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### Integrative taxonomic reassessment of *Odontophrynus* populations in Argentina and phylogenetic relationships within Odontophrynidae (Anura)

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Amphibians are the most vulnerable vertebrates to biodiversity loss mediated by habitat destruction, climate change and diseases. Informed conservation management requires to improve the taxonomy of anurans to assess reliably the species ' geographic range. In this study, we applied robust integrative taxonomic methods combining genetic (allozymes, mitochondrial 16S gene), morphological and behavioural data (advertisement call structure) to delimit species of the genus *Odontophrynus* sampled from throughout their centre of diversity in Argentina. The combined evidence used to assess the validity of the nominal taxa demonstrates one case of cryptic diversity and another of overestimation of species richness. The tetraploid populations referred to as *O. americanus* comprise at least two species. In contrast, *O. achalensis* and *O. barrioi* represent junior synonyms of the phenotypically plastic species *O. occidentalis*. We conclude that each of the four species occurring in Argentina possesses networks of populations in medium to large areas. Red list classification is currently "least concern". We also propose a phylogenetic hypothesis for the genus and associated genera *Macrogenioglottus* and *Proceratophrys* (Odontophrynidae) and discuss its implications on advertisement call evolution.

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#### 24 ABSTRACT

25 Amphibians are the most vulnerable vertebrates to biodiversity loss mediated by habitat 26 destruction, climate change and diseases. Informed conservation management requires to 27 improve the taxonomy of anurans to assess reliably the species' geographic range. In this study, 28 we applied robust integrative taxonomic methods combining genetic (allozymes, mitochondrial 29 16S gene), morphological and behavioural data (advertisement call structure) to delimit species 30 of the genus Odontophrynus sampled from throughout their centre of diversity in Argentina. The 31 combined evidence used to assess the validity of the nominal taxa demonstrates one case of 32 cryptic diversity and another of overestimation of species richness. The tetraploid populations 33 referred to as O. americanus comprise at least two species. In contrast, O. achalensis and O. 34 *barrioi* represent junior synonyms of the phenotypically plastic species O. occidentalis. We 35 conclude that each of the four species occurring in Argentina possesses networks of populations in medium to large areas. Red list classification is currently "least concern". We also propose a 36 phylogenetic hypothesis for the genus and associated genera Macrogenioglottus and 37 38 Proceratophrys (Odontophrynidae) and discuss its implications on advertisement call evolution.

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40 Keywords: Species delimitation, Integrative taxonomy, Morphometry, Advertisement call,

41 Allozymes, 16S rRNA sequences, Macrogenioglottus, Proceratophrys, Odontophrynus

#### 43 INTRODUCTION

44 Patterns of tropical and subtropical amphibian diversity are not well understood because of 45 incomplete information on taxonomy and distribution (e.g., Vieites et al., 2009; Winter et al., 46 2016). Yet amphibians are of high conservation concern, with almost 43% the currently known species being globally threatened and another 25% data deficient (Stuart et al., 2004). 47 48 Taxonomic uncertainty stems partially from the prevalence of the morphospecies concept in 49 most original descriptions of amphibian species (Frost, 2018). Morphological characters alone often fail to differentiate among species due to the conservatism in the morphological evolution 50 51 of anurans and to environmental constraints posed by adaptations to a specific mode of living 52 (e.g., Elmer, Dávila & Lougheed, 2007; Vences et al., 2010; Kaefer et al., 2012; Rojas et al., 53 2018). Advertisement calls as powerful tools of premating isolation can reveal morphologically 54 cryptic species in sympatry, but in allopatry distinct species may give almost identical calls as do Hyperolius castaneus, H. constellatus and H. lateralis (e.g., Schneider & Sinsch, 2007; Sinsch et 55 al., 2011, 2012; Greenbaum et al. 2013; Köhler et al., 2017). Delimiting species solely based on 56 57 genetic distances obtained by barcoding approaches may inflate real species numbers by overestimating the taxonomic importance of genetic structuring (e.g., Sukumaran & Knowles, 58 59 2017). Therefore, species delimitation in morphologically conserved groups should attempt to unite several lines of evidence to provide robust taxonomic hypotheses (e.g. Dayrat, 2005; Padial 60 61 & De La Riva, 2010; Rojas et al., 2018).

The South American Anura provide several examples for morphologically highly conserved genera in which recently integrative taxonomy led to reliable species delimitation and subsequent priorities for conservation measure (e.g., Von May, Lehr & Rabosky, 2018; Rojas et al., 2018). Osteological, histological and molecular data sets in combination have proved useful

66 to re-evaluate the uncertain taxonomic status of allopatric populations in stream-inhabiting 67 Telmatobius frogs that occur in remote Andean highland valleys (e.g., Sinsch & Lehr, 2010; 68 Barrionuevo, 2013; Sáez et al., 2014). The semi fossorial toads of the genus *Odontophrvnus* pose 69 a similar challenge because all original species descriptions are morphology based and often too ambiguous for a reliable species distinction (Cei, Ruiz & Becak, 1982; di Tada et al., 1984; Cei, 70 71 1985; Martino & Sinsch, 2002; Rosset et al., 2006, 2007; Rosset, 2008; Caramaschi & Napoli, 72 2012; Rocha et al., 2017). Nevertheless, extant populations are currently assigned to eleven species which are placed into three phenetic groups based on overall morphological similarities 73 74 (Frost, 2018): The O. americanus group including the O. americanus, O. cordobae, O. juquinha, 75 O. lavillai, and O. maisuma, the O. occidentalis group including O. achalensis, O. barrioi, and 76 O. occidentalis, and the O. cultripes group including O. carvalhoi, O. cultripes and O. 77 monachus. Odontophrynus toads inhabit a latitudinal range of 5°S to 41°S west of the Andes covering an altitudinal range from sea level to montane valleys of about 2,200m above sea level 78 79 (Turazzini, Taglioretti & Gomez, 2016; Santos-Silva et al., 2017).

80 Taxonomic assignment of populations to the currently recognized species is hampered by 81 the overall similarity of external morphology, and corresponding geographic ranges are bear a 82 high degree of uncertainty. Therefore, the red list status and resulting conservation needs are at least debatable, with six species considered as "least concern", one as "vulnerable" and four as 83 84 "data deficient" (IUCN, 2018). The three disjunct areas inhabited by the tetraploid O. 85 *americanus* may indicate the presence of cryptic species (Rosset et al., 2006). Highland taxa 86 such as O. achalensis may not occur exclusively in the Pampa de Achala plateau in the Sierras de 87 Cordoba, but also in similar habitats of the Sierra de San Luis (di Tada et al., 1984). Diploid O. 88 americanus-like populations reported from the vicinity of the disjunct O. americanus ranges

89 have been recently described as three distinct species O. cordobae in Central Argentina (Martino 90 & Sinsch, 2002), O. maisuma in coastal Uruguay and Brazil (Rosset, 2008) and O. juquinha in 91 Minas Gerais, Brazil (Rocha et al., 2017). It remains controversial, if diploids of the O. 92 americanus group derived from tetraploids or tetraploids several times independently from diploids (Becak & Becak, 1974; Becak, 2014). With respect to these issues and the validity of 93 the phenetic groups within Odontophrynus, the most recent molecular phylogeny of 94 95 Odontophrynus is inconclusive (Pyron & Wiens, 2011). Only five of the 11 nominal taxa (achalensis, americanus, carvalhoi, cultripes, occidentalis) were included and bootstrap values 96 97 lean weak support to proposed nodes. Still, this and an earlier phylogeny proposed by Amaro, 98 Pavan & Rodrigues (2009) agree in that the Odontophrynidae are monophyletic and that 99 Macrogenioglottus and Odontophrynus are the sister taxa.

