UNIVERSIDAD DE CHILE-FACULTAD DE CIENCIAS-ESCUELA DE PREGRADO

"¿Qué está desaparecido, poblaciones o una especie? Antiguas grabaciones de llamadas revelan similitud entre las dos presuntas especies de Ranita de Darwin"

Seminario de Título entregado a la Universidad de Chile en cumplimiento parcial de los requisitos para optar al Título de: **Biólogo con Mención en Medio Ambiente**

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ESCUELA DE PREGRADO – FACULTAD DE CIENCIAS – UNIVERSIDAD DE CHILE

INFORME DE APROBACIÓN SEMINARIO DE TITULO

Se informa a la Escuela de Pregrado de la Facultad de Ciencias, de la Universidad de Chile

que el Seminario de Título, presentado por el Sr:

Gabriel Bidart Enriquez.

"¿Qué está desaparecido, poblaciones o una especie? Antiguas grabaciones de llamadas revelan similitud entre las dos presuntas especies de Ranita de Darwin"

Ha sido aprobado por la Comisión de Evaluación, en cumplimiento parcial de los requisitos para optar al Título de Biologo con Mencion en Medio Ambiente

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Santiago de Chile, …………………………

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Resumen

El género *Rhinoderma* tiene dos especies putativas, la Ranita de Darwin del norte y del sur que tiene una simpatría histórica entre 36 y 27° S en Chile central. La identidad de las dos taxones se basa en estudios que muestran variación en caracteres morfológicos, evolutivos, cariológicos y moleculares. La Ranita de Darwin del norte no se ha avistado desde 1981. Las señales acústicas de atracción de pareja están sujetas a la variación geográfica y son herramientras importantes para aclarar estatus sistemáticos entre especies, dado que facilitan barreras reproductivas entre especies. Para investigar la diferenciación acústica entre las Ranitas de Darwin, hemos analizado grabaciones antiguas de llamadas de anuncio de Ranitas de Darwin del norte, un taxón actualmente perdido, junto con más grabaciones recientes de tres poblaciones de la Ranita de Darwin del sur. Combinamos pruebas GLM y análisis discrminante para evaluar las diferencias de la Ranita de Darwin del norte y la formación de conjuntos acústicos entre todas las poblaciones de las dos presuntas especies. El análisis mostró diferencias limitadas en las llamadas de la especie del norte en comparación con las del sur que se relacionan, probablemente, con las disimilitudes en las condiciones climáticas y el tamaño del cuerpo entre las poblaciones. El análisis discriminante arrojo dos grupos separados, uno para la población de Tantauco de la *R. darwinii* de la Isla de Chiloé y otro para las poblaciones continentales que incluyeron Las Carmelitas, cerca de la ciudad de Concepción (*R.rufum*), Huilo Huilo y Quelulat (*R.dariwini*). Luego de repetir este análisis excluyendo a la población de la isla, las poblaciones continentales de la Ranita de Darwin del norte y del sur permanecieron en un conjunto único. Proponemos que la divergencia entre poblaciones de la Ranita de Darwin relacionada a los rasgos acústicos informados en este estudio y otros caracteres informados en estudios previos se debe a una variación geográgica

26   que depende de regímenes climáticos a lo largo del rango histórico de *Rhinoderma*. Las

27   medidas de conservación recomendadas por la Estrategia Binacional de Conservación de las

28   Ranitas de Darwin debiesen considerar una falta potencial de diferenciación específica a lo

29   largo de la distribución geográfica de las poblaciones de la Ranita de Darwin en Chile.

Research Article - Animal Conservation

Is it a species or populations that are missing? vintage call recordings reveal similarity

between the two alleged species of Darwin's frogs

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Abstract

The genus Rhinoderma comprises two putative species, the northern and southern Darwin's frogs that had a historical sympatric area between 36 and 37 ° S in central Chile. The identity of the two taxa is based in studies showing variation in morphological, developmental, karyological and molecular characters. The northern Darwin's frog has not been seen since 1981. Mate attracting acoustic signals are subjected to geographical variation and are important tools to resolve systematic status between species as these signals provide reproductive barriers between species. To investigate acoustic differentiation in Darwin`s frogs we analysed vintage recordings of the advertisement calls of northern Darwin's frogs, currently a missing taxon, together with more recent recordings of three populations of southern Darwin's frogs. We combined GLM tests and discriminant analysis to assess the differences of the northern Darwin's frog and the formation of acoustic clusters between all populations of the two presumed species. The analysis showed limited differences in the calls of the northern species compared to the southern species that are probably related to dissimilarities in climate conditions and body size between populations. The discriminant analysis yielded two separate clusters, one for the Tantauco population of R. darwinii on the island of Chiloé and another for populations from the mainland that included Las Carmelitas, near the city of Concepción (R. rufum) Huilo Huilo and Queulat (R. darwinii). After repeating this analysis excluding the island population, the continental populations of the northern and southern Darwin's frogs remained in a single cluster. We propose that the divergence between populations of Darwin's frogs in acoustic traits reported in this study and other characters reported in previous studies is due to geographical variation? dependent on climate regimes along the historical range of Rhinoderma. The conservation measures recommended by the Binational Conservation Strategy for Darwin's Frogs should account for

67 a potential lack of specific differentiation along the historical geographic distribution of

68 Darwin's frog populations in Chile.

71 **Introduction**

72 Acoustic signals of animals can reveal insights on their evolution, providing relevant tools for

73 promoting their conservation (reviewed in Laiolo, 2010). Species producing vocal signals

74 usually have particular characteristics that allow recognition by conspecifics (Tyack, 2000;

75 Feng et al., 2009; Araya-Salas et al., 2019). These signals are produced in different contexts

76 such as mate attraction, territorial defence or in response to stressful situations (Obrist et al.,

77 2010; Bradbury & Vehrencamp, 2011). In mate attraction contexts, the signal structure is

78 affected by coevolutionary processes between sender and receiver, promoting species-specific

79 communication (e.g. Tang et al., 2001; Wolley, 2011).

80   As such, different patterns of variation of acoustic signals can occur depending on the

81 geographic distance between populations and the identity of the species involved. For

82 instance, related species living in areas that have been apart for long time spans can show

83 divergence between populations that depends on the geographic distance among localities

84 (Nevo & Capranica, 1985; Wilczynski & Ryan, 1999; Castellano et al., 2000; Giacoma and

85 Castellano, 2001; Bernal et al., 2005; Prohl et al., 2006, 2007; Weaver et al., 2020). However,

86 a contrasting condition corresponds to allopatric populations having limited differentiation in

87 their acoustic signals, for which a specific status can be established by genetic evidence only

88 (Heyer et al., 2005; Greenbaum et al., 2013). Among anurans, morphologically cryptic

89 species occur frequently, but combined genetic and bioacoustic evidence has allowed to

90   detect unnoticed diversity among sympatric species (e.g., Funk et al., 2012) or to merge taxa

91   formerly considered as morphologically distinct species (e.g., Kuramoto, 1997).

