A peer-reviewed version of this preprint was published in PeerJ on 25 February 2019.

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Martino AL, Dehling JM, Sinsch U. 2019. Integrative taxonomic reassessment of *Odontophrynus* populations in Argentina and phylogenetic relationships within Odontophrynidae (Anura) PeerJ 7:e6480 https://doi.org/10.7717/peerj.6480

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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ABSTRACT

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.

Keywords: Species delimitation, Integrative taxonomy, Morphometry, Advertisement call, Allozymes, 16S rRNA sequences, *Macrogenioglottus, Proceratophrys, Odontophrynus*

INTRODUCTION

Patterns of tropical and subtropical amphibian diversity are not well understood because of incomplete information on taxonomy and distribution (e.g., Vieites et al., 2009; Winter et al., 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). Taxonomic uncertainty stems partially from the prevalence of the morphospecies concept in 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 of anurans and to environmental constraints posed by adaptations to a specific mode of living (e.g., Elmer, Dávila & Lougheed, 2007; Vences et al., 2010; Kaefer et al., 2012; Rojas et al., 2018). Advertisement calls as powerful tools of premating isolation can reveal morphologically 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 al., 2011, 2012; Greenbaum et al. 2013; Köhler et al., 2017). Delimiting species solely based on genetic distances obtained by barcoding approaches may inflate real species numbers by overestimating the taxonomic importance of genetic structuring (e.g., Sukumaran & Knowles, 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 & 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

to re-evaluate the uncertain taxonomic status of allopatric populations in stream-inhabiting *Telmatobius* frogs that occur in remote Andean highland valleys (e.g., Sinsch & Lehr, 2010; Barrionuevo, 2013; Sáez et al., 2014). The semi fossorial toads of the genus *Odontophrynus* pose a similar challenge because all original species descriptions are morphology based and often too ambiguous for a reliable species distinction (Cei, Ruiz & Beçak, 1982; di Tada et al., 1984; Cei, 1985; Martino & Sinsch, 2002; Rosset et al., 2006, 2007; Rosset, 2008; Caramaschi & Napoli, 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 (Frost, 2018): The *O. americanus* group including the *O. americanus, O. cordobae, O. juquinha, O. lavillai*, and *O. maisuma*, the *O. occidentalis* group including *O. achalensis*, *O. barrioi*, and O. *occidentalis, and* the *O. cultripes* group including *O. carvalhoi, O. cultripes* and *O. 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 (Turazzini, Taglioretti & Gomez, 2016; Santos-Silva et al., 2017).

Taxonomic assignment of populations to the currently recognized species is hampered by the overall similarity of external morphology, and corresponding geographic ranges are bear a 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 “data deficient” (IUCN, 2018). The three disjunct areas inhabited by the tetraploid *O. americanus* may indicate the presence of cryptic species (Rosset et al., 2006). Highland taxa such as *O. achalensis* may not occur exclusively in the Pampa de Achala plateau in the Sierras de Cordoba, but also in similar habitats of the Sierra de San Luis (di Tada et al., 1984). Diploid *O. americanus*-like populations reported from the vicinity of the disjunct *O. americanus* ranges

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

Consequently, a reliable delimitation of *Odontophrynus* species, an assessment of their geographical ranges, conservation needs and phylogenetic relationships require an integrative taxonomic approach critically evaluating information derived from morphology, behaviour and genes. In this long-term study covering more than 20 years of field and laboratory work, we focus geographically on Argentina, the centre of *Odontophrynus* diversity with six recognized 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, allozyme patterns and partial 16S rRNA sequences, all providing meaningful taxonomic information. Data refer to 34 populations, among them those at the type localities for reference. 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*) 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.

MATERIALS AND METHODS

Study area and field sampling

Since 1995, we identified and sampled 34 local populations of toads pertaining to the genus *Odontophrynus* in Argentina (Table S1). The type localities of the nominal taxa *O. achalensis* di Tada, Barla, Martori, and Cei, 1984 (Pampa de Achala, Cordoba province), *O. barrioi* Cei, Ruiz, and Beçak, 1982 (Aguadita springs, Sierra de Famatina, La Rioja province), *O. cordobae* Martino and Sinsch, 2002 (Villa General Belgrano, Cordoba province) and *O. lavillai* Cei, 1985 (Villa de la Punta, Santiago del Estero province) were sampled to obtain topotypical individuals for taxonomic comparison. Unfortunately, the type localities of the most wide-spread species *O. 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 Aires" and that the holotype of *O. occidentalis* was collected in an "arroyo agrario" in the Neuquén province (Frost, 2018). Still, populations of tetraploid *O. americanus* were readily distinguished from those of the diploid taxa by erythrocyte size (Rosset et al., 2006; Otero et al., 2013). Populations of uncertain taxonomic assignment were assigned as *O.* cf. *achalensis* (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.

Morphological data

In a first step, presumed ploidy (diploid/tetraploid) was verified by measuring the erythrocyte size, which correlates with the DNA content. Smears of fresh blood were air-dried and light-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 carcasses preserved in 4% formaldehyde. Use of vertebrate animals was approved by the Ethics Committee (COEDI) of the Universidad Nacional de Rio Cuarto. (https: //www.unrc. 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 Ethical Committee of Investigation of the National University of Río Cuarto (Nº 38/11). The external morphology of 256 specimens was described quantitatively by measuring fifteen morphometric distances (to the nearest 0.1 mm; Martino & Sinsch, 2002): (1) Snout-vent length (SVL); (2) maximal head width (HW); (3) head length (HL); (4) snout-eye distance (SED); (5) internarinal distance (IND); (6) interocular distance (IOD); (7) eye-narinal distance (END); (8) 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
161 fixed number of three PCs extracted. By this means, we explored the morphometric variability
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
164 were run on the taxa of the phenetic groups. Assignment of populations to a phenetic group was
165 based on the advertisement call structure (*O. americanus*-group: simple pulsed calls; *O.*
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
170 consider a correct taxon classification of at least 80% of the individuals studied as indicative for
171 taxonomic implications. Due to the low resolution among taxa of the *O. occidentalis* group we
172 tested for clinal variation of PCs along latitudinal and altitudinal gradients by a multiple
173 regression analysis (Procedure: backward selection at F=4.0). Significance level was set to
174 alpha=0.05. All calculations were performed using the statistical package statgraphics centurion,
175 version XVIII (Statpoint Inc., 2018).