100 Consequently, a reliable delimitation of *Odontophrynus* species, an assessment of their geographical ranges, conservation needs and phylogenetic relationships require an integrative 101 102 taxonomic approach critically evaluating information derived from morphology, behaviour and 103 genes. In this long-term study covering more than 20 years of field and laboratory work, we 104 focus geographically on Argentina, the centre of *Odontophrynus* diversity with six recognized 105 species and several populations of still undetermined taxonomic status. The character complexes included in the re-assessment of taxa are quantitative morphometrics, advertisement call features, 106 allozyme patterns and partial 16S rRNA sequences, all providing meaningful taxonomic 107 108 information. Data refer to 34 populations, among them those at the type localities for reference. 109 Sites of sympatry (O. americanus/O. occidentalis, O. cordobae/O. occidentalis) are contrasted with those in narrow contact zones (O. americanus/O. cordobae, O. achalensis/O. occidentalis) 110 111 and sites of allopatry. Additional data on the molecular *Odontophrynus* diversity in Brazil are

used for a broader phylogenetic view on Odontophrynidae (Amaro, Pavan & Rodrigues, 2009).
Specifically, we test the following hypotheses: (1) Phenotypic variation in morphology and acoustic communication used for taxon description is associated with corresponding genetic differentiation; (2) The phenetic groups within *Odontophrynus* represent distinct phylogenetic lineages; (3) Current red list classification does not reflect genetic diversity and geographical range of taxa.

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#### 119 MATERIALS AND METHODS

#### 120 Study area and field sampling

121 Since 1995, we identified and sampled 34 local populations of toads pertaining to the genus 122 Odontophrynus in Argentina (Table S1). The type localities of the nominal taxa O. achalensis di 123 Tada, Barla, Martori, and Cei, 1984 (Pampa de Achala, Cordoba province), O. barrioi Cei, Ruiz, 124 and Beçak, 1982 (Aguadita springs, Sierra de Famatina, La Rioja province), O. cordobae 125 Martino and Sinsch, 2002 (Villa General Belgrano, Cordoba province) and O. lavillai Cei, 1985 126 (Villa de la Punta, Santiago del Estero province) were sampled to obtain topotypical individuals 127 for taxonomic comparison. Unfortunately, the type localities of the most wide-spread species O. 128 americanus (Duméril and Bibron, 1841) and O. occidentalis (Berg, 1896) are unknown because the original descriptions only state that the holotype of O. americanus was "sent from Buenos 129 Aires" and that the holotype of O. occidentalis was collected in an "arroyo agrario" in the 130 Neuquén province (Frost, 2018). Still, populations of tetraploid O. americanus were readily 131 132 distinguished from those of the diploid taxa by erythrocyte size (Rosset et al., 2006; Otero et al., 133 2013). Populations of uncertain taxonomic assignment were assigned as O. cf. achalensis 134 (Locality: La Carolina, San Luis province) or O. cf. barrioi (Localities: Aguada de Molle, Huerta

de Guachi, San Juan province; Table S1). Material and data collected at the study sites were: (1)
blood smears for ploidy assessment; (2) adult specimens for morphometric measurements, (3)
records of advertisement calls, (4) muscle and liver homogenates for allozyme analyses, and (5)
alcohol preserved tissue for barcoding (partial sequences of the mitochondrial 16S rRNA gene).
The carcasses of specimens studied were deposited in museum collections; Table S1). The
Córdoba Environment Agency (A.C.A.S.E.), Environmental Secretary of Córdoba Government
[A01-2013], authorized our study and issued research and collecting permits.

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#### 143 Morphological data

In a first step, presumed ploidy (diploid/tetraploid) was verified by measuring the erythrocyte 144 size, which correlates with the DNA content. Smears of fresh blood were air-dried and light-145 146 microscopically examined at a magnification of 1000x using an OLYMPUS BX50 following the procedures described in Otero et al. (2013). Specimens were sacrificed, tissues sampled, and 147 carcasses preserved in 4% formaldehyde. Use of vertebrate animals was approved by the Ethics 148 149 Committee (COEDI) of the Universidad Nacional de Rio Cuarto. (https://www.unrc. 150 edu.ar/unrc/coedi/index.html). The investigation was conducted according to the state law "Protection and Conservation of Wild Fauna" (Argentina National Law Nº 22.421) and the 151 Ethical Committee of Investigation of the National University of Río Cuarto (Nº 38/11). The 152 external morphology of 256 specimens was described quantitatively by measuring fifteen 153 154 morphometric distances (to the nearest 0.1 mm; Martino & Sinsch, 2002): (1) Snout-vent length 155 (SVL); (2) maximal head width (HW); (3) head length (HL); (4) snout-eye distance (SED); (5) 156 internarinal distance (IND); (6) interocular distance (IOD); (7) eye-narinal distance (END); (8) 157 rostronarinal distance (RND); (9) eye diameter (ED); (10) humerus length (HL); (11) length of

158 3rd finger (F3L); (12) femur length (FL); (13) tibia length (TL); (14) foot length (FOL); (15)
159 length of 4th toe (T4L). All measurements were taken by the first author.

160 All variables were standardized and subjected to a principal component analysis with a fixed number of three PCs extracted. By this means, we explored the morphometric variability 161 162 independent of taxonomic assignment and reduced the information to statistically unrelated 163 factors. PC1 represents size-related features, PC2 and PC3 shape-related ones. Separate PCAs were run on the taxa of the phenetic groups. Assignment of populations to a phenetic group was 164 based on the advertisement call structure (O. americanus-group: simple pulsed calls; O. 165 166 occidentalis-group: complex calls consisting of several pulse groups; Salas & di Tada, 1994; 167 Martino & Sinsch, 2002). The morphospace built by three PC-axes was used to evaluate 168 partitioning among taxa. A discriminant analysis with a priori taxon assignment was applied to 169 quantify the partitioning of morphospace with respect to PC1-3 for each phenetic group. We consider a correct taxon classification of at least 80% of the individuals studied as indicative for 170 taxonomic implications. Due to the low resolution among taxa of the O. occidentalis group we 171 172 tested for clinal variation of PCs along latitudinal and altitudinal gradients by a multiple regression analysis (Procedure: backward selection at F=4.0). Significance level was set to 173 174 alpha=0.05. All calculations were performed using the statistical package statgraphics centurion, version XVIII (Statpoint Inc., 2018). 175

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#### 177 Bioacoustic data

Series of 11-116 advertisement calls given by 302 individuals were recorded in field using a
DAT recorder Sony TCD-100<sup>©</sup> with stereo microphone ECM-MS907 Sony<sup>©</sup> and tapes TDK
DA-RGX 60<sup>©</sup> (Table 1). Ambient temperature (to the nearest 0.5<sup>o</sup>C) was registered at the

181 individual calling sites (usually shallow water near shore) immediately after recording. 182 Whenever possible, specimens were collected to obtain tissue samples and for morphometric 183 measurements. Oscillograms, sonograms and power spectra of the call series were prepared with 184 the Medav Mosip 3000 Signal Processing System or the PC program Adobe Audition 1.0. Each 185 call series was characterized by ten parameters which were measured in three calls per series 186 (terminology and procedure according to Martino & Sinsch, 2002; Schneider & Sinsch, 2007): (1) call duration [ms]; (2) number of pulse groups per call [N]; (3) duration of pulse group [ms]; 187 (4) interval between pulse groups; (5) pulses per pulse group [N]; (6) pulse duration [ms]; (7) 188 189 interpulse interval [ms]; (8) pulse rate [pulses/s]; (9) pulse quotient (=pulse duration/interpulse 190 interval); (10) dominant frequency [Hz].