92   Reproductive signals provide an isolation mechanism to prevent hybridization among

93   related sympatric species (Claridge, 1990; Freeman & Montgomery, 2017; Weaver et al.,

94   2020). As such, sexual signals contribute to evolutionary processes supporting species

95   recognition and delimitation (Mace, 2004; Padial et al., 2010; Cardoso et al., 2012;

96   Tishechkin & Vedenina, 2016; Bolaños-Sittler et al., 2020; Escalona et al., 2019). Therefore,

97   assessing how acoustic variables change along geographic distribution of a species is relevant

98   to understand the role of these traits in divergence and adaptation processes, and they are

99   useful as taxonomical tools (Köhler et al., 2017; Miller et al., 2020), and their pertinence for

100   conservation initiatives has been acknowledged (Laiolo, 2010).

101   To relate different acoustic variables with divergence processes of anurans, Gerhardt

102   (1991) proposed a classification along the continuum of variation of acoustic traits, using a

103   predetermined value of the coefficient of variation as a threshold to identify these variables as

104   static or dynamic. The two types of variables are likely to evolve under different pressures as

105   static traits (those in which the coefficient of variation is lower than 5%) are relevant for

106   species recognition whereas dynamic traits (those in which the coefficient of variation is

107   higher than 5%) are related to social interactions. Typically, dominant frequency and pulse

108   rate are static variables, whereas call rate and call duration are dynamic variables (Gerhardt,

109   1991; Castellano & Giacoma, 1998; Zimmitti, 1999; Castellano et al., 2000, 2002; Friedl,

110   2006; Reinhold, 2011, Köhler et al., 2017).

111   The number of notes in particular is considered a dynamic variable because it is

112   largely under behavioural and physiological control (Jehle & Arak, 1998; Gerhardt et al.,

113   2000). In some species, more or less notes in the repertoire may be associated with a

114   communication system that indicates territoriality (Toledo et al., 2015). But this diversity of

115   sequences differing in the number of notes may also reflect divergence in the call repertoires

116   (Höbel & Fellows, 2016; Serrano, 2018; Weaver et al., 2020). In contrast, dominant

117   frequency is a typical static acoustic variable because it is usually a species-specific trait that

118   can vary with body size both at the intra-population (Gerhardt & Huber, 2002; Gingras et al.,

119   2013) and inter-specific level (Penna & Veloso, 1990; Bosch and de la Riva, 2004; Serrano et

120   al., 2020).

121   *Rhinoderma* is an anuran genus endemic to the South American Patagonia,

122   comprising the species *Rhinoderma darwinii* and *Rhinoderma rufum*, commonly known as

123   southern and northern Darwin's frogs, respectively (Azat et al., 2020). The historical

124   distribution of *R. rufum* was restricted to small and isolated populations located mainly along

125   the coastal mountains of Chile, from Zapallar (32°33' S) to the south of the city of

126   Concepción (36°45' S) (Cuevas, 2014), while the distribution of *R. darwinii* ranges from

127   Concepción to Aysen (46°44' S) in Chile and also distributes in Argentina (Azat et al., 2020).

128   Interestingly, a sympatric zone of the two *Rhinoderma* species occurred in the region of

129   Concepción and Nahuelbuta mountains (37°47' S) (Bourke et al., 2012; Soto-Azat et al.,

130   2013; Fig. 1). Despite of its extended historical distribution, *R. darwinii* is currently in a high

131   risk of extinction due to its fragmented occurrence and populations decrease (Valenzuela-

132   Sánchez et al., 2017), while *R. rufum* is apparently extinct since 1981, as recent efforts to find

133   populations have been unsuccessful (Cuevas, 2014; Bourke, 2012; Soto-Azat et al., 2013;

134   IUCN SSC Amphibian Specialist Group, 2015).
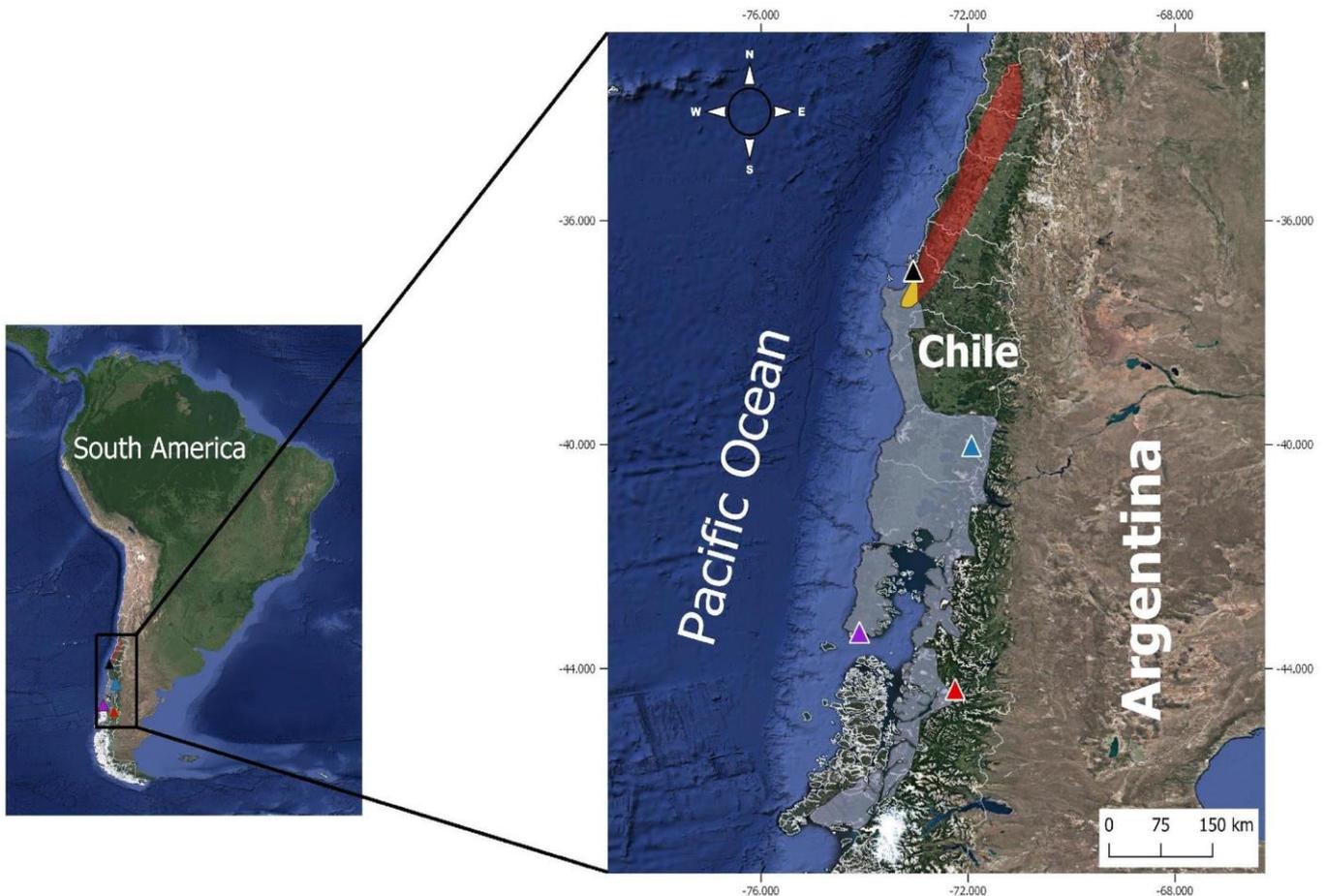
Figure 1. Geographic distribution of the two *Rhinoderma* species according to the UICN red list. The historical distribution of *R. rufum* and *R. darwinii* are depicted in red and grey, respectively, and the area of sympatry of the two species is in yellow. The population where the advertisement calls of *R. rufum* were recorded is indicated by a black triangle (Las Carmelitas) and the populations of *R. darwinii* are indicated by blue, red and purple triangles (Huilo Huilo, Queulat and Tantauco, respectively).