176

177 **Bioacoustic data**

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

individual calling sites (usually shallow water near shore) immediately after recording. Whenever possible, specimens were collected to obtain tissue samples and for morphometric measurements. Oscillograms, sonograms and power spectra of the call series were prepared with the Medav Mosip 3000 Signal Processing System or the PC program Adobe Audition 1.0. Each call series was characterized by ten parameters which were measured in three calls per series (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]; (4) interval between pulse groups; (5) pulses per pulse group [N]; (6) pulse duration [ms]; (7) interpulse interval [ms]; (8) pulse rate [pulses/s]; (9) pulse quotient (=pulse duration/interpulse interval); (10) dominant frequency [Hz].

The arithmetic means of these call parameters were calculated for each series (=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 corresponding ambient temperature) with N=304 observations. As several call variables co-vary with ambient temperature, we calculated linear regression models of call parameter *vs.* temperature and used the standardised residuals to obtain a temperature-adjusted data set for 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 with seven variables *vs.* complex calls with ten variables) to explore the bioacoustic differentiation among the taxa of each phenetic group. The three PCs explaining the most of the variance were extracted to describe the sound space utilized by *Odontophrynus* and its partitioning among taxa. Moreover, a discriminant analysis was applied to quantify the partitioning of among-taxon sound space, again applying the 80% criterion on the rates of the

204 correct classification of call. Again, we tested for clinal variation of PCs along latitudinal and
205 altitudinal gradients by a multiple regression analysis (Procedure: backward selection at F=4.0).
206
207 **Allozyme data**
208 Liver samples were obtained from 147 individuals (Table S2). Samples were dissolved in 1ml
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,
211 5.7x14.0cm) and submitted to a continuous horizontal electrophoresis (Hebert & Beaton, 1993).
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
214 7.0 and constant 130V. Following electrophoresis, each gel was stained using standard recipes
215 (Murphy et al., 1996).
216 The allozyme pattern of liver tissue consisted of 10 enzyme systems controlled by a total
217 of 14 presumptive loci: aspartate amino transferase (2 loci, AAT, EC 2.6.1.1), carboxylesterase
218 (1, EST, 3.1.1.1), glycerol-3-phosphate dehydrogenase (1, G3PD, 1.1.1.8), glucosephosphate
219 isomerase (1, GPI, 5.3.1.9), isocitrate dehydrogenase (2, IDH, 1.1.1.42), lactate dehydrogenase
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,
222 2.7.5.1). Mitochondrial and cytoplasmatic loci were distinguished by prefixes (m/c),
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.
225 Statistical analyses of data included the calculation of allele frequencies (Table S2) and
226 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).

Molecular phylogenetic analysis

We compared the partial sequence of the mitochondrial 16S rRNA gene of the samples from the different localities in Argentina to assess the number of species present in the country and their phylogenetic relationships (Table S3). The 16S barcoding gene has been demonstrated to contain a strong phylogenetic signal and to be especially informative in topology resolution (Vences et al., 2005; Zhang et al., 2013). DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Polymerase Chain Reaction (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 (5'-CCGGTCTGAACTCAGATCACGT-3'). Amplification followed the standard PCR 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. 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 Kit (Roche Diagnostics). Sequencing of both strands was performed with the DYEnamic ET Terminator Cycle Sequencing Premixkit (GE Healthcare, Munich, Germany) for sequencing reactions run on a MegaBACE 1000 automated sequencer (GE Healthcare). Chromas lite 2.1.1 software (Technelysium Pty Ltd, http://www.technelysium.com.au) was used to check and read 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).

The general time-reversible model with proportion of invariable sites and gamma-distributed rate variation among sites (GTR + I + G) was chosen as the best-fitting model of 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 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 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 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, and the remaining trees from both runs were used to calculate a consensus tree and Bayesian posterior probabilities (BPP). Treegraph2 (Stöver & Müller, 2010) was used to draw trees.

RESULTS

Morphological variation

All nominal taxa of *Odontophrynus* resemble each other considerably in colouration and external morphology reflecting their semi fossorial mode of living (Fig. 1). Quantitative morphometric analyses based on 15 measured variables still demonstrated a significant morphological variation among some taxa. The three principal component representing the axes of morphospace explained 77.2% of total variance in the *O. americanus* group and 80.1% in the *O. occidentalis* group, respectively (Table 1). The morphospace of the *O. americanus* group was partitioned 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 separation of *O. lavillai* at 82.1% correct classification rate mainly based on its larger size (PC1; Table 2).

In contrast, resolution among taxa in the *O. occidentalis* group was lower with *O. occidentalis, O. achalensis* and *O.* cf. *achalensis* being indistinguishable among each other (Fig. 3; Table 2). *O. barrioi* and *O.* cf. *barrioi* differed from these mainly by their larger size (PC1) and among each other by head shape (PC2/3) at a 70% and 81%, respectively, correct classification rate (Table 2). A significant proportion of morphometric variability among individuals assigned to the *O. occidentalis* group was caused by a clinal variation along altitudinal and latitudinal gradients. Size-related variation (PC1) was significantly correlated with altitude and latitude (Multiple regression model, R^2=32.1%, $F_{2,102}$=24.03, P<0.00001), i.e. size of individuals increased with elevation and from south to north. PC2 (position of nares and 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, R^2=10.0%, $F_{1,103}$=11.42, P=0.001).