The arithmetic means of these call parameters were calculated for each series 191 192 (=individual) and used for further analyses. Thus, the basic data set describing the advertisement calls of the populations studied consisted of eleven variables (ten call parameters and the 193 194 corresponding ambient temperature) with N=304 observations. As several call variables co-vary 195 with ambient temperature, we calculated linear regression models of call parameter vs. 196 temperature and used the standardised residuals to obtain a temperature-adjusted data set for 197 further analysis. Analogous to the treatment of morphometric data, a principal component analysis was run on call data subsets of populations with homologous call structure (simple calls 198 with seven variables vs. complex calls with ten variables) to explore the bioacoustic 199 200 differentiation among the taxa of each phenetic group. The three PCs explaining the most of the 201 variance were extracted to describe the sound space utilized by Odontophrynus and its 202 partitioning among taxa. Moreover, a discriminant analysis was applied to quantify the 203 partitioning of among-taxon sound space, again applying the 80% criterion on the rates of the

correct classification of call. Again, we tested for clinal variation of PCs along latitudinal and
altitudinal gradients by a multiple regression analysis (Procedure: backward selection at F=4.0).

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#### 207 Allozyme data

Liver samples were obtained from 147 individuals (Table S2). Samples were dissolved in 1ml 208 209 homogenate buffer (Tris-EDTA-NADP at pH 7.0) and stored at -65°C until use. Aliquots of 0.3-210 3µl liver homogenate were applied to commercial cellulose acetate plates (PHERO-cel, 5.7x14.0cm) and submitted to a continuous horizontal electrophoresis (Hebert & Beaton, 1993). 211 212 Buffer systems and duration of electrophoresis were 30-40min at room temperature: (1) Tris-213 Glycine at pH 8.5 and constant 200V; (2) CAAPM (Citric acid aminopropyl morpholine) at pH 7.0 and constant 130V. Following electrophoresis, each gel was stained using standard recipes 214 215 (Murphy et al., 1996).

216 The allozyme pattern of liver tissue consisted of 10 enzyme systems controlled by a total of 14 presumptive loci: aspartate amino transferase (2 loci, AAT, EC 2.6.1.1), carboxylesterase 217 218 (1, EST, 3.1.1.1), glycerol-3-phosphate dehydrogenase (1, G3PD, 1.1.1.8), glucosephosphate isomerase (1, GPI, 5.3.1.9), isocitrate dehydrogenase (2, IDH, 1.1.1.42), lactate dehydrogenase 219 220 (1, LDH, 1.1.1.27), malate dehydrogenase (2, MDH, 1.1.1.37), malic enzyme (1, ME, 1.1.1.40), 221 6-phosphogluconate dehydrogenase (1, 6PGD, 1.1.1.44), phosphoglucomutase (2, PGM, 2.7.5.1). Mitochondrial and cytoplasmatic loci were distinguished by prefixes (m/c), 222 223 electromorphs (presumptive alleles) of each locus were designated alphabetically from cathode 224 to anode. For reference, we used a sample of one *O. americanus* individual in each run.

Statistical analyses of data included the calculation of allele frequencies (Table S2) and
Nei's unbiased genetic distances (Nei, 1972). Distances >0.1 were considered indicative for

differentiation at species level (e.g., Highton ,1999; Scillitani & Picariello, 2000). Calculation
was performed using the program GENDIST of the Phylogeny Inference Package (PHYLIP,
version 3.695) by Felsenstein (2008).

230

#### 231 Molecular phylogenetic analysis

232 We compared the partial sequence of the mitochondrial 16S rRNA gene of the samples from the 233 different localities in Argentina to assess the number of species present in the country and their 234 phylogenetic relationships (Table S3). The 16S barcoding gene has been demonstrated to contain 235 a strong phylogenetic signal and to be especially informative in topology resolution (Vences et 236 al., 2005; Zhang et al., 2013). DNA was extracted using Qiagen DNeasy Blood and Tissue Kit 237 (Qiagen, Hilden, Germany) following the manufacturer's protocol. Polymerase Chain Reaction 238 (PCR) was used to amplify fragments of approximately 560 base pairs of 16S mitochondrial rRNA using the standard primers 16SAL (5'-CGCCTGTTTACTAAAAACAT-3'), and 16SBH 239 (5'-CCGGTCTGAACTCAGATCACGT-3'). Amplification followed the standard PCR 240 241 conditions (Palumbi, 1996) with the following thermal cycle profile: 120 s at 94 °C, followed by 33 cycles of 94 °C for 30 s, 49 °C (12S) / 53 °C (16S) for 30 s, and extension at 65 °C for 60 s. 242 243 All amplified PCR products were verified using electrophoresis on a 1.4% agarose gel stained with ethidium bromide. PCR products were purified using Highpure PCR Product Purification 244 Kit (Roche Diagnostics). Sequencing of both strands was performed with the DYEnamic ET 245 246 Terminator Cycle Sequencing Premixkit (GE Healthcare, Munich, Germany) for sequencing 247 reactions run on a MegaBACE 1000 automated sequencer (GE Healthcare). Chromas lite 2.1.1 248 software (Technelysium Pty Ltd, http://www.technelysium.com.au) was used to check and read 249 the chromatograms of the sequences. The obtained sequences were compared with those in

GenBank using a standard nucleotide-nucleotide BLAST search. Homologous sequences of *Odontophynus* as well as from species of the closely related genera *Macrogenioglottus* and *Proceratophrys* were downloaded from GenBank and incorporated in an alignment. A sequence of *Ceratophrys cornuta* was used as outgroup (Table S3). The sequences were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in MEGA 7 (Tamura et al., 2016). The final alignment consisted of 552 base pairs. Pairwise distances were calculated in MEGA7. Distances >1% were considered indicative for differentiation at species level (e.g., Sáez et al., 2014).