135   The two species of *Rhinoderma* differ in morphological features, including interdigital

136   membranes of the hindlimbs, parental care phenology in adults and mouth and digestive tract

137   structures in tadpoles (Formas et al., 1975; Jorquera et al., 1981, 1982; Formas, 2013).

138   Furthermore, isoenzyme and chromosomal differences contribute to place the two species

139   apart (Formas, 1976; Díaz & Veloso, 1979; Díaz, 1986). In addition, Formas et al (1975)

140   describe qualitative call differences not supported by recordings between the two species, and

141   Penna & Veloso (1990) contributed a preliminary analysis of the calls of *R. darwinii* and *R.*

142   *rufum* indicating species distinctiveness. However, that description of *Rhinoderma*

143   advertisement calls was based on advertisement calls of just four and eight individuals of *R.*

144   *darwinii* and *R. rufum*, respectively. Differences in number of notes, notes and call duration

145   and dominant frequency were reported, but no statistical analyses were applied. One

146   additional evidence on vocal divergence between the two species is the production of release

147   calls by *R. rufum* males, a vocalization that is apparently absent in the congeneric species

148   (Serrano et al., accepted). In this context, to define the taxonomic delimitation of *Rhinoderma*

149   is priority for the current Binational Conservation Strategy of the Darwin's Frogs (Azat et al.,

150   2020) that considers *R. darwinii* and *R. rufum* as two units under different negative pressures,

151   and for which different conservation measures are planned.

152   The main purpose of this study is to compare the acoustic properties of the

153   advertisement calls of *R. rufum* and *R. darwinii* to assess potential divergence between both

154   species. We carried out a detailed description of the advertisement calls of *R. rufum* recorded

155   in 1980 (Penna & Veloso, 1990) and contrasted these data with large samples of

156   advertisement calls from three *R. darwinii* populations recorded recently (Serrano et al.,

157   2020). We analysed distinctiveness of the advertisement calls of the two species addressing

158   the hypothesis that they correspond to distinct taxonomic entities, in agreement with evidence

159   reported in comparative developmental, biochemical and karyological studies. Alternatively,

160   a lack of distinctiveness in the advertisement calls between the two species would contribute

161   to assess their similarity within *Rhinoderma.* Our study is also meant to provide relevant

162   information to support contemporary conservation efforts of these endangered or missing

163   taxa.

Materials and Methods

Study site and general procedures

The advertisement calls of *R. rufum* were recorded by Mario Penna during spring (October 1980) in a fragment of native forest located in the vicinity of Las Carmelitas convent, approximately 10 km south from the city of Concepción, Chile (36°53'39.48'', S 73°3'37.349'' W; Fig 1). Advertisement calls of nine individuals (presumably males) were recorded during day and night times. No individuals were captured and the air temperature during recordings was 14-18 °C, but temperatures for recordings of individual recordings were not registered. These recordings were partially reported in an early study on the description of vocalizations of anurans from the temperate austral forest (Penna & Veloso, 1990). Advertisement calls of *R. darwinii* were recorded in three localities during spring along the distribution of this frog (Huilo Huilo, n=12, Tantauco, n=13 and Queulat, n=12) between 2011 and 2014 and their signal variation patterns have been reported recently (Serrano et al., 2020; Fig. 1).

Acoustic recordings

Advertisement calls of *R. rufum* were recorded with an omnidirectional microphone (Uher M517) and a reel tape recorder (Uher 4400 Report Stereo IC) at a tape speed of 19 cm/s. The reel tapes were digitized in WAV format using the analogue-digital converter tools of Sequoia 14 (Magix Software GmbH, Berlin, Germany) at a sampling rate of 44,100 Hz and 24-bit resolution. The calls were analysed with Raven PRO v1.5 software. Five acoustic variables were analysed in oscillograms: call duration (s), note duration (ms), inter-note interval (ms), notes per call and call rate (calls/min); and two variables were obtained from spectrograms in which we measured the dominant frequency (Hz) and the proportion of note

187   duration segments containing the non-linear phenomenon of chaos. Figures of sound

188   spectrograms and oscillograms were obtained using the Seewave package (Sueur et al., 2019).

189   *Data analysis*

190   A data matrix containing the average values of calls of the *R. rufum* population and three

191   populations of *R. darwinii* was constructed. We calculated coefficients of variation (CV) of

192   advertisement calls within populations and within individuals of the four *Rhinoderma*

193   populations as a measure of the intra-population and intra-individual variation of the acoustic

194   characteristics.

195   A Generalized Linear Model (GLM) was constructed to assess differences between

196   the advertisement calls of *R. rufum* (at Las Carmelitas) and the three populations of *R.*

197   *darwinii* studied, using acoustic variables as dependent variables and populations as

198   predictors. To perform all the statistical analysis properly, the data matrix was analysed for

199   normality using the Mardia test for multivariate normality (Korkmaz, 2014). Call variability

200   corresponding to different numbers of notes per call, was analysed separately using a

201   Kolmogorov-Smirnov pared test (to maximize the test power as concluded by Böhm &

202   Hornik, 2012) to explore inter-population differences.

203   A linear discrimination analysis (LDA) was performed using the principal

204   components (PC) obtained from a principal component analysis (PCA) in order to study if the

205   acoustic variables discriminate the populations (Liang et al., 2010; Bolaños-Sittler et al.,

206   2020). The PCA was performed using a correlation matrix and a linear combination (z

207   transformation) to deal with non-normalities in some of the acoustic variables. The PCs

208   reaching above 80% of the cumulative explained variance (Jackson, 1993) were used as the

209   entry data into the LDA. This procedure has the advantage of reducing the effect of the great

210   variance of some variables that the LDA could mistake for observed variability, discard

211   collinearity effects and eliminate the "noise" that variables such as dominant frequency could

212 cause by having much higher values than the other analysed variables (Mardia et al, 1979;

213 Næs & Mevik, 2001) and has been employed in other evaluations of acoustic data (Funk et

214 al., 2012; Bolaños-Sittler et al., 2020). Finally, to validate the discriminant model, the same

215 individuals of all populations were reclassified using the linear model generated by the LDA.

