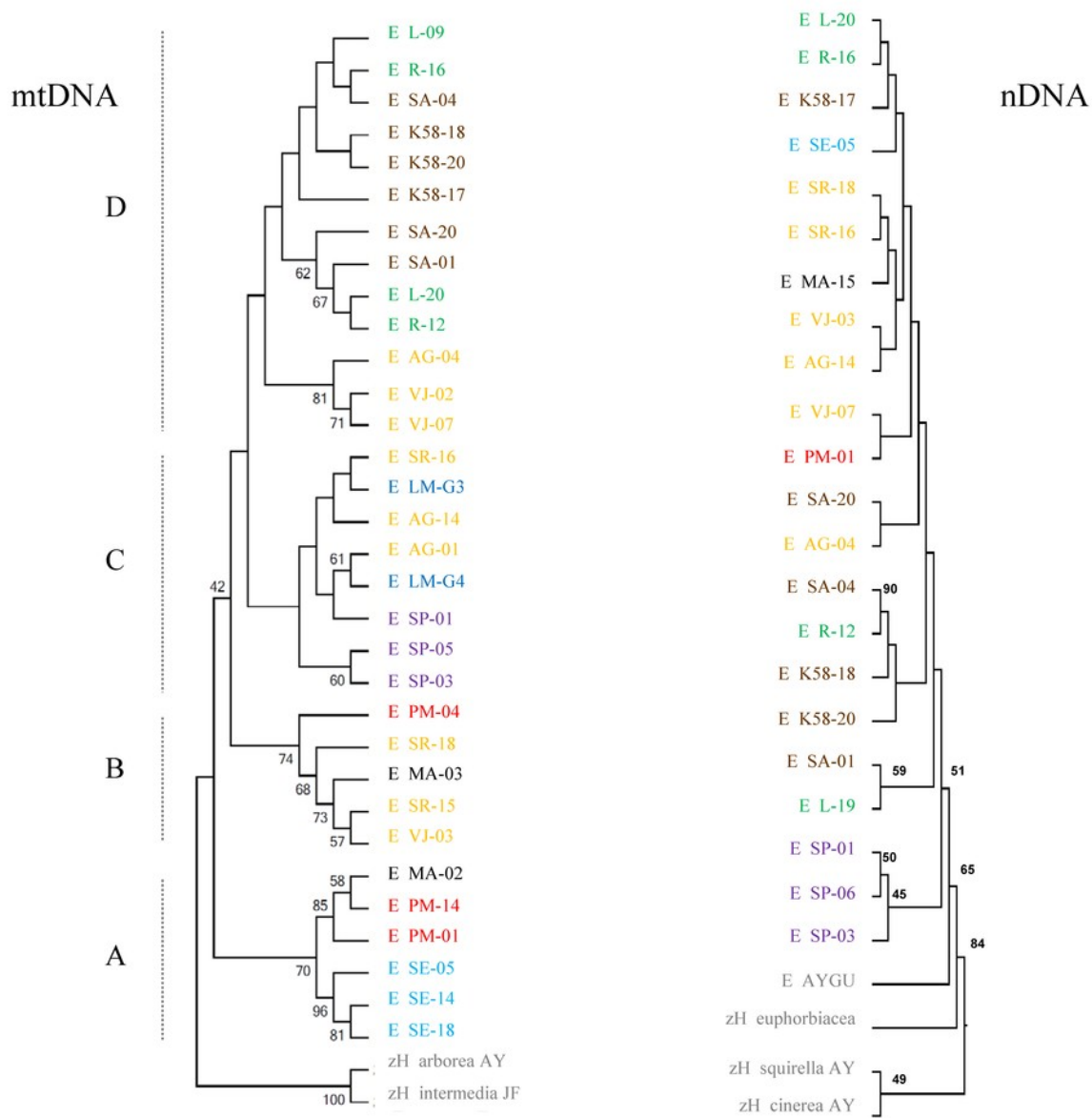
Figure 6

Haplotype network of concatenated mitochondrial genes Panuco

Haplotypes correspond to each of the basins' sub-regions.
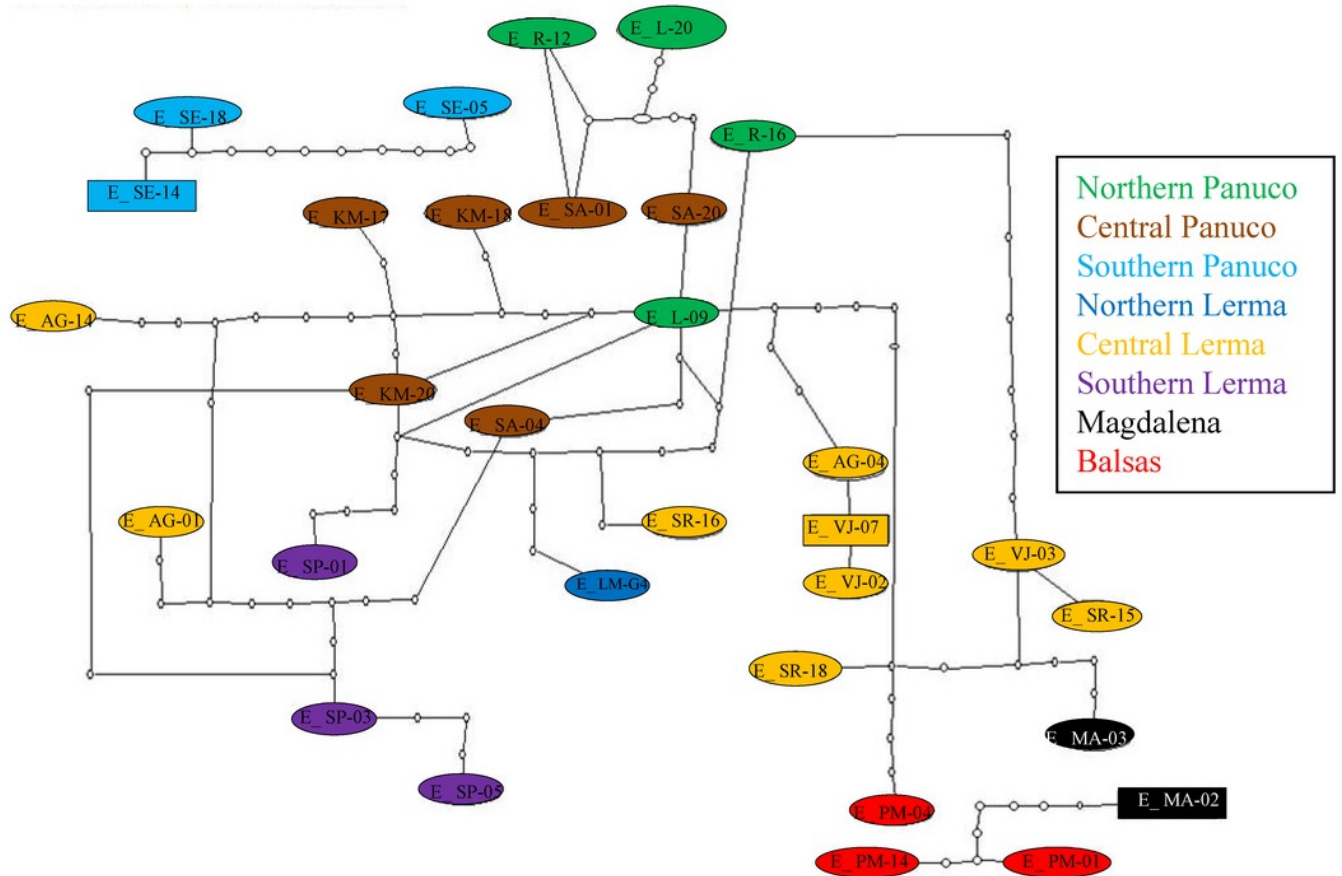
Figure 7

Population structure of sampled individuals of *H. eximia*

A) Plot of Delta K (change in the probability of K) as a function of K (= number of groups). B) Population structure infered using POMC nuclear gene. C) Population structure inferred using Rhod nuclear gene. Barplots in B and C show the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular population for K= 3, determined with the Evanno method.