Amphibia-Reptilia 39 (2018): 1-10

Naturally occurring triploids in contact zones between diploid/tetraploid *Odontophrynus cordobae* and *O. americanus* (Anura, Odontophrynidae)

Pablo Grenat[1,2,*], Nancy Salas[1], Favio Pollo[1,2], Manuel Otero[1,2], Mariana Baraquet[1,2], Ulrich Sinsch[3], Adolfo Martino[1]

Abstract. Polyploidization plays an important role in speciation and evolution in anurans. However, a few stable triploid populations and some isolated triploid individuals have been reported. Here, we report the discovery of naturally occurring triploids in contact zones between diploid *Odontophrynus cordobae* and tetraploid *O. americanus* from Central Argentina, and propose values of erythrocyte area for the distinction of ploidy levels. A total of 101 individuals from three contact zones were studied and ploidy of each specimen was identified by mean chromosome count and erythrocyte size. Twenty three adult triploid specimens (males: $n = 21$; females: $n = 2$) from two contact sites were identified (percentage of individuals per ploidy level: site S2, $2n = 40.6\%$, $3n = 12.5\%$, $4n = 46.9\%$; site S3: $2n = 44.7\%$, $3n = 40.4\%$, $4n = 14.9\%$). The limit values of erythrocyte nuclear area used to distinguish between different ploidy levels were 23.62 μm^2 (probability to be assigned to a respective ploidy level = 94.78%) for separating diploids and triploids and 27.67 μm^2 (98.62%) for triploids and tetraploids. The high number of adult triploids occurring in more than one contact site between *O. cordobae* and *O. americanus* indicates that is not an isolated event. However, further studies are necessary to provide a hypothesis on the origin and evaluate the possible maintenance of triploids in syntopy with *O. cordobae* and *O. americanus*.

Keywords: amphibian, Argentina, hybridization, ploidy level, syntopy, triploidy.

Introduction

While polyploidy is generally rarer in animals than in plants; it has been reported in several invertebrate and vertebrate organisms (see Gregory and Mable, 2005). Particularly, polyploidy plays an important role in speciation and evolution in anurans (Bogart, 1980) with about 50 polyploid species described in multiple families, including bisexually reproducing species with several ploidy levels (Mable, Alexander and Taylor, 2011; Evans, Pyron and Wiens, 2012). However, few stable triploid species or populations and some isolated triploid individuals have been reported in anurans (Evans, Pyron and Wiens, 2012). Consequently, the occurrences of new cases of natural polyploidy, as well as the coexistence and potential reproductive interactions with other species with different ploidy level are particularly interesting from ecology and evolution perspective (Petit, Bretagnolle and Felber, 1999).

Tetraploid *Odontophrynus americanus* (Duméril and Bibron, 1841) was the first record of a naturally occurring polyploid ($4n = 44$ chromosomes) found in a bisexual vertebrate species (Beçak, Beçak and Rabello, 1966). Later, morphologically cryptic diploid populations ($2n = 22$) were also detected (Beçak, Beçak and Vizotto, 1970). Currently, tetraploid and diploid *O. americanus* populations is widespread in Brazil, Paraguay, Uruguay and Argentina (Barrio and Pistol de Rubel, 1972; Martino and Sinsch 2002; Rosset et al., 2006). In Argentina, where tetraploid populations are widely distributed (Martino and Sin-

1 - Ecología, Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional N° 36 – km 601, (X5804BYA) Río Cuarto, Argentina
2 - CONICET
3 - Department of Biology, Zoology Group, University of Koblenz-Landau, Koblenz, Germany
*Corresponding author;
e-mail: pablogrenat@gmail.com;
pgrenat@exa.unrc.edu.ar