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
302 of sound space (Table 1). The sound space was partitioned into three discrete groups
303 representing *O. americanus, O. cordobae,* and *O. lavillai* individuals, respectively (Figure 2).
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
308 indistinguishable among each other (Fig. 3; Table 3). The acoustic niches of *O. barrioi* and *O.* cf.
309 *barrioi* were better resolved from the continuum formed by the other taxa, but showed a slight
310 overlap between each other. Still, the correct classification rates for *O. barrioi* and *O.* cf. *barrioi*
311 were at a 91% and 86%, respectively (Table 3). Temperature-adjusted advertisement call
312 variation was also influenced by geographical clines. PC1 (size-related dominant frequency) and
313 PC2 (call duration) were significantly correlated with latitude (Multiple regression models:
314 R^2=9.8%, $F_{1,74}$=7.94, P=0.0062, and R^2=14.1%, $F_{1,74}$=11.97, P=0.0009, respectively), and PC3
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,

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

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.*

342 *barrioi*. The relationships within this clade remained largely unresolved and the three nominal
343 taxa did not resolve into distinct phylogenetic lineages. The second clade contained the
344 remaining species and splitted into two subclades, one consisting of *O. carvalhoi* and *O.*
345 *cultripes*, the other one containing *O. americanus*, *O. cordobae*, and *O. lavillai*. The two samples
346 of *O. americanus* did not form a monophyletic clade but the topotypic Argentinian sample
347 appeared to be more closely related to *O. cordobae* and *O. lavillai* than to the Brazil sample
348 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
354 plasticity may result in an overestimation of species diversity (*O. occidentalis* group), whereas
355 molecular data may reveal unexpected cryptic diversity in morphologically uniform populations
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
358 and propose a model of the phylogenetic relationships within the genus *Odontophrynus*.
359

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

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

americanus group (distinct by advertisement call variation and 16S sequences) do not differ 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 variation following environmental gradients (e.g., Sinsch, Pelster & Ludwig, 2015) renders the support of taxonomic decision by SVL differences alone unreliable (e.g., Rakotoarison et al., 2015; Rojas et al., 2016). Ploidy distinguishes *O. lavillai* from *O. americanus*, but not from *O. 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 on dorsum, lacking a light middorsal stripe; Cei, 1985; Rosset & Baldo, 2014) are diagnostic and allow for an unequivocal identification (diagnostic characters listed for *O. juquinha* are widely the same; Rocha et al., 2017). Nevertheless, combined evidence derived from the four character 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 Aires province, Argentina and a specimen from Minas Gerais, Brazil, indicates that they differ at species level. The close relationship between *O. cordobae* and *O. americanus* from Argentina with rare hybridization in nature suggests a common genetic stock (Grenat et al., 2018). Future 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 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
395 the absence of significant genetic differentiation from *O. occidentalis* is contrasted by
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.
400 Advertisement call variation is mainly based on differences in dominant frequency, again an
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).

408 In conclusion, hypothesis 1 is verified for the species of the *O. americanus* group, but not
409 for the nominal taxa of the *O. occidentalis* group. Conflicting evidence from phenotypic and
410 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 phylogenetic**
416 **lineages**
417 Our phylogram indeed resolves three clades within the monophyletic genus *Odontophrynus*
418 representing the morphologically defined *O. americanus*, *O. cultripes* and *O. occidentalis* groups
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. goyana, P.*
425 *rotundipalpebra,* Martins & Giaretta, 2013; *P. carranca,* Godinho et al., 2013) have
426 independently evolved *O. occidentalis*-like complex advertisement calls. Unfortunately, no
427 barcoding sequences are available for these species, so it remains presently unknown, if
428 evolution of complex calls happened one or several times in *Proceratophrys*. Within
429 *Odontophrynus* with simple calls there is a deep lineage divergence between the members of the
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*,
432 and the topotypical tetraploids *O. americanus* are closely related, but the sister species
433 relationship between *O. americanus* and *O. cordobae* is well resolved possibly indicating an

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 yet).

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.

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

Our reassessment of *Odontophrynus* spp. demonstrates that all taxa considered as valid species 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 to north (*barrioi* phenotype) and to east (*achalensis* phenotype). *O. occidentalis* is endemic to 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. americanus* and *O. cordobae*. Thus, the red list classification "least concern" seems justified, whereas the associated ecotypes "achalensis" and "barrioi" ("vulnerable" and "data deficient") do not deserve classifications apart. With respect to the tetraploids referred to as *O. americanus* our study suggests strongly that there is more than one species involved. The western taxon, identical with the nominal species *O. americanus*, is certainly widespread in Argentina (16 provinces and ca. 67% of the territory) and extends to Bolivia and Paraguay in the north. The status "least concern" seems appropriate. The exact range of this taxon and of the eastern taxa in

Brazil remains to be assessed using barcoding for species identification. Most probably, the easternmost locality in Misiones pertains rather to the *O.* aff. *americanus* of Brazil than to the nominal taxon of Argentina. *O. cordobae* has smallest area of distribution of the four species occurring exclusively in the central part of the Cordoba province, and thus, being endemic to 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 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 extends further north to Bolivia and Paraguay (Rosset & Baldo, 2014). The classification "least 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.

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.

CONCLUSIONS

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. Genotypic variation among the nominal taxa of the *O. occidentalis* group did not correspond to the phenotypic plasticity in response to altitude and latitudinal gradients found in the ecotypes "achalensis", "barrioi" and "occidentalis". Consequently, molecular evidence melts down the *O. occidentalis* group to a single, polymorphic species *O. occidentalis*. Whereas the species diversity was grossly overestimated in this case, considerable genetic divergence between *O. americanus* originating from a topotypical population (Argentina) and from Brazil indicates

cryptic diversity currently subsumed in a single tetraploid species. Tetraploids may have arisen from distinct diploid stocks possibly by alloploidy as already suggested by Beçak and Beçak (1974). Phylogenetic relationships among *Odontophrynus* species suggests that *O. occidentalis* 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 assessment of the corresponding geographical ranges and for an informed basis of red list classification. The four species occurring in Argentina do not seem endangered currently, but the small geographic range of *O. cordobae* may require a future reassessment of the species´ status.