257 The general time-reversible model with proportion of invariable sites and gamma-258 distributed rate variation among sites (GTR + I + G) was chosen as the best-fitting model of 259 sequence evolution on the basis of the Akaike information criterion as implemented in jModelTest 2 (Darriba et al., 2012) and was applied in Maximum Likelihood (ML) and Bayesian 260 261 Inference (BI) analyses. ML was performed in MEGA 7 with heuristic searches with stepwise addition and TBR branch-swapping algorithm, generating 1,000 bootstrap replicates. BI was 262 263 performed using MrBayes 3.2.5 (Ronquist et al., 2012). Two independent Metropolis-coupled Monte Carlo Markov Chain (Larget & Simon, 1999) analyses were run for 10 Million 264 265 generations, each with one hot and three cold chains and the temperature set at 0.2. Trees were sampled every 5000 generations. The first 500 samples of each run were discarded as burn-in, 266 and the remaining trees from both runs were used to calculate a consensus tree and Bayesian 267 posterior probabilities (BPP). Treegraph2 (Stöver & Müller, 2010) was used to draw trees. 268

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270 RESULTS
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#### 272 Morphological variation

273 All nominal taxa of *Odontophrynus* resemble each other considerably in colouration and external 274 morphology reflecting their semi fossorial mode of living (Fig. 1). Quantitative morphometric 275 analyses based on 15 measured variables still demonstrated a significant morphological variation 276 among some taxa. The three principal component representing the axes of morphospace 277 explained 77.2% of total variance in the O. americanus group and 80.1% in the O. occidentalis 278 group, respectively (Table 1). The morphospace of the O. americanus group was partitioned 279 between O. lavillai on one side and the indistinguishable pair O. americanus/O. cordobae on the other side (Figure 2). The discriminant analysis based on PC1-3 confirmed a significant 280 281 separation of O. lavillai at 82.1% correct classification rate mainly based on its larger size (PC1; 282 Table 2).

283 In contrast, resolution among taxa in the O. occidentalis group was lower with O. 284 occidentalis, O. achalensis and O. cf. achalensis being indistinguishable among each other (Fig. 285 3; Table 2). O. barrioi and O. cf. barrioi differed from these mainly by their larger size (PC1) 286 and among each other by head shape (PC2/3) at a 70% and 81%, respectively, correct 287 classification rate (Table 2). A significant proportion of morphometric variability among 288 individuals assigned to the O. occidentalis group was caused by a clinal variation along 289 altitudinal and latitudinal gradients. Size-related variation (PC1) was significantly correlated 290 with altitude and latitude (Multiple regression model,  $R^2=32.1\%$ ,  $F_{2.102}=24.03$ , P<0.00001), i.e. 291 size of individuals increased with elevation and from south to north. PC2 (position of nares and 292 eyes) was significantly correlated with latitude (Multiple regression model,  $R^2=16.6\%$ ,  $F_{1,103}$ =20.52, P<0.00001), PC3 (head length) with altitude (Multiple regression model, 293 R<sup>2</sup>=10.0%, F<sub>1.103</sub>=11.42, P=0.001). 294

295

#### 296 Advertisement call variation

297 The taxa of the O. americanus group emit simple and short pulsed advertisement calls, whereas 298 those of the O. occidentalis group produce long and complex advertisement calls consisting of a 299 variable number of short pulse groups (Fig. 4). Quantitative analyses of the advertisement calls 300 based on seven temperature-adjusted variables in the O. americanus group showed a significant 301 variation among the three taxa. Three PCs explained 85.1% of total variance represented the axes of sound space (Table 1). The sound space was partitioned into three discrete groups 302 representing O. americanus, O. cordobae, and O. lavillai individuals, respectively (Figure 2). 303 304 The discriminant analysis based on PC1-3 correctly assigned all calls except four two to the 305 corresponding taxon (Table 3).

306 Analogous to morphometric variation, sound space partitioning was low among the taxa 307 of the O. occidentalis group, with O. occidentalis, O. achalensis and O. cf. achalensis being indistinguishable among each other (Fig. 3; Table 3). The acoustic niches of O. barrioi and O. cf. 308 barrioi were better resolved from the continuum formed by the other taxa, but showed a slight 309 310 overlap between each other. Still, the correct classification rates for O. barrioi and O. cf. barrioi were at a 91% and 86%, respectively (Table 3). Temperature-adjusted advertisement call 311 312 variation was also influenced by geographical clines. PC1 (size-related dominant frequency) and PC2 (call duration) were significantly correlated with latitude (Multiple regression models: 313 R<sup>2</sup>=9.8%, F<sub>1.74</sub>=7.94, P=0.0062, and R<sup>2</sup>=14.1%, F<sub>1.74</sub>=11.97, P=0.0009, respectively), and PC3 314 315 (pulse group duration) with altitude ( $R^2=9.1\%$ ,  $F_{1.74}=7.31$ , P=0.0085).

316

#### 317 Genetic distances: allozymes and barcoding

318 Fourteen presumptive loci were scored in the nominal taxa (Table S2). Two loci (mAAT,

319 mMDH) were monomorphic in all taxa. The overall degree of allele fixation was high and varied 320 between 5 loci in *O. americanus* and 11 in *O. lavillai*. Five taxa possessed one private allele 321 each: *O. americanus* cIDH a, *O. lavillai* cAAT a, *O. achalensis* LDH d, *O.* cf. *achalensis* GPI d 322 and *O. barrioi* cMDH a. The pairwise Nei distances among the taxa were clearly below species 323 level in four taxon pairs (Table 4): 0.0220 in *O. americanus/O. cordobae*, 0.0232 in *O.* 324 *achalensis/O. occidentalis*, 0.0292 in *O.* cf. *achalensis/O. occidentalis*, and 0.0351 in *O.* 325 *achalensis/O.* cf. *achalensis*.

The 19 samples from eight nominal *Odontophrynus* species differed from each other in the 16S sequences by uncorrected pairwise distances of 0.0–5.3 % (Table 5). The divergence between samples of *O. achalensis*, *O. barrioi*, *O.* cf. *barrioi*, and *O. occidentalis* were minimal (0.0–0.9 %) and we regard them as belonging to a single species. The distances among the three nominal species *O. americanus*, *O. cordobae* and *O. lavillai* collected in Argentina were small (1.8–2.7 %), but at species level. Interestingly, the distance (2.4 %) between the topotypic *O. americanus* from Argentina and the *O. americanus* from Brazil was also at species level.

333

#### 334 Phylogenetic relationships among the Odontophrynidae

The topologies derived from the two phylogenetic analysis methods were largely congruent. We show the BI phylogeny with bootstrap values from ML and posterior probabilities from BI (Figure 5). The monophyly of the three genera within Odontophrynidae is strongly supported as well as the sister group relationship between *Macrogenioglottus* and all *Odontophrynus* taxa. The *Proceratophrys* clade resolved as the sister group to the clade formed by the other two genera. The samples of *Odontophrynus* resolved into two major clades with strong node support. The first one contained the samples of *O. occidentalis* as well as those of *O. achalensis* and *O.* 

*barrioi.* The relationships within this clade remained largely unresolved and the three nominal taxa did not resolve into distinct phylogenetic lineages. The second clade contained the remaining species and splitted into two subclades, one consisting of *O. carvalhoi* and *O. cultripes*, the other one containing *O. americanus*, *O. cordobae*, and *O. lavillai*. The two samples of *O. americanus* did not form a monophyletic clade but the topotypic Argentinian sample appeared to be more closely related to *O. cordobae* and *O. lavillai* than to the Brazil sample assigned to *O. americanus*.

349

#### 350 DISCUSSION

351 Lines of evidence obtained from phenotypic and genotypic character complexes in 352 Odontophrynus toads exemplify the common dilemma of taxonomy – which level of character 353 differentiation requires taxonomic consequences? Our case study demonstrates that phenotypic plasticity may result in an overestimation of species diversity (O. occidentalis group), whereas 354 molecular data may reveal unexpected cryptic diversity in morphologically uniform populations 355 356 (tetraploid O. americanus populations). The following discussion of the three hypotheses basic to 357 our investigation will present a completely revised view on the actual *Odontophrynus* diversity and propose a model of the phylogenetic relationships within the genus Odontophrynus. 358

359

# 360 Hypothesis 1: Phenotypic variation in morphology and acoustic communication used for 361 taxon description is associated with corresponding genetic differentiation

Phenotypic variability among the currently recognized *Odontophrynus* species in Argentina is
very low with respect to morphology suggesting that taxonomic assignments based exclusively
on this character complex should be treated with caution. The well-defined species of the *O*.