216 For this, the probability of pertaining to a population was calculated for each individual

217 according to the model predictors; we a priori considered as correctly classified the

218 individuals that reached a belonging percentage higher than 50% relative to their original

219 locality. All the analyses were performed with the packages MVN (Korkmaz et al., 2014),

220 ade4 (Dray et al., 2016), ggplot2 (Wickham et al., 2016) and MASS (Ripley et al., 2013) to R

221 (R Core Team, 2019).

222 **Results**

223 The advertisement calls of *R. darwinii* and *R. rufum* are whistle-like sounds composed by two

224 to six notes.

225 Notes usually show a slight amplitude and frequency modulation, however in some

226 individuals these modulations can be pronounced. Non-linear phenomena, namely chaos

227 occurred preferentially at the beginning or end of the notes (Fig. 2).

228   In the continuum of variation of vocal traits proposed by Gerhardt (1991), we found

229 that in *R. rufum* dominant frequency had the lowest within population CV (below 5%) (Table

230 1), therefore this was considered as a static variable. Call duration, note duration, chaos

231 proportion, inter-note interval duration and call rate had higher CV's (from 17% to 59.1%)

232 and therefore were considered as dynamic variables (Table 1, for inter-individual CVs see

233 Table S1).
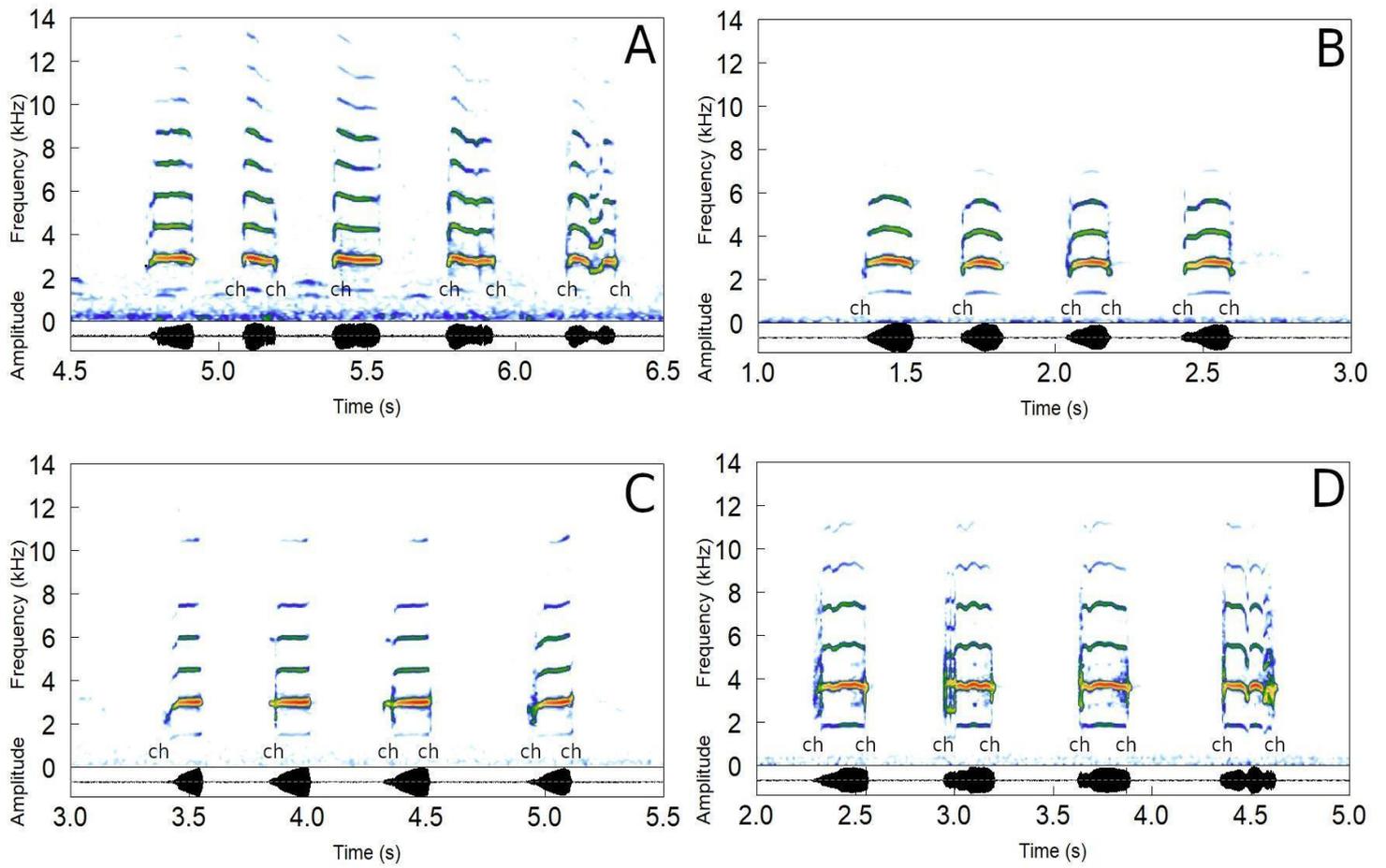
Figure 2. Spectrograms and oscillograms of representative advertisement calls of *Rhinoderma rufum* recorded in Las Carmelitas (A) and *Rhinoderma darwinii* recorded in Huilo Huilo (B), Queulat (C) and Tantauco (D). Chaos (ch) segments are indicated in spectrograms.

Table 1. Average values and coefficients of variation (in cursive) of the acoustic variables in Rhinoderma rufum and Rhinoderma darwinii populations. Asterisks indicate significance levels of GLMs resulting from comparisons between R. rufum population of Las Carmelitas versus each of the populations of R. darwinii (*: p <0.05, ***: p <0.001).