ACKNOWLEDGMENTS

We acknowledge the help of R. Buff, I.E. di Tada, J.C. Acosta, E. Sanabria, J. Marinero, M. Olivares, C. Martino, P. Grenat, and J. Valetti during fieldwork, and that of B. Nilow-Lange and L. Sinsch during allozyme analyses.

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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.

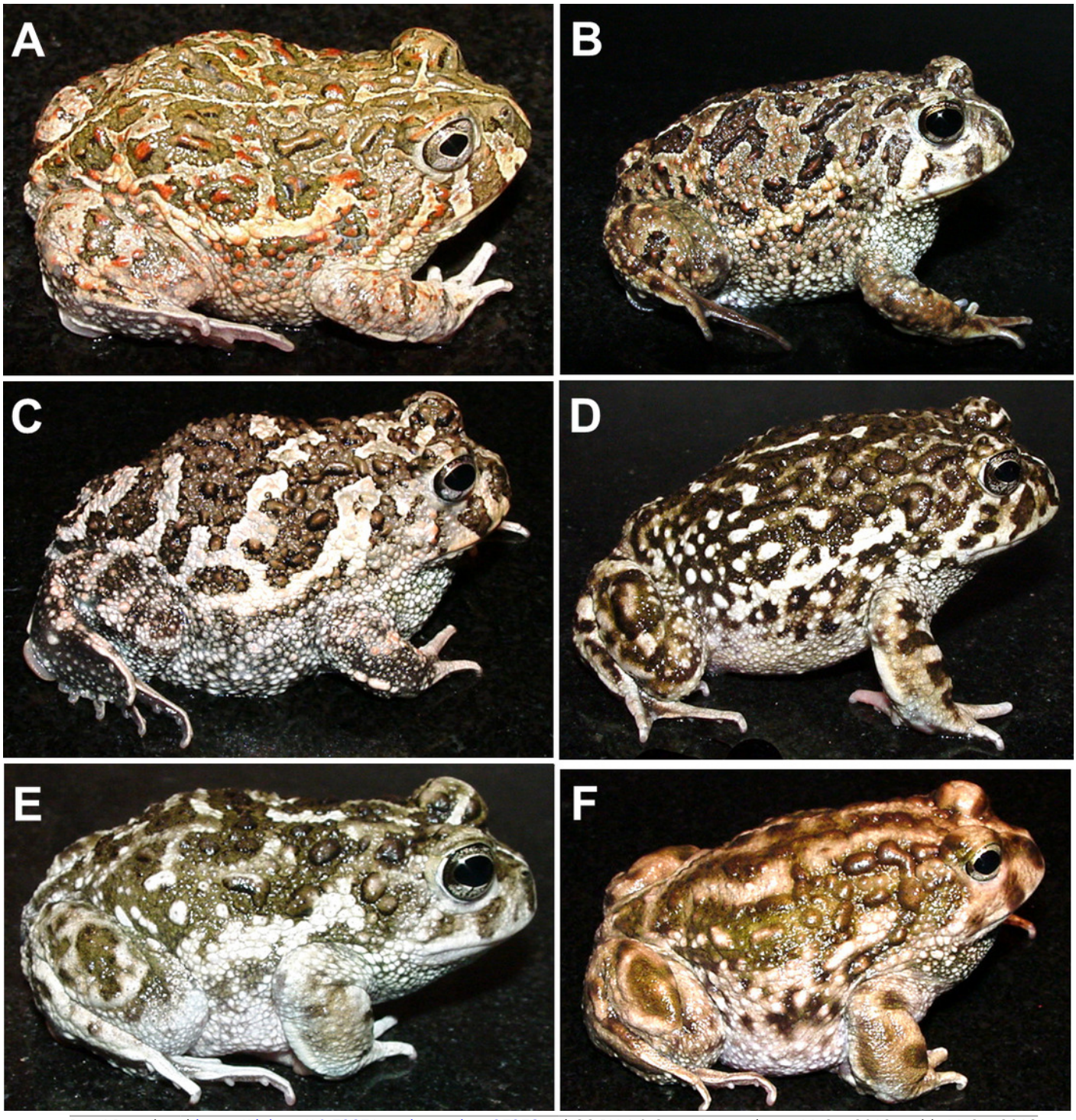

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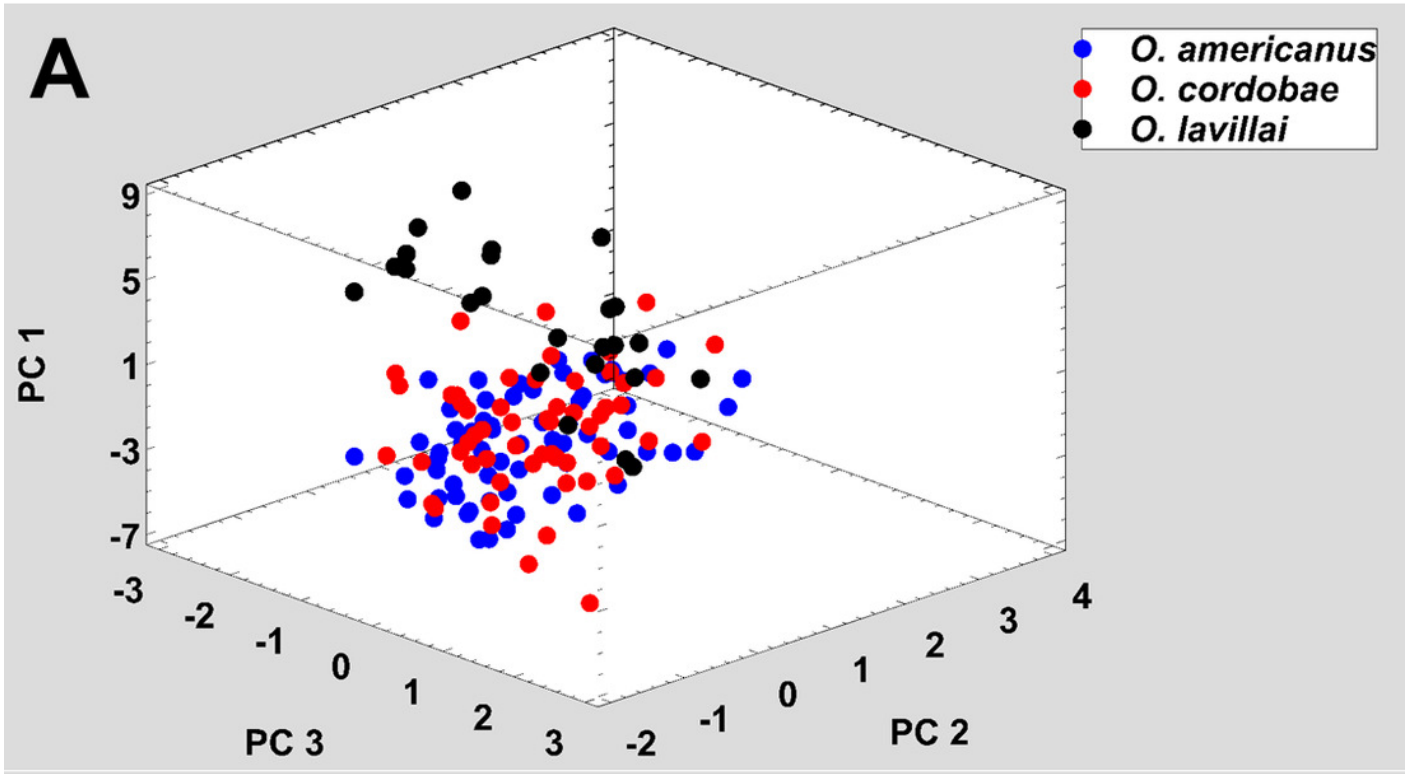
Figure 2

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.



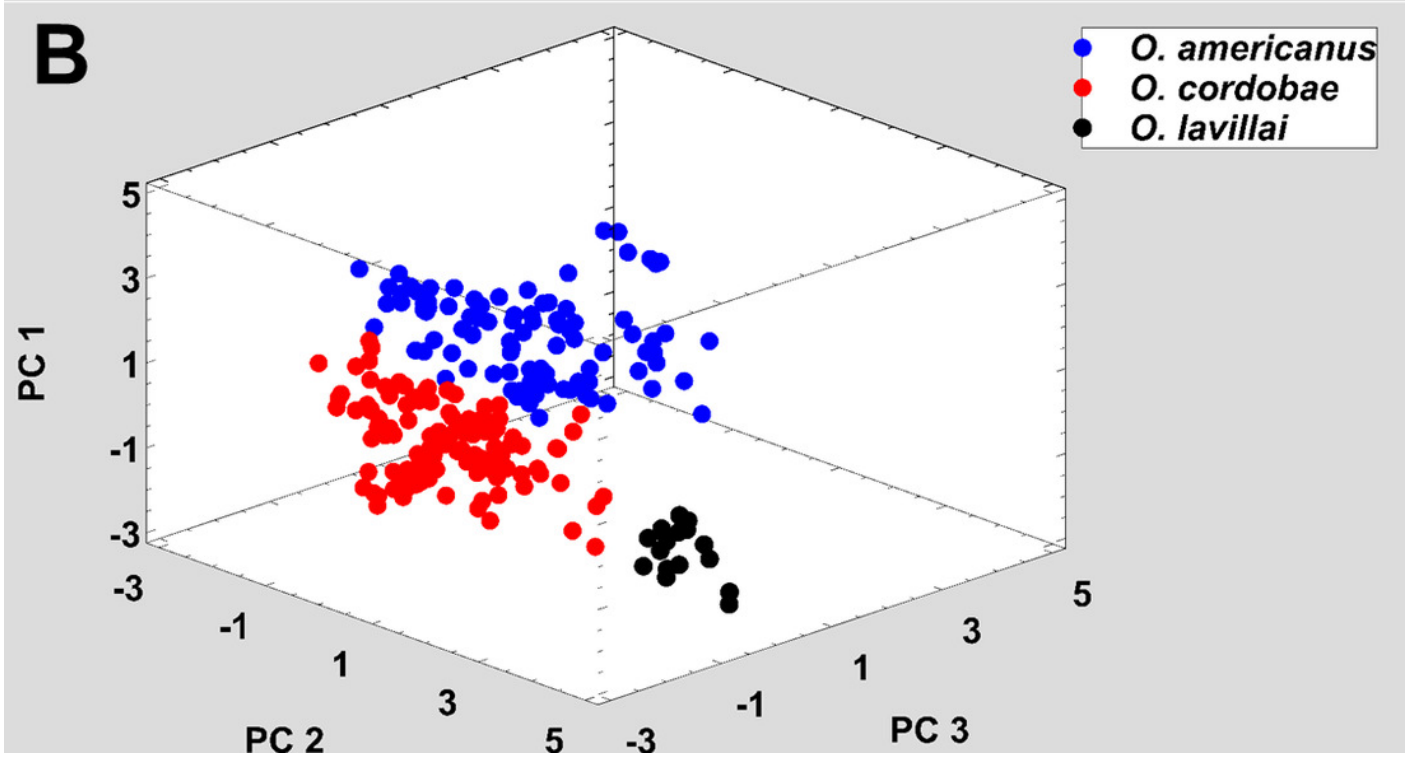


Figure 3

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.