365 americanus group (distinct by advertisement call variation and 16S sequences) do not differ 366 morphometrically at all (O. americanus/O. cordobae, but age-adjusted size differences are significant; Martino & Sinsch, 2002) or by size alone (O. lavillai, this study). Within-species size 367 368 variation following environmental gradients (e.g., Sinsch, Pelster & Ludwig, 2015) renders the 369 support of taxonomic decision by SVL differences alone unreliable (e.g., Rakotoarison et al., 370 2015; Rojas et al., 2016). Ploidy distinguishes O. lavillai from O. americanus, but not from O. 371 cordobae, O. juquinha or O. maisuma. It seems doubtful that qualitative morphological features (e.g., the skin on dorsum heavily granular and glandular, three transversal dark brown blotches 372 373 on dorsum, lacking a light middorsal stripe; Cei, 1985; Rosset & Baldo, 2014) are diagnostic and 374 allow for an unequivocal identification (diagnostic characters listed for *O. juquinha* are widely 375 the same; Rocha et al., 2017). Nevertheless, combined evidence derived from the four character 376 complexes analysed allows for unequivocal diagnosis and clearly supports species status in the diploids. Molecular evidence on the tetraploids, a topotypical O. americanus from the Buenos 377 378 Aires province, Argentina and a specimen from Minas Gerais, Brazil, indicates that they differ at 379 species level. The close relationship between O. cordobae and O. americanus from Argentina 380 with rare hybridization in nature suggests a common genetic stock (Grenat et al., 2018). Future 381 research should focus on the identification of the diploid counterparts of O. americanus from Brazil. O. americanus may resolve as complex of cryptic species, which has evolved by 382 383 polyploidization of distinct diploid source species.

The most surprising taxonomic implication resulted from the reassessment of the taxa included in the *O. occidentalis* group. Broadly overlapping variation in all character complexes surveyed demonstrates that *O. achalensis* from the Sierra de Cordoba and associated populations from the Sierra de San Luis are phenotypically and genetically indistinguishable from *O*.

388 occidentalis. Ranges overlap in the Sierra de Cordoba suggesting ongoing gene flux between 389 lowland and highland phenotypes. The taxonomic conclusion is straightforward - O. achalensis 390 does not deserve species status and is a junior synonym of O. occidentalis. Consequently, the 391 morphological features put forward to support species status of O. achalensis apart from O. 392 occidentalis (e.g., the dorsal blotch pattern with whitish background colour, the elongated snout, 393 dorsal gland size; di Tada et al., 1984; Rosset el al. 2007) simply describe the highland ecotype 394 variation of a phenotypically plastic species. The case of O. barrioi is more complicated because the absence of significant genetic differentiation from O. occidentalis is contrasted by 395 396 morphometric, bioacoustics and allozyme differentiation. Morphometric differentiation is 397 exclusively based on size whereas the shape variation is the same as in the O. occidentalis/O. 398 achalensis continuum. Within-species altitudinal and latitudinal size variation is well known in 399 anurans (e.g., Sinsch, Pelster & Ludwig, 2015) and does not support an own taxonomic status. Advertisement call variation is mainly based on differences in dominant frequency, again an 400 401 indicator of size of the calling individual and thus, of low taxonomic significance. Again, the 402 diagnostic features to distinguish morphologically among O. achalensis, O. barrioi and O. 403 occidentalis by Rosset el al. (2007) only represent the extremes of a continuum between lowland 404 and highland ecotypes, and between southern and northern variants of the same species. For the 405 same reason González et al. (2014) failed to detect significant morphological differences among 406 the tadpoles within the O. occidentalis group. Moreover, defensive behaviour of adults is also 407 indistinguishable (Borteiro et al., 2018).

In conclusion, hypothesis 1 is verified for the species of the *O. americanus* group, but not for the nominal taxa of the *O. occidentalis* group. Conflicting evidence from phenotypic and genotypic variation in the taxa of the *O. occidentalis* group demonstrates that adaptation to

411 altitude and geographic isolation from conspecific populations (allopatry) may result in
412 phenotypes that erroneously were referred to as distinct species. Molecular evidence melts down
413 the *O. occidentalis* group to single, polymorphic and highly adaptable species *O. occidentalis*.

414

# 415 Hypothesis 2: The phenetic groups within *Odontophrynus* represent distinct phylogenetic416 lineages

Our phylogram indeed resolves three clades within the monophyletic genus Odontophrynus 417 representing the morphologically defined O. americanus, O. cultripes and O. occidentalis groups 418 419 (Fig. 5). The basal splitting of lineages separates O. occidentalis, the only species with complex 420 advertisement call consisting of several pulse groups, from the two lineages with simple pulsed 421 calls. The ancestral character state of advertisement call structure in Odontophrynidae is 422 undoubtly a simple pulsed call, present in the sister group *Macrogenioglottus alipioi* (Abravaya 423 & Jackson, 1978) and in Proceratophrys. In fact, most of the studied species of Proceratophrys 424 share this call feature, but four species (P. vielliardi, Martins & Giaretta, 2011; P. govana, P. rotundipalpebra, Martins & Giaretta, 2013; P. carranca, Godinho et al., 2013) have 425 426 independently evolved O. occidentalis-like complex advertisement calls. Unfortunately, no barcoding sequences are available for these species, so it remains presently unknown, if 427 evolution of complex calls happened one or several times in Proceratophrys. Within 428 Odontophrynus with simple calls there is a deep lineage divergence between the members of the 429 430 O. cultripes group and of the O. americanus group indicating an early splitting of the ancestral 431 stock. The species occurring in Argentina and Bolivia, the diploids O. cordobae and O. lavillai, and the topotypical tetraploids O. americanus are closely related, but the sister species 432 433 relationship between O. americanus and O. cordobae is well resolved possibly indicating an

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autopolyploid origin of these tetraploids. The eastern tetraploids in Brazil, still referred to as *O*. *americanus* as well, represent another lineage, possibly related to *O*. *juquinha* (no sequences
available vet).

In conclusion, hypothesis 2 is verified with respect to genetic base of the phenetic groups.
Our reconstruction of phylogenetic relationships among these groups suggests that *O*. *occidentalis* evolved from the ancestral stock before the diversification of the *O*. *americanus* and *O*. *cultripes* group occurred.