Species and Populations	Call rate (Calls / min)	Call duration (s)	Note duration (s)	Inter-note interval (s)	Chaos proportion (%)	Dominant frequency (Hz)
R. rufum Las Carmelitas (n= 9)	1.479± 0.64 *43.0*	1.5 ± 0.46 *30.6*	0.17 ± 0.03 *17.0*	0.19 ± 0.05 *24.5*	27.8 ± 16.45 *59.1*	2946 ± 113.53 *3.9*
R. darwinii Huilo Huilo (n= 12)	1.468 ± 0.45 *31.0*	1.8 ± 0.31 *39.6*	0.16 ± 0.02 *22.9*	0.32 ± 0.05*** *37.3*	58.11 ± 8.0 *16.0*	2806 ± 40.47 *5.4*
R. darwinii Queulat (n= 12)	1.522 ± 0.48 *30.0*	1.4 ± 0.17 *21.1*	0.16 ± 0.02 *18.4*	0.3 ± 0.04*** *24.7*	67.27 ± 21.7 *13.0*	3006 ± 45.62 *4.1*
R. darwinii Tantauco (n= 13)	1.752 ± 0.60 *34.0*	1.8 ± 0.36* *29.8*	0.18 ± 0.02 *25.7*	0.37 ± 0.07*** *23.2*	77.41 ± 12.6 *19.0*	3749 ± 96.58*** *6.0*

234 *Acoustic differences between species*

235 The GLM shows that advertisement calls of *R. rufum* from Las Carmelitas mainly differed

236 from *R. darwinii* from Tantauco population in their call duration ($t = 2.169$, $P = 0.036$), inter-

237 note interval ($t = 5.992$, $P < 0.001$) and dominant frequency ($t = 12.218$, $P < 0.001$). In

238 addition, inter-note interval of calls from Las Carmelitas show significant differences with the

239 calls of Huilo Huilo ($t = 3.996$, $P < 0.001$) and Queulat ($t = 3.797$, $P < 0.001$). No differences

240 between the *R. rufum* population and the three *R. darwinii* populations occurred in call rate,

241 note duration and proportion of chaos (Table 1). Although the most common call issued in the

242 *R. rufum* population is the five-note call and in the three *R. darwinii* populations it is the four-

243 note call (Fig. 3), the Kolmogorov–Smirnov test showed no differences in this feature among

244 the four populations ($D < 0.67$, $P > 0.13$ for all comparisons).
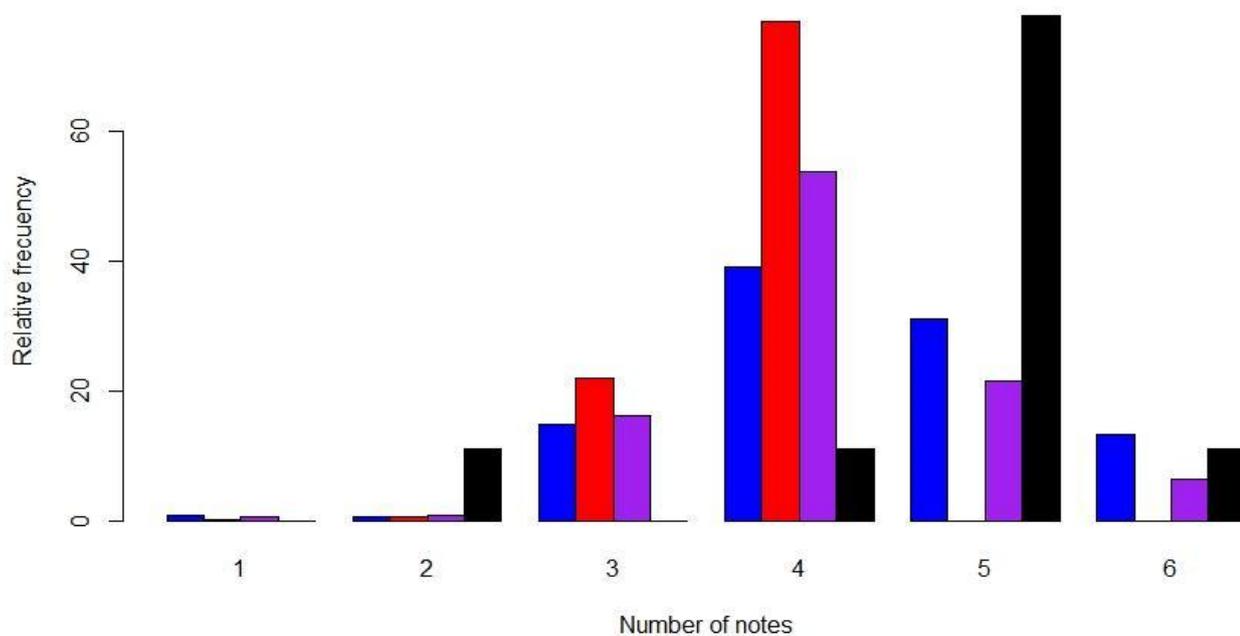
Figure 3. Proportions of number of notes in the calls of each population of *Rhinoderma*. Blue: Huilo Huilo, red: Queulat, purple: Tantauco, black: Las Carmelitas

245   *Distinctiveness analysis for all populations*

246   The PCA grouped the acoustic variables in six principal components from which the first four

247   were considered, as they accumulated 86.7% of the explained variance (Table 2). The

248   variables with higher weight (scores higher than 0.4) for PC1 after the z transformation were

249   call duration, note duration, inter-note interval and dominant frequency (Table 2). Dominant

250   frequency and chaos proportion had the highest weights for PC2, call rate and note duration

251   for PC3 and note duration and inter-note interval for PC4 (Table 2).

Table 2. Loadings of the acoustic variables, eigenvalues and explained variances for each principal component (PC), resulting from the analysis including the four populations studied. Since the cumulative explained variance reaches an 86.77% with PC4, the first four PCs were considered for the discrimination function analysis.

Acoustic characteristics	PC1	PC2	PC3	PC4	PC5	PC6
Call rate	-0.142	0.269	-0.859	0.224	-0.293	0.184
Call duration	-0.542	0.236	0.114	-0.313	-0.454	-0.577
Note duration	-0.410	0.220	0.448	0.642	-0.191	0.366
Inter-note interval	-0.534	0.091	-0.038	-0.535	0.375	0.527
Chaos proportion	-0.149	-0.808	-0.027	-0.092	-0.503	0.251
Dominant frequency	-0.458	-0.403	-0.217	0.380	0.527	-0.399
Eigenvalues	2.196	1.223	1.053	0.733	0.502	0.290
Explained variance (%)	36.610	20.390	17.560	12.22	8.383	4.837
Cumulative explained variance (%)	36.610	57.000	74.560	86.780	95.163	100.000

252   The LDA correctly classified the individuals from Tantauco (*R. darwinii*), with 85%

253   of success, followed by 50% for Queulat (*R darwinii*), 50% for Huilo Huilo (*R. darwinii*) and

254   33% of success for Las Carmelitas (*R. rufum*). The LDA indicated that the most important

255   components for discrimination among groups were PC1 and PC2 in the first linear

256   discriminant (LD1), which explained 93% of the variation, and PC4 was the most important