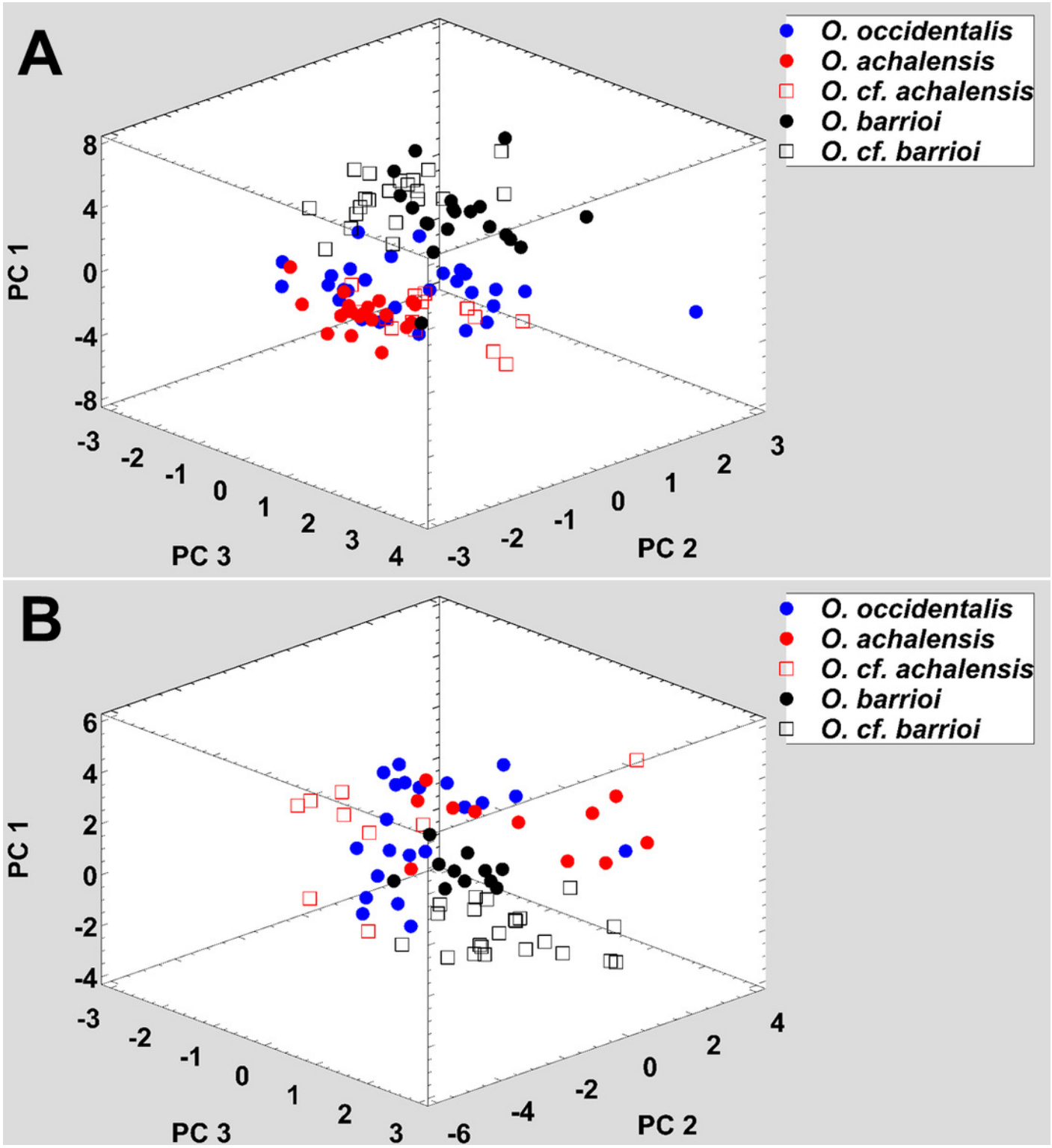


Figure 4

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).