441

### 442 Hypothesis 3: Current red list classification does not reflect genetic diversity and 443 geographical range of taxa

Our reassessment of *Odontophrynus* spp. demonstrates that all taxa considered as valid species 444 445 are present in many localities forming a continuous geographical range (Fig. 6). The geographical distribution of *O. occidentalis* is even larger than previously appreciated extending 446 to north (barrioi phenotype) and to east (achalensis phenotype). O. occidentalis is endemic to 447 448 Argentina inhabiting many localities in eight provinces covering about 16% of the territory. This species is highly adaptable to wide range of habitats, and tolerant to local sympatry with O. 449 450 americanus and O. cordobae. Thus, the red list classification "least concern" seems justified, whereas the associated ecotypes "achalensis" and "barrioi" ("vulnerable" and "data deficient") 451 do not deserve classifications apart. With respect to the tetraploids referred to as O. americanus 452 453 our study suggests strongly that there is more than one species involved. The western taxon, 454 identical with the nominal species O. americanus, is certainly widespread in Argentina (16 455 provinces and ca. 67% of the territory) and extends to Bolivia and Paraguay in the north. The 456 status "least concern" seems appropriate. The exact range of this taxon and of the eastern taxa in

457 Brazil remains to be assessed using barcoding for species identification. Most probably, the 458 easternmost locality in Misiones pertains rather to the O. aff. americanus of Brazil than to the 459 nominal taxon of Argentina. O. cordobae has smallest area of distribution of the four species 460 occurring exclusively in the central part of the Cordoba province, and thus, being endemic to 461 Argentina (Fig. 6). Recent assessment of localities inhabited demonstrates that there is a viable network of probably connected populations (Grenat et al., 2018). Therefore, we propose the 462 463 classification "least concern" as long as there is no further shrinkage of its geographical range. Finally, O. lavillai inhabits eight provinces of Argentina as does O. occidentalis, but its range 464 465 extends further north to Bolivia and Paraguay (Rosset & Baldo, 2014). The classification "least 466 concern" seems reasonable for this species as well. The red list status of newly described species from Brazil and Uruguay and those of the O. cultripes group are outside the scope of this study. 467

In conclusion, hypothesis 3 is verified with respect to cryptic diversity within *O*. *americanus*. The invalid species status of *O*. *achalensis* renders its status "vulnerable" obsolete.

470

#### 471 CONCLUSIONS

472 Integrative taxonomy has proved to be the appropriate tool to cope with distinct levels of character differentiation in the morphologically highly conserved genus of Odontophrynus toads. 473 Genotypic variation among the nominal taxa of the O. occidentalis group did not correspond to 474 the phenotypic plasticity in response to altitude and latitudinal gradients found in the ecotypes 475 "achalensis", "barrioi" and "occidentalis". Consequently, molecular evidence melts down the O. 476 477 occidentalis group to a single, polymorphic species O. occidentalis. Whereas the species diversity was grossly overestimated in this case, considerable genetic divergence between O. 478 479 *americanus* originating from a topotypical population (Argentina) and from Brazil indicates

480 cryptic diversity currently subsumed in a single tetraploid species. Tetraploids may have arisen 481 from distinct diploid stocks possibly by alloploidy as already suggested by Beçak and Beçak (1974). Phylogenetic relationships among Odontophrynus species suggests that O. occidentalis 482 483 evolved from the ancestral stock before the diversification of the O. americanus and O. cultripes group occurred. Reliable taxonomic delimitation of Odontophrynus taxa allows for a precise 484 assessment of the corresponding geographical ranges and for an informed basis of red list 485 classification. The four species occurring in Argentina do not seem endangered currently, but the 486 small geographic range of *O. cordobae* may require a future reassessment of the species' status. 487

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## Figure 1

The nominal Odontophrynus taxa of Argentina.

(A) *O. americanus*, (B) *O. cordobae*, (C) *O. lavillai*, (D) *O. occidentalis*, (E) *O. achalensis*, (F) *O. barrioi*. Dorsolateral view.



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## Figure 2

Phenotypic variation among the three nominal taxa included in the *Odontophrynus americanus* group.

(A) Morphometric variation, (B) advertisement call variation. Each data point represents one individual.

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## Figure 3

Phenotypic variation among the five nominal taxa included in the *Odontophrynus occidentalis* group.

(A) Morphometric variation, (B) advertisement call variation. Each data point represents one individual.





## Figure 4

Advertisement calls of *O. americanus* (A) and *O. occidentalis* (B, C) as representatives of the two phenetic groups of *Odontophrynus* in Argentina.

Oscillograms show calls recorded at 19.5°C water temperature (A) and at 17.5°C water temperature (B). Three pulse groups of the complex advertisement call of *O. occidentalis* (B) are presented in (C).



Relative Amplitude [dB]

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## Figure 5

Bayesian phylogram on Odontophrynidae inferred from mitochondrial nucleotide sequence data of 16S rRNA (560 BP).

Numbers above branches are non-parametric bootstrap support values from MP and ML, respectively, numbers below branches are Bayesian posterior probabilities.



0.020

## Figure 6

Geographic distribution of *Odontophrynus* species for 8 provinces and 34 different localities sampled.

*Odontophrynus americanus* (green label). Córdoba province (9 localities): A = Achiras; BA = Barreto; K619 = Km 619, National Road #8; K624 = Km 624, National Road #8; K657 = Km 657, National Road #8; LE = La Escondida; P = Punilla; PB = Piedra Blanca; RC = Río Cuarto. Buenos Aires province (1 locality): C = Chivilcoy. *O. cordobae* (red label). Córdoba province (8 localities): AP = Athos Pampa; Be = Berrotarán; CS = Cañada del Sauce; RDLS = Río de los Sauces; SC = San Clemente; SR = Santa Rosa; T = Tanti; VGB = Villa General Belgrano. *O. lavillai* (orange label). Santiago del Estero province (2 localities): MQ = Monte Quemado; VLP = Villa La Punta. Salta province (3 localities): LC = Los Colorados; P = Pocitos; SJ = San Javier. *O. occidentalis* (blue label). Córdoba province (7 localities): A = Achiras; AP = Alpa Corral; LA = Las Albahacas; LT = Est. Los Tabaquillos; PA = Pampa de Achala; RV = Rodeo Viejo: VGB = Villa General Belgrano. San Luis province (2 localities): C = Carolina; ET = El Trapiche. San Juan province (2 localities): AM = Aguada del Molle, Sierra de Pié de Palo; HH = Huerta de Huachi. La Rioja (1 locality): AS = Aguadita Springs. Catamarca province (1 locality): RC = Río El Carrizal, Condor Huasi. Details on localities are given in S1.

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### Table 1(on next page)

Principal component analyses of morphometric and call data sets.

For details see text.

1

| (A) | Individuals | of the ( | 0. americanus | s-group |
|-----|-------------|----------|---------------|---------|
|-----|-------------|----------|---------------|---------|