257   in the second linear discriminant (LD2), which explains 7% of the variation (Fig. 4A). As

258   such, the variables having larger relevance in the LDA because of their weights for principal

259   components were dominant frequency, call duration, inter-note interval and chaos proportion,

260   as shown in Table 2. As such, the LDA grouped the individuals in two clear clusters separated

261   mainly across LD1. These groupings did not show a separation between species. The left

262   cluster was completely formed by individuals from the *R. darwinii* insular population of

263   Tantauco and the right cluster was formed by the three mainland populations, comprising *R.*

264   *darwinii* and *R. rufum* (Fig. 4A).
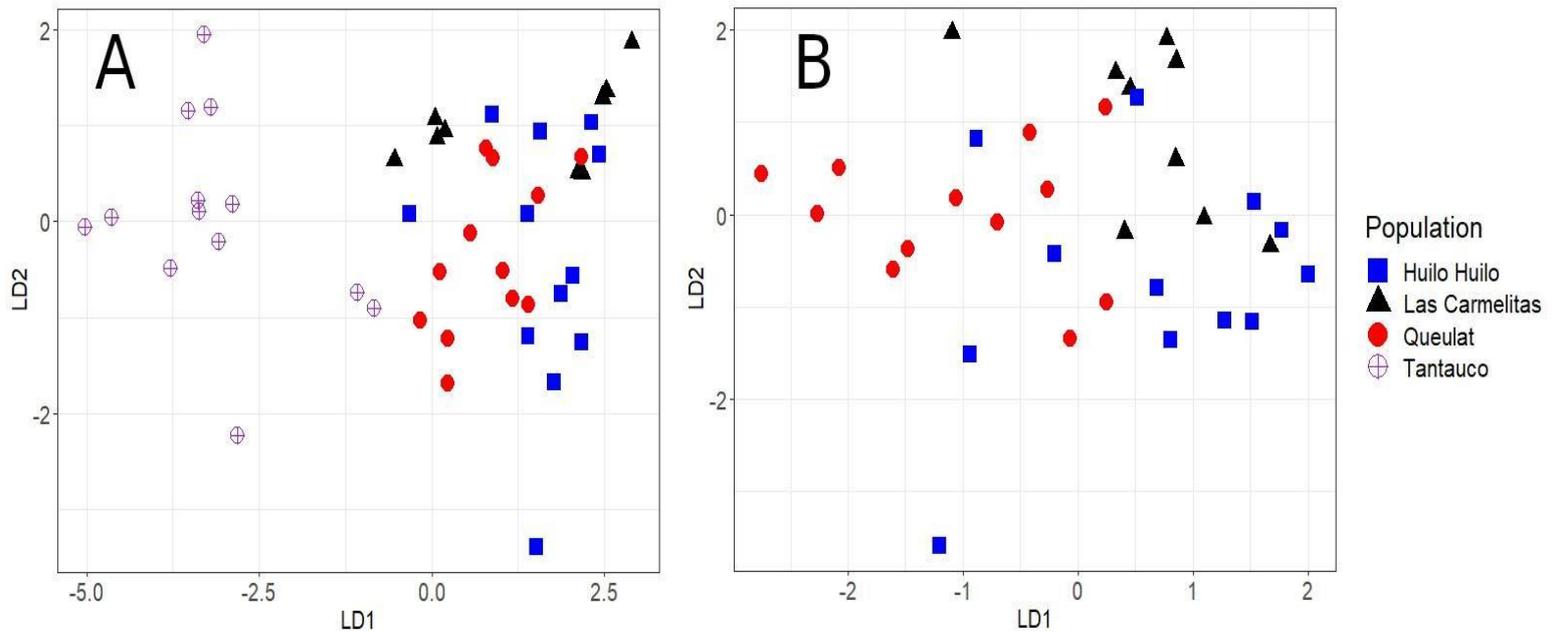
Figure 4. Discriminant analysis of call characteristics of individuals of *R. darwinii* and *R. rufum*: A) biplot of four localities showing that LD1 and LD2 account for 93 and 6.5 % of the total variance, respectively, and B) biplot excluding individuals from the insular population of Tantauco. showing that LD1 and LD2 account for 58% and 42% of the total variance, respectively.

265   *Distinctiveness analysis for mainland populations*

266   As populations were grouped indistinctly of the species to which they belong and because

267   discrimination showed separation among mainland and insular populations (Fig. 4A), we

268   performed a re-classification following the initial procedure, but excluding the individuals

269   from the insular population of Tantauco. This procedure intended to preclude the large

270   difference between mainland populations masked by the large variance of Tantauco. This

271   second classification increased the percentages of correct classification to 56%, 58% and 67%

272   for Las Carmelitas, Queulat and Huilo Huilo, respectively. Even though distinctiveness of the

273   mainland populations improved after excluding the insular population, the grouping did not

274   separate populations based on their species identity (Fig. 4B).

Table 3. Loadings of the acoustic variables, eigenvalues and explained variances for each principal component (PC), resulting from the analysis excluding the insular population of Tantauco. Since the cumulative explained variance reaches an 85.03% for PC4, the first four PCs were considered for the discrimination function analysis.

Acoustic characteristics	PC1	PC2	PC3	PC4	PC5	PC6
Call rate	-0.046	0.688	-0.067	0.506	0.361	-0.366
Call duration	-0.627	-0.021	-0.257	0.135	0.359	0.627
Note duration	-0.473	-0.041	0.308	0.480	-0.667	-0.069
Inter-note interval	-0.512	0.098	-0.459	-0.492	-0.157	-0.501
Chaos proportion	-0.055	-0.717	-0.170	0.399	0.336	-0.426
Dominant frequency	0.341	0.030	-0.772	0.307	-0.398	0.187
Eigenvalues	1.875	1.383	0.990	0.857	0.642	0.250
Explained variance (%)	31.260	23.060	16.510	14.290	10.710	4.182
Cumulative explained variance (%)	31.260	54.320	70.830	85.110	95.820	100.000

Discussion

Geographic variation of Rhinoderma advertisement calls and body size

Overall, advertisement calls of both *Rhinoderma* species were acoustically similar. Our results indicate that the calls of *R. rufum* recorded in the sympatric zone did not differ substantially from the calls of other mainland populations of *R. darwinii*. Among the populations analysed, the insular population of *R. darwinii* showed larger divergence relative to the population of *R. rufum*. Geographic variation of calls observed within mainland populations of *Rhinoderma* (including *R. rufum* and *R. darwinii*) assimilates to the one reported for *P. thaul,* a species having a wide distribution and environmental range in Chile (Velásquez, 2014). Similar instances of geographic variation occur in *Hyla cinerea* in the USA (Asquith et al., 1988) and *Allobates femoralis* in Brazil (Simões, 2008; Amézquita et al., 2009). In these cases, geographic distance and geological barriers play a key role limiting gene flow between populations, contributing to acoustic divergence. The lack of divergence

288 between the calls of *R. rufum* and continental *R. darwinii* populations contrasts with the

289 notable divergence of the island population of Tantauco, differing in several acoustic

290 properties from the mainland populations of *Rhinoderma*. This mode of geographic variation

291 in acoustic traits does not support the divergence process causing isolation between *R. rufum*

292 and *R. darwinii*, as suggested by morphological, karyological and molecular evidence in

293 earlier studies.