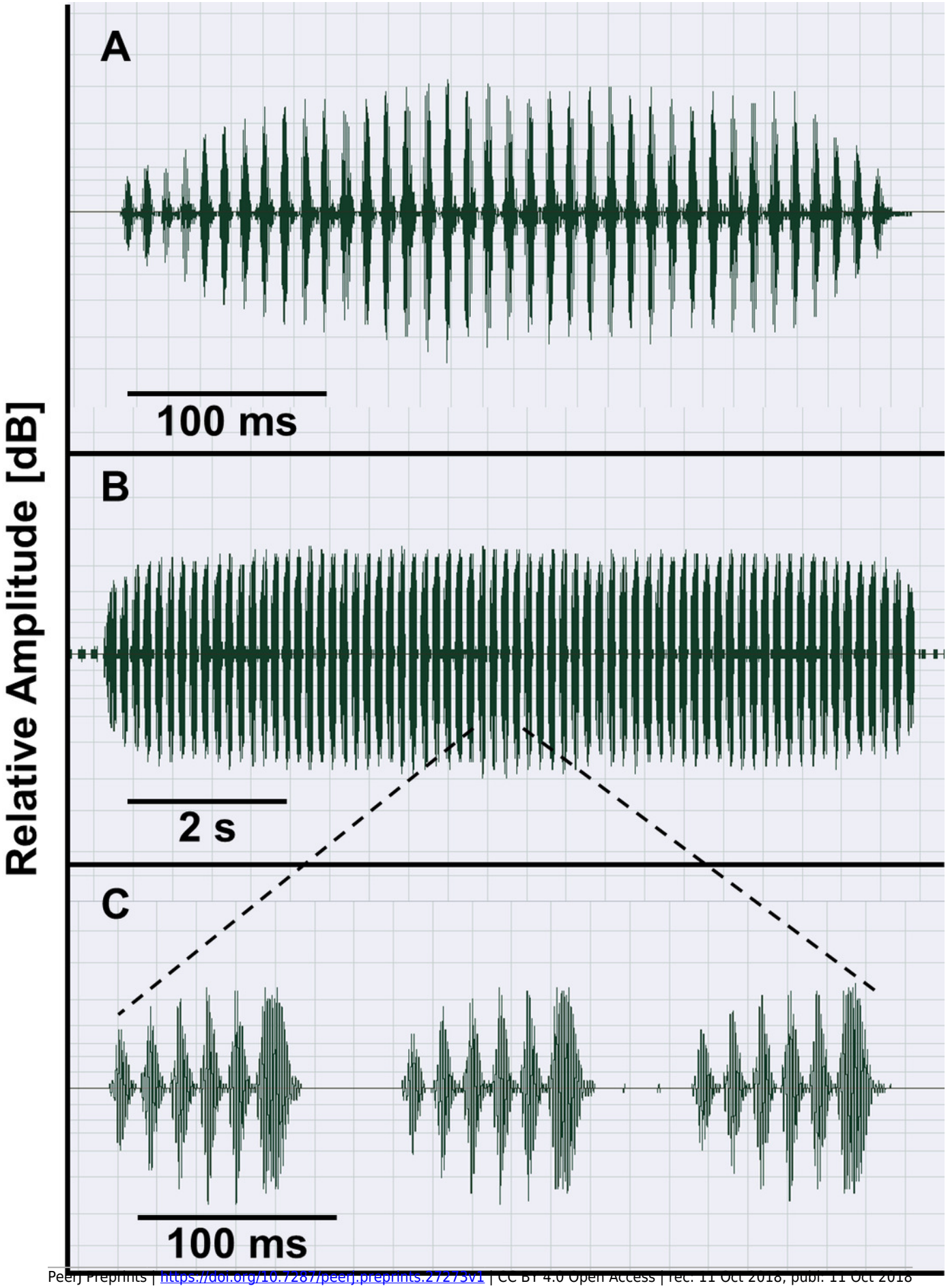


Figure 5

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.

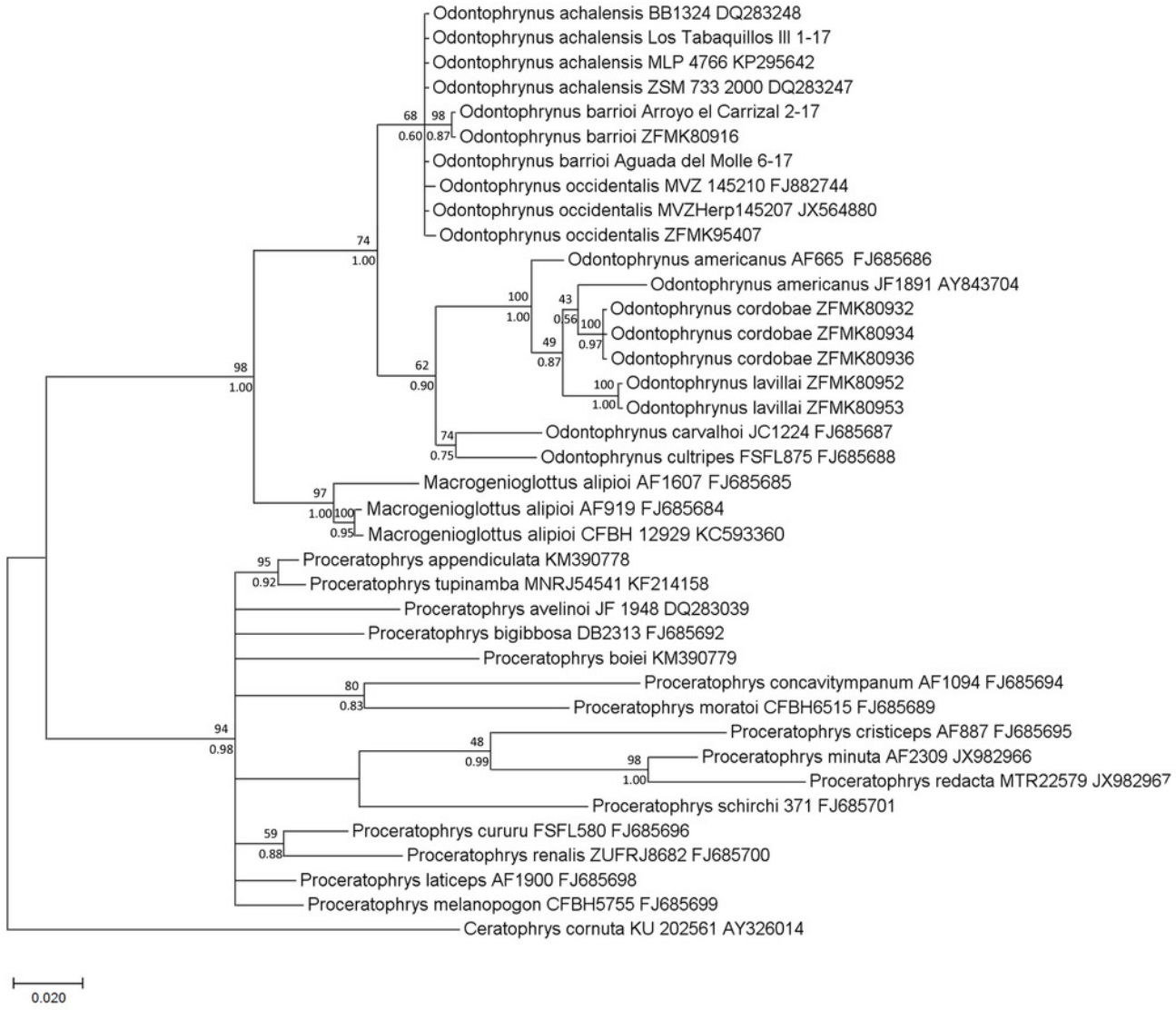


Figure 6

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.