| Morphometric   | PC 1             | PC2             | PC3             | Call variables with N=227 | PC 1             | PC2              | PC3              |
|----------------|------------------|-----------------|-----------------|---------------------------|------------------|------------------|------------------|
| variables with | Eigenwert: 9.71  | Eigenwert: 0.97 | Eigenwert: 0.90 | observations              | Eigenwert: 2.72  | Eigenwert: 2.22  | Eigenwert: 1.01  |
| N=105          | Variance         | Variance        | Variance        |                           | Variance         | Variance         | Variance         |
| observations   | explained: 64.7% | explained: 6.5% | explained: 6.0% |                           | explained: 38.9% | explained: 31.8% | explained: 14.4% |
| SVL            | 0.289            | -0.129          | 0.025           | Call duration             | 0.238            | 0.342            | 0.638            |
| HW             | 0.295            | -0.103          | 0.096           | Pulses per call           | -0.340           | -0.075           | 0.712            |
| HL             | 0.227            | -0.182          | 0.348           | Pulse duration            | 0.382            | 0.450            | -0.139           |
| SED            | 0.226            | -0.510          | -0.067          | Interpulse duration       | 0.533            | -0.240           | 0.113            |
| IND            | 0.221            | 0.067           | -0.364          | Pulse rate                | -0.590           | -0.040           | -0.107           |
| IOD            | 0.165            | -0.039          | 0.754           | Pulse quotient            | -0.162           | 0.604            | -0.191           |
| END            | 0.258            | -0.047          | 0.106           | Dominant frequency        | 0.153            | -0.501           | -0.073           |
| RND            | 0.220            | -0.463          | -0.303          |                           |                  |                  |                  |
| ED             | 0.246            | -0.269          | -0.150          |                           |                  |                  |                  |
| HL             | 0.296            | 0.144           | -0.080          |                           |                  |                  |                  |
| FL             | 0.278            | 0.216           | -0.056          |                           |                  |                  |                  |
| TL             | 0.298            | 0.162           | 0.092           |                           |                  |                  |                  |
| FOL            | 0.289            | 0.218           | -0.026          |                           |                  |                  |                  |
| F3L            | 0.264            | 0.371           | -0.134          |                           |                  |                  |                  |
| T4L            | 0.261            | 0.324           | -0.047          |                           |                  |                  |                  |

### (B) Individuals of the *O. occidentalis*-group

| Morphometric   | PC 1             | PC2             | PC3             | Call variables with N=75  | PC 1             | PC2              | PC3              |
|----------------|------------------|-----------------|-----------------|---------------------------|------------------|------------------|------------------|
| variables with | Eigenwert: 10.53 | Eigenwert: 0.79 | Eigenwert: 0.69 | observations              | Eigenwert: 3.39  | Eigenwert: 2.24  | Eigenwert: 1.84  |
| N=76           | Variance         | Variance        | Variance        |                           | Variance         | Variance         | Variance         |
| observations   | explained: 70.2% | explained: 5.3% | explained: 4.6% |                           | explained: 37.9% | explained: 22.4% | explained: 18.4% |
| SVL            | 0.286            | -0.001          | 0.010           | Call duration             | 0.310            | 0.357            | 0.277            |
| HW             | 0.291            | -0.009          | 0.020           | Pulse groups per call     | 0.294            | 0.428            | 0.208            |
| HL             | 0.222            | -0.286          | 0.551           | Pulse group duration      | -0.192           | -0.315           | 0.439            |
| SED            | 0.221            | -0.650          | -0.168          | Interpulse group interval | 0.083            | -0.325           | 0.526            |
| IND            | 0.223            | 0.485           | -0.381          | Pulses per pulse group    | -0.395           | -0.239           | 0.061            |
| IOD            | 0.196            | 0.016           | 0.326           | Pulse duration            | -0.320           | 0.342            | 0.348            |
| END            | 0.255            | -0.093          | 0.012           | Interpulse duration       | 0.398            | -0.377           | 0.036            |
| RND            | 0.234            | -0.328          | -0.530          | Pulse rate                | -0.332           | 0.037            | -0.457           |

| ED  | 0.247 | -0.057 | -0.270 | Pulse quotient     | -0.369 | 0.382 | 0.199  |
|-----|-------|--------|--------|--------------------|--------|-------|--------|
| HL  | 0.288 | 0.075  | -0.036 | Dominant frequency | 0.334  | 0.145 | -0.192 |
| FL  | 0.272 | 0.237  | 0.099  |                    |        |       |        |
| TL  | 0.289 | 0.045  | 0.166  |                    |        |       |        |
| FOL | 0.284 | 0.052  | 0.121  |                    |        |       |        |
| F3L | 0.270 | 0.236  | -0.033 |                    |        |       |        |
| T4L | 0.270 | 0.135  | 0.094  |                    |        |       |        |

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### Table 2(on next page)

Discriminant functions based on the three Principal Components describing morphometric variation.

Analyses were run separately on the two phenetic *Odontophrynus* groups. For details see text.

| Discriminant<br>function | Eigenwert | Percentage | Canonical correlation | Wilks<br>Lambda | Chi-squared | Degrees of<br>freedom | P-value   |  |
|--------------------------|-----------|------------|-----------------------|-----------------|-------------|-----------------------|-----------|--|
| O. americanus-group      |           |            |                       |                 |             |                       |           |  |
| 1                        | 1.47      | 99.9       | 0.772                 | 0.404           | 133.2       | 6                     | < 0.00001 |  |
| 2                        | 0.001     | 0.1        | 0.033                 | 0.999           | 0.2         | 2                     | 0.9234    |  |
| O. occidentalis-group    |           |            |                       |                 |             |                       |           |  |
| 1                        | 4.03      | 86.8       | 0.895                 | 0.117           | 214.3       | 12                    | < 0.00001 |  |
| 2                        | 0.42      | 9.0        | 0.543                 | 0.590           | 52.7        | 6                     | < 0.00001 |  |
| 3                        | 0.20      | 4.2        | 0.404                 | 0.836           | 17.8        | 2                     | 0.0001    |  |

|           |         | Standardized discriminant functions |                       |        |       |  |  |  |  |  |
|-----------|---------|-------------------------------------|-----------------------|--------|-------|--|--|--|--|--|
|           | O. amer | ricanus-group                       | O. occidentalis-group |        |       |  |  |  |  |  |
| Variables | 1       | 2                                   | 1 2                   |        | 3     |  |  |  |  |  |
| PC 1      | 1.007   | -0.016                              | 1.063                 | -0.188 | 0.015 |  |  |  |  |  |
| PC 2      | -0.203  | 0.655                               | 0.554                 | 0.903  | 0.182 |  |  |  |  |  |
| PC 3      | 0.226   | 0.748                               | -0.183                | -0.201 | 0.973 |  |  |  |  |  |

|                             |                 | Predicted species |                   |              |                |
|-----------------------------|-----------------|-------------------|-------------------|--------------|----------------|
| Actual species              | O. americanus   | O. cordobae       | O. lavillai       |              |                |
| <i>O. americanus</i> (n=66) | 54.5% (n=36)    | 43.9% (n=29)      | 1.5% (n=1)        |              |                |
| <i>O. cordobae</i> (n=57)   | 45.6% (n=26)    | 49.1% (n=28)      | 5.3% (n=3)        |              |                |
| <i>O. lavillai</i> (n=28)   | -               | 17.9% (n=5)       | 82.1% (n=23)      |              |                |
|                             |                 | Ì                 | Predicted species |              |                |
| Actual species              | O. occidentalis | O. achalensis     | O. cf. achalensis | O. barrioi   | O. cf. barrioi |
| O. occidentalis (n=29)      | 69.0% (n=20)    | 6.9% (n=2)        | 17.2% (n=5)       | 3.5% (n=1)   | 3.5% (n=1)     |
| O. achalensis (n=20)        | 10.0% (n=2)     | 75.0% (n=15)      | 15.0% (n=3)       | -            | -              |
| O. cf. achalensis (n=15)    | 6.6% (n=1)      | 26.7% (n=4)       | 66.7% (n=10)      | -            | -              |
| O. barrioi (n=20)           | 5.0% (n=1)      | -                 | 5.0% (n=1)        | 70.0% (n=14) | 20% (n=4)      |
| O. cf. barrioi (n=21)       | 4.8% (n=1)      | -                 | -                 | 14.3% (n=3)  | 81.0% (n=17)   |

5

### Table 3(on next page)

Discriminant functions based on the three Principal Components describing advertisement call variation.