294 The calls of *R. rufum* differed from the three populations of *R. darwinii* in having shorter

295 inter-note interval duration, and from the Tantauco population in having shorter call duration.

296 Temporal variables in frog calls can result from environmental influences. For example, in

297 some anurans, relationships between body temperature and pulse rate (Gerhardt, 1978; Ryan,

298 1988; Giacoma & Castellano, 2001), and between air temperature and call duration (Gayou,

299 1984; Bee et al., 2013; Ziegler et al., 2016) have been reported. It is not possible to assess the

300 influence of temperature on the call differences between *Rhinoderma* species because of the

301 lack of precise temperature measurements for each individual of *R. rufum* recorded, however

302 it is unlikely that this factor is relevant in this particular case, as the temperature range of

303 recordings of *R. rufum* (14-18 °C) are within the ranges of those of the three populations of *R.*

304 *darwinii* recorded (Serrano et al. 2020).

305   Among the *Rhinoderma* populations analysed, the island population of *R. darwinii* in

306 Tantauco has the most divergent call characteristics relative to *R. rufum*, including inter-note

307 interval, call duration and dominant frequency. The Tantauco population of *R. darwinii* is

308 characterized by its smaller body size relative to mainland populations (Valenzuela-Sánchez

309 et al., 2015), and this morphometric feature could account for the variation of inter-note

310 interval duration and dominant frequency, as these traits are strongly related to body size of

311 this species (Serrano et al., 2020). In particular, the inverse relationship between body size

312 and dominant frequency of the advertisement call occurs extensively in anurans (Castellano &

313 Giacoma, 2000; Gerhardt & Huber, 2002).

314   The smaller size of the Tantauco population could be related to different geographical

315 and environmental factors. Populations of different vertebrates inhabiting insular territories

316 have a tendency to small size relative to continental counterparts, a tendency known as the

317 Island Rule (Van Valen, 1973), however this relationship does not occur regularly in anurans

318 (Rebouças et al., 2018). Another environmental determinant of anuran size is temperature

319 seasonality, which is inversely related to size of *R. darwinii* populations. The low temperature

320 seasonality in Tantauco is in correspondence with the relatively small size of animals of this

321 population (Valenzuela et al 2015).

322   Divergence in acoustic signals of populations not related to geographic distance has

323 been proposed to depend on genetic drift (Wilczynski & Ryan, 1999; Amézquita et al., 2009;

324 Velásquez et al., 2013) or multiple evolutionary forces such as inter and intra-sexual selection

325 (Asquith et al., 1988; Littlejohn, 1999; Petrusková et al., 2010; Velásquez, 2014). In

326 *Rhinoderma*, calls show a pattern of variation concordant with geographic isolation. Indeed,

327 after excluding the island population of Tantauco from distinctiveness analysis,

328 reclassification percentages of the mainland populations increased, but do not contribute to

329 improve the distinctiveness of the *R. rufum* population from Las Carmelitas. This result

330 indicates that the status of distinct species is unlikely for continental populations of

331 *Rhinoderma* (Funk et al., 2012). Such tenet is strengthened by considering that the population

332 of *R. rufum* analyzed is within the area of sympatry with *R. darwinii* (Bourke et al., 2012), as

333 in these zones, signal divergence between related species is in diverse cases reinforced,

334 promoting reproductive isolation and preventing deleterious hybridization (Littlejohn, 1999,

335 2001; Verrell, 1999; Höbel & Gerhardt, 2003; Rodriguez-Tejeda et al., 2014; Köhler et al.,

336 2017). As such, the lack of differentiation between the advertisement calls of *R. rufum* and *R.*

337 *darwinii* in the sympatric points to the absence of a main pre-mating isolation barrier

338 characteristic of anurans.

339   Our data indicate that the greatest acoustic divergence in *Rhinoderma* has taken place

340 in the island of Chiloé. Call differentiation related to geographic isolation, concurrent

341 morphological differentiation and exposure to extreme environments are likely to result in

342 speciation processes in anurans (Nevo & Capranica, 1985; Pröhl et al., 2006; Amézquita et

343 al., 2009; Funk et al., 2012; Rebouças et al., 2020). The historical geographic isolation of

344 Chiloé island dates from the late Pleistocene (Heusser, 1982, Villagrán et al., 1986; Denton et

345 al., 1999), and could account for the acoustic differentiation of the Tantauco population. The

346 distinctiveness analysis concurs to identify Tantauco as the only population that stands out

347 forming a cluster apart from the rest of populations. It is noteworthy that dominant frequency,

348 the only acoustic trait categorized as a static variable for all the populations analysed

349 (Gerhardt 1991) and likely having taxonomical relevance relative to dynamic variables, had a

350 high contribution to the separation between Tantauco and the mainland populations.

351 Similarly, Rebouças et al. (2020) found that call frequency is a static variable in *Boana*

352 *albomarginata* but having a lower frequency in island respect to mainland populations related

353 to larger body size. In contrast, the lack of distinctiveness observed between continental

354 populations of the two species of *Rhinoderma*, indicates an absence of reproductive barriers.

355 *Variation in other characters in Rhinoderma*

356   Studies of phenotypic and genetic characters have stressed the differences between *R.*

357 *darwinii* and *R. rufum* comprising morphological traits in adults and tadpoles and larval phase

358 and parental care phenology (Formas et al., 1975; Jorquera et al., 1981, 1982; Formas, 2013).

359 However, variation in such traits can result from phenotypic plasticity, as has been shown to

360 occur in other anurans some anuran species (Noble, 1925; Zippel, 1997; Relyea, 2003). The

361 expansion of interdigital membranes and the vascularization of folds in the integument can

vary according to the water level and other environmental conditions during development

(Cuello et al., 2008). The length of the larval stage can vary in response to environmental

conditions such as temperature, population density and food availability (Vences et al., 2001;

Cuello et al., 2014). Even the digestive tract development can vary in response to

temperature, as seen in tadpoles of *Calyptocephalella gayi* (Castañeda et al., 2006). Growth

rate of tadpoles and size at metamorphosis has shown to have effects on oral structures in

North American frogs (Venesky et al., 2010; Vences et al., 2001).