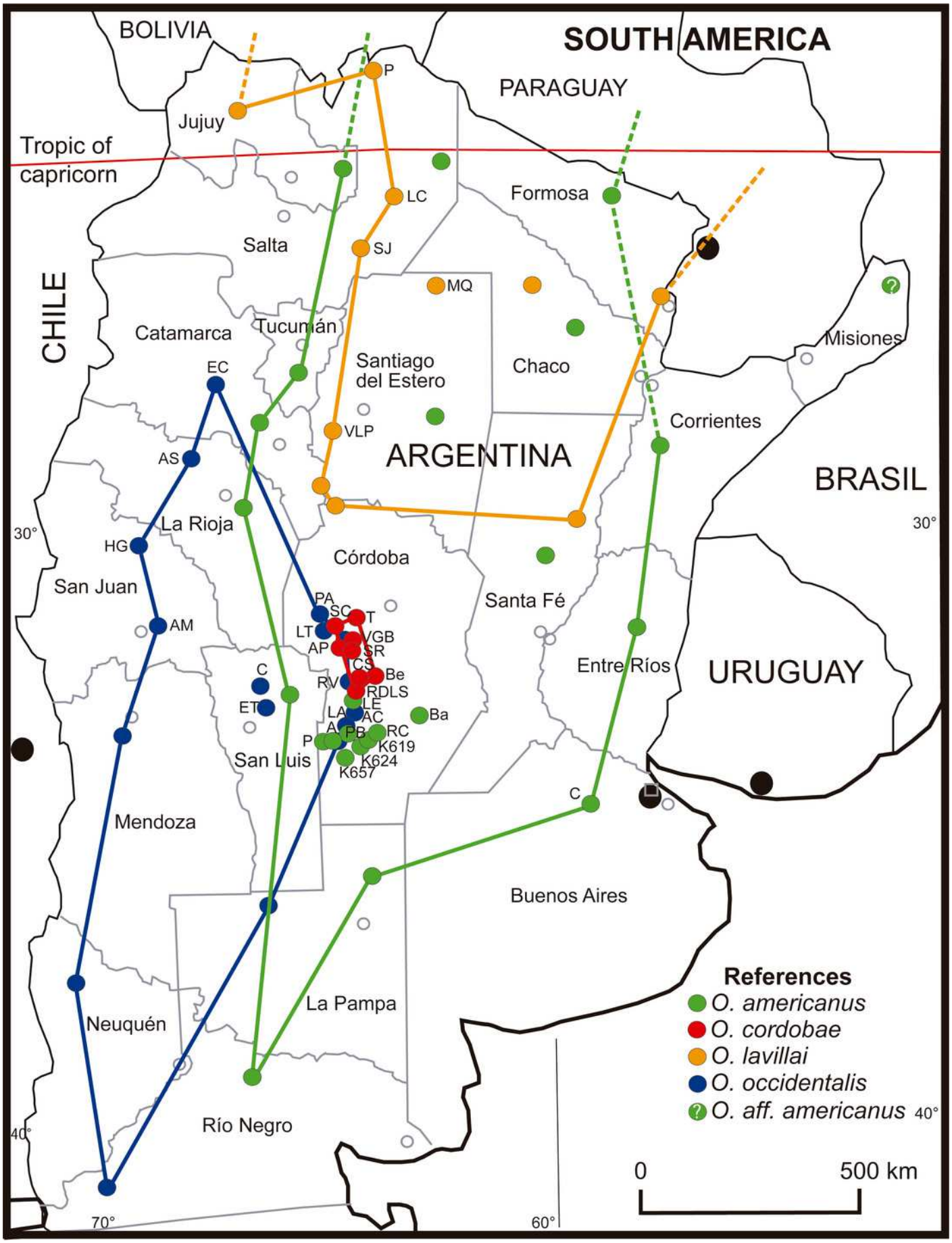


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Table 1(on next page)

Principal component analyses of morphometric and call data sets.

For details see text.

1

(A) **Individuals of the *O. americanus*-group**

Morphometric variables with N=105 observations	**PC 1** Eigenwert: 9.71 Variance explained: 64.7%	**PC2** Eigenwert: 0.97 Variance explained: 6.5%	**PC3** Eigenwert: 0.90 Variance explained: 6.0%	**Call variables with N=227 observations**	**PC 1** Eigenwert: 2.72 Variance explained: 38.9%	**PC2** Eigenwert: 2.22 Variance explained: 31.8%	**PC3** Eigenwert: 1.01 Variance 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 variables with N=76 observations	**PC 1** Eigenwert: 10.53 Variance explained: 70.2%	**PC2** Eigenwert: 0.79 Variance explained: 5.3%	**PC3** Eigenwert: 0.69 Variance explained: 4.6%	**Call variables with N=75 observations**	**PC 1** Eigenwert: 3.39 Variance explained: 37.9%	**PC2** Eigenwert: 2.24 Variance explained: 22.4%	**PC3** Eigenwert: 1.84 Variance 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				

2
3

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.

1

Discriminant function	*Eigenwert*	*Percentage*	*Canonical correlation*	*Wilks Lambda*	*Chi-squared*	*Degrees of 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

2

	Standardized discriminant functions				
	O. americanus-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

3

	Predicted species				
Actual species	***O. americanus***	***O. cordobae***	***O. lavillai***		
***O. americanus* (n=66)**	54.5% (n=36)	43.9% (n=29)	1.5% (n=1)		
***O. cordobae* (n=57)**	45.6% (n=26)	49.1% (n=28)	5.3% (n=3)		
***O. lavillai* (n=28)**	-	17.9% (n=5)	82.1% (n=23)		
	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)

4

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.

1

Discriminant function	*Eigenwert*	*Percentage*	*Canonical correlation*	*Wilks Lambda*	*Chi-squared*	*Degrees of 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

2

	Standardized discriminant functions				
	O. americanus-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

3

	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)		
***O. lavillai* (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)

4

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

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	*O. americanus* (Brazil)	*O. cordobae*	*O. lavillai*	*O. occidentalis*	*O. achalensis*	*O. barrioi*	*O.* cf. *barrioi*	*M. alipioi*	*P. bigibossa*	*C. cornuta*
O. americanus (Argentina)	2.4	2.0	2.7	4.7-4.9	4.7	5.3	4.2	5.9-6.2	8.6	10.8
O. americanus (Brazil)		1.6	2.4	4.2-4.4	4.2	4.7	4.7	6.2-6.4	9.6	11.0
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

2

3