Analyses were run separately on the two phenetic *Odontophrynus* groups.For details see text.

| Discriminant<br>function | Eigenwert | Percentage | Canonical correlation | Wilks<br>Lambda | Chi-squared | Degrees of<br>freedom | P-value   |  |  |
|--------------------------|-----------|------------|-----------------------|-----------------|-------------|-----------------------|-----------|--|--|
| O. americanus-group      |           |            |                       |                 |             |                       |           |  |  |
| 1                        | 4.68      | 78.9       | 0.908                 | 0.078           | 568.2       | 6                     | < 0.00001 |  |  |
| 2                        | 1.25      | 21.1       | 0.746                 | 0.444           | 181.0       | 2                     | < 0.00001 |  |  |
| O. occidentalis-group    |           |            |                       |                 |             |                       |           |  |  |
| 1                        | 3.18      | 85.0       | 0.872                 | 0.149           | 133.4       | 12                    | < 0.00001 |  |  |
| 2                        | 0.46      | 12.3       | 0.561                 | 0.622           | 33.2        | 6                     | < 0.00001 |  |  |
| 3                        | 0.10      | 2.7        | 0.305                 | 0.907           | 6.8         | 2                     | 0.0331    |  |  |

|           | Standardized discriminant functions |               |                       |        |       |  |  |
|-----------|-------------------------------------|---------------|-----------------------|--------|-------|--|--|
|           | O. amer                             | ricanus-group | O. occidentalis-group |        |       |  |  |
| Variables | 1 2                                 |               | 1                     | 2      | 3     |  |  |
| PC 1      | 1.067 -0.250                        |               | 1.050                 | 0.149  | 0.117 |  |  |
| PC 2      | 0.504                               | 0.916         | -0.377                | -0.076 | 0.964 |  |  |
| PC 3      | 0.505                               | 0.070         | -0.395                | 0.953  | 0.016 |  |  |

|                           |                   | Predicted species |                   |              |                |  |  |  |
|---------------------------|-------------------|-------------------|-------------------|--------------|----------------|--|--|--|
| Actual species            | O. americanus     | O. cordobae       | O. lavillai       |              |                |  |  |  |
| O. americanus (n=91)      | 98.9% (n=90)      | -                 | 1.1% (n=1)        |              |                |  |  |  |
| O. cordobae (n=119)       | 0.8% (n=1)        | 97.5% (n=116)     | 1.7% (n=2)        |              |                |  |  |  |
| <i>O. lavillai</i> (n=17) | -                 | -                 | 100% (n=17)       |              |                |  |  |  |
|                           | Predicted species |                   |                   |              |                |  |  |  |
| Actual species            | O. occidentalis   | O. achalensis     | O. cf. achalensis | O. barrioi   | O. cf. barrioi |  |  |  |
| O. occidentalis (n=21)    | 61.9% (n=13)      | 4.8% (n=1)        | 19.1% (n=4)       | 14.3% (n=3)  | -              |  |  |  |
| O. achalensis (n=11)      | -                 | 72.7% (n=8)       | 27.3% (n=3)       | -            | -              |  |  |  |
| O. cf. achalensis (n=10)  | 30.0% (n=3)       | 20.0% (n=2)       | 40.0% (n=4)       | -            | 10.0% (n=1)    |  |  |  |
| O. barrioi (n=11)         | 9.1% (n=1)        | -                 | -                 | 90.9% (n=10) | -              |  |  |  |
| O. cf. barrioi (n=22)     | -                 | -                 | -                 | 13.6% (n=3)  | 86.4% (n=19)   |  |  |  |

### Table 4(on next page)

Nei's genetic distances among eight Odontophrynus taxa.

Distances were calculated from the allele frequencies listed in S2.

1

| Taxon             | O. cordobae | O. lavillai | O. occidentalis | O. achalensis | O. cf. achalensis | O. barrioi | O. cf. barrioi |
|-------------------|-------------|-------------|-----------------|---------------|-------------------|------------|----------------|
| O. americanus     | 0.0220      | 0.1853      | 0.1821          | 0.1942        | 0.2452            | 0.4196     | 0.5471         |
| O. cordobae       |             | 0.2224      | 0.2084          | 0.2146        | 0.2707            | 0.4160     | 0.5943         |
| O. lavillai       |             |             | 0.2781          | 0.4126        | 0.4982            | 0.6705     | 0.5608         |
| O. occidentalis   |             |             |                 | 0.0232        | 0.0292            | 0.1846     | 0.2604         |
| O. achalensis     |             |             |                 |               | 0.0351            | 0.1660     | 0.3422         |
| O. cf. achalensis |             |             |                 |               |                   | 0.1772     | 0.3406         |
| O. barrioi        |             |             |                 |               |                   |            | 0.2186         |

2 3

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### Table 5(on next page)

Uncorrected P-distances [%] among seven nominal *Odontophrynus* taxa and *Macrogenioglottus alipioi, Proceratophrys bigibossa* and *Ceratophrys cornuta* (outgroups).

Distances were calculated using the partial sequences of the 16S rRNA gene (560 bp) listed in S3.

1

| Taxon           | О.         | О.       | О.       | <i>O</i> .   | О.         | О.      | О.          | М.      | <i>P</i> . | С.       |
|-----------------|------------|----------|----------|--------------|------------|---------|-------------|---------|------------|----------|
|                 | americanus | cordobae | lavillai | occidentalis | achalensis | barrioi | cf. barrioi | alipioi | bigibossa  | cornuta  |
|                 | (Brazil)   |          |          |              |            |         |             | -       | _          |          |
| O. americanus   | 2.4        | 2.0      | 2.7      | 4.7-4.9      | 4.7        | 5.3     | 4.2         | 5.9-6.2 | 8.6        | 10.8     |
| (Argentina)     |            |          |          |              |            |         |             |         |            |          |
| O. americanus   |            | 1.6      | 2.4      | 4.2-4.4      | 4.2        | 4.7     | 4.7         | 6.2-6.4 | 9.6        | 11.0     |
| (Brazil)        |            |          |          |              |            |         |             |         |            |          |
| O. cordobae     |            |          | 1.8      | 4.6-4.7      | 4.6        | 5.1     | 4.6         | 5.7-6.0 | 8.8        | 10.6     |
| O. lavillai     |            |          |          | 4.6-4.7      | 4.6        | 5.1     | 4.6         | 6.8-6.9 | 9.2        | 11.2     |
| O. occidentalis |            |          |          |              | 0.0-0.2    | 0.7-0.9 | 0.2         | 3.8-5.1 | 8.8-9.0    | 9.9-10.1 |
| O. achalensis   |            |          |          |              |            | 0.7     | 0.0         | 3.8-4.9 | 8.8        | 9.9      |
| O. barrioi      |            |          |          |              |            |         | 0.7         | 3.8-5.3 | 8.6        | 9.7      |
| O. cf. barrioi  |            |          |          |              |            |         |             | 3.8-4.9 | 8.8        | 9.9      |
| M. alipioi      |            |          |          |              |            |         |             |         | 8.8-9.0    | 9.5-10.6 |
| P. bigibossa    |            |          |          |              |            |         |             |         |            | 11.0     |