In particular, concerning the free aquatic living larval period that characterizes and

distinguishes *R. rufum* from *R. darwinii*, Wilhelm (1927) and Pflaumer (1935) successfully

raised adults from tadpoles taken from the vocal sac of *R. darwinii's* specimens, showing the

plasticity of parental care, in contrast with the assertion of Goicoechea et al. (1986) that

tadpoles of *R. darwinii* are unable to develop in water. Parental care in vertebrates is a labile

behaviour and its variation in closely related species relates to the degree of parental

investment (Reynolds et al., 2002), as behavioural decisions to care for tadpoles are a trade-

off that compensate for higher costs of diverse selective pressures affecting reproduction

(Schulte et al., 2020). Reproductive site selection in the Brazilian treefrog *Aparasphenodon*

arapapa (Lantyer-Silva et al., 2018) and hatching age in glass frogs and tree frogs

(Warkentin, 1995; Delia et al. 2019) can be accounted for by plasticity.

The geographical distribution of the two *Rhinoderma* species suggests that differences

between the two taxa could relate to different environmental pressures as the boundary

between the distributions of *R. rufum* and *R. darwinii* coincides with the transition of

Mediterranean and temperate forests (Luebert & Pliscoff, 2006).

The use of chromosomal karyotypes was widely used for taxonomic delimitation of

amphibians in the 60s (Hillis, 2019), and in Chilean amphibian as well, contributing to clarify

inter-genera and inter-family systematics, but having limited resolution between congeneric

species (Díaz & Veloso, 1979). The karyotypic structures of the two species of *Rhinoderma*

are basically similar, showing minor differences in chromosomal arms length and centromeric

fusions (Formas, 1976; Díaz & Veloso, 1979). This karyological technique as unique criteria

is not used in present days to set species relationships, as if not contrasted with banding

techniques to identify nucleolus organizer regions (Utsumi & Takehisa, 1974) it is not

appropriate to identify synapomorphies and can misidentify truly homologous characters

(Dobigny et al., 2002). Historically, chromosomal rearrangements (for example, between

haploid and diploid species) provide stronger evidence for reproductive isolation rather than

comparisons in chromosomal structures (Hillis, 2019). Instead, the use of one or more

combined DNA molecular markers (Funk et al., 2012; Hillis, 2019) would provide more

repeatable and comparable evidence to integrate genetics in delimiting species boundaries in

Rhinoderma.

In recent years, several other traits have been shown to vary widely within *R.

darwinii*, for example, climate-dependent colour variation (Bourke et al., 2011), geographic

variation of body size and nasal appendix extension (Valenzuela-Sánchez et al., 2015), and

population variation of the advertisement call (Serrano et al., 2020), including the occurrence

of release calls (Serrano et al., accepted). We propose that the morphological differences

observed in *Rhinoderma* could be related to specific environmental conditions influencing the

development of local morphotypes instead of speciation, as formulated in former studies.

Conservation implications

Our study is framed within the objectives of the Binational Conservation Strategy for

Darwin's Frogs, for which priorities are to define 1) the taxonomic status of *R. rufum* and 2)

relevant units for the conservation of *R. darwinii* (Azat et al., 2020). These definitions are

important to focus on prospective conservation actions, especially since *Rhinoderma*

411   populations are largely isolated among them (Bourke et al., 2018) and dispersal ability of the

412   species is likely limited (Uribe-Rivera et al., 2017). Such isolation restricts the genetic

413   diversity of the species and can jeopardize its survival while facing an alarming climate

414   change rate (Cummins et al., 2019), which has been identified as a main factor of Darwin's

415   frogs' decline (Bourke et al., 2018). In this scenario, a managed re-localization of populations

416   could be effective for in-situ conservation actions (Gascon, 2007; Olden et al., 2011). The

417   acoustic characterization that we have conducted for *Rhinoderma* can contribute to identify

418   populations and individuals sampled by means of automated acoustic monitoring (Gilbert et

419   al., 2002; Tripp and Otter, 2006; Vögeli et al., 2008), and also provide management tools in

420   wild sites using attracting or repelling signals for wildlife (Aubin, 1990; Buxton et al., 2018;

421   Santema et al., 2019).

422   The bioacoustic evidence contributed by our study and the critical review of

423   phenotypic and genetic traits studied formerly in *Rhinoderma* indicates that *R. rufum* may not

424   be a species different from *R. darwinii*, suggesting instead that *Rhinoderma* should be

425   considered as a monospecific genus. Thus, the divergence between populations of

426   *Rhinoderma* more likely corresponds to an instance of geographic variation dependent on

427   climate regimes along the historical distribution range of the genus. Assessing the plasticity

428   supporting the behavioural and morphological differentiation between northern and southern

429   populations of *Rhinoderma* would be important to implement adaptive management measures

430   (Caro, 2007; Gascon, 2007), such as creating common gardens in protected areas where

431   *Rhinoderma* has disappeared. This management would also allow to confront the challenges

432   that climate change, habitat loss and chytridiomycosis impose on *Rhinoderma* in the near

433   future (Uribe-Rivera et al., 2017; Bourke et al., 2018) and hopefully will contribute new

434   measures for long-term conservation initiatives for this unique anuran entity.

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Supplementary information

Table S 1. Averages and coefficients of intra-individual variation (in cursive) of the acoustic variables analysed in the advertisement calls of Rhinoderma rufum from Las Carmelitas. Coefficient of variation was not computed for Call rate as a single recording of successive calls was carried out for most individuals. Call rate was not computed for one individual for which a single call was recorded.

Individuals	Number of calls	Notes per call	Call rate (Calls/min)	Call duration (s)	Note duration (ms)	Inter-note interval (ms)	Dominant frequency (Hz)	Chaos proportion (%)
1	4	5		1.23	0.14	0.13	2939.3	NR
			1.805	*2.9*	*7.8*	*26.8*	*2.1*	*NR*
2	1	5		1.22	0.14	0.13	2894.1	35.2
			–	*NR*	*6.2*	*18.9*	*2.7*	*51.1*
3	4	2		0.64	0.18	0.26	2885.5	NR
			1.116	*0.41*	*6.7*	*22.27*	*1.6*	*NR*
4	4	5		1.57	0.17	0.18	3130.9	21.7
			1.846	*20.7*	*8.1*	*18.6*	*2.1*	*1.4*
5	4	5		1.57	0.15	0.20	2851	4.1
			2.308	*3.0*	*13.7*	*24.9*	*1.9*	*0*
6	3	5		1.67	0.16	0.21	2968.7	NR
			1.488	*1.5*	*11.9*	*18.0*	*3.1*	*NR*
7	3	6		2.08	0.20	0.18	2990.7	15.6
			1.978	*1.5*	*7.5*	*10.4*	*2.2*	*28.4*
8	3	5		1.93	0.21	0.22	2876.8	38.1
			0.865	*1.4*	*6.8*	*10.5*	*2.5*	*47.2*
9	2	5		1.92	0.20	0.23	2842.4	38.8
			1.667	*0*	*6.8*	*12.2*	*0*	*33.3*