 DOI:10.1163/15685381-00003141

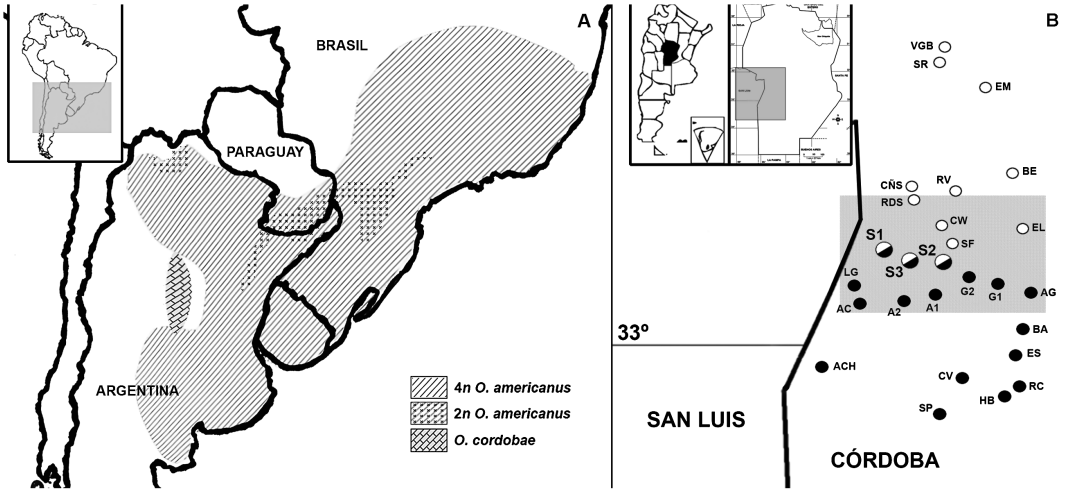

Figure 1. Geographic location of the known localities for *Odontophrynus cordobae* (white circles) and *O. americanus* (black circles) from south-central Córdoba according to Martino and Sinsch (2002) and Grenat et al. (2009, 2013). *O. cordobae*: VGB, Villa General Belgrano; SR, Santa Rosa de Calamuchita; EM, Embalse; BE, Berrotarán; EL, Elena; CÑS, Cañada del Sauce; RDS, Río de los Sauces; RV, Rodeo Viejo; CW, Camino W; SF, A° San Francisco. *O. americanus*: SP, Sampacho; HB, Holmberg; ACH, Achiras; CV, Camino Cuatro Vientos; RC, Río Cuarto; ES, Espinillo; BA, Baigorria; AG, Alcira Gigena; G1, Camino Gigena 1; G2, Camino Gigena 2; A1, Camino Alpa 1; A2, Camino Alpa 2; AC, Alpa Corral; LG, Las Guindas. Syntopic sites between the two species reported in this study are S1, S2 and S3 (black and white circles).

sch, 2002; Rosset et al., 2006; Grenat, Salas and Martino, 2012), diploid populations of *O. americanus* were reported only in three geographically distant points: northeastern, northwestern and central Argentina (fig. 1A; Beçak, Beçak and Vizotto, 1970; Barrio and Pistol de Rubel, 1972; Bogart and Wasserman, 1972; Martino and Sinsch, 2002; Rosset et al., 2006). While the taxonomic status of most diploid populations remains unresolved, diploid populations of central Argentina were revised by Martino and Sinsch (2002) and using morphological, ethological and molecular characters were finally described as a new species, *Odontophrynus cordobae* Martino and Sinsch, 2002. In the south-central part of Córdoba province, tetraploid *O. americanus* and diploid *O. cordobae* occur as a mosaic of populations which coexist in syntopy across the distribution boundaries of both species, representing so far the only known contact area between these two species (fig. 1B; Martino and Sinsch, 2002; Grenat et al., 2009; Grenat, Valetti and Martino, 2013).

Because these species are morphologically indistinguishable, numerous populations along the distribution range of *O. americanus* and *O. cordobae* have been cytogenetically studied and most show a conservative karyotype with small variations in the morphology of some groups of chromosomes, specifically in *O. cordobae* (Beçak, Beçak and Rabello, 1966; Barrio and Pistol de Rubel, 1972; Ruiz, 1980; Schmid et al., 1985; Roset et al., 2006; Salas and Martino, 2007). The location of secondary constrictions varies between diploid and tetraploid karyotypes being found mainly in chromosomes of groups four and 11, respectively (Roset et al., 2006). To date, only a single case of natural triploidy potentially arising by autopolyploidy in a diploid population of *O. americanus* from northeastern Argentina was reported (Rosset et al., 2006).

Most populations inhabiting the contact area between *O. cordobae* and *O. americanus* were visited on several occasions and new cytogenetic and erythrometric analyzes were performed (Barale et al., 1981; Martino and Sinsch, 2002; Grenat, Salas and Martino, 2009, 2012; Otero et al., 2013). Here, we report the first evidence for the presence of many natural triploid

individuals in two localities which might have originated from either autopolyploidy from *O. cordobae* or hybridization with *O. americanus* in central Argentina. At both localities, triploid specimens occur in syntopy with diploid *O. cordobae* and tetraploid *O. americanus*. Furthermore, because these species and the novel reported triploids are morphologically cryptic, we developed a method based on erythrocyte nuclear size for the distinction of ploidy levels (Grenat, Salas and Martino, 2009; Otero et al., 2013) that does not require sacrificing or karyotyping individuals.

Materials and methods

Study area and sampling

Odontophrynus cordobae and *O. americanus* breed during austral spring-summer months (September-March) and daily reproductive activity takes place mainly between 2000 and 0500 hours. Previous samplings covering an area of about 45.000 km^2 by our research group yielded 30 populations of *O. cordobae* and *O. americanus* across south central Córdoba, Argentina (fig. 1B; Martino and Sinsch, 2002, 2009; Grenat, Salas and Martino, 2012; Grenat, Valetti and Martino, 2013). Particularly, the species distribution boundaries, and consequently, the potential contact area between these species were identified (fig. 1B, gray rectangle: NW corner coordinates: 32°31′S, 64°62′W; SE corner coordinates: 32°46′S, 64°16′W). This area is mainly characterized by an agricultural landscape, which is fragmented by rural roads and vegetated deep gullies with a typical dendritic drainage net. To the west, the study area is limited by the piedmont of the southern end of Sierra de Comechingones. Here, *O. americanus* and *O. cordobae* are distributed as a mosaic of populations and coexist in some breeding sites (Grenat, Salas and Martino, 2009; Grenat, Valetti and Martino, 2013). Between 2006 and 2015, we identified three syntopic breeding sites distanced about 8-10 km apart and collected 101 individuals (S1, $n = 22$; S2, $n = 32$; S3, $n = 47$) that were analyzed for ploidy level. Two sites correspond to semi-permanent artificial ponds related to pig farming and the remaining site was located in a flood depression of an agricultural field.

Karyotyping

Ploidy of individuals was analyzed by means of cytogenetics and erythrometry (Grenat, Salas and Martino, 2009; Grenat et al., 2009; Otero et al., 2013). We conducted cytogenetic analyzes on 49 of 101 syntopic specimens. In some cases, karyotyping did not yield satisfactory metaphase preparations; therefore, successful spreads were obtained from 42 individuals (S1: $n = 7$; S2: $n = 27$; S3: $n = 8$). Each individual was intraperitoneally injected with 0.2 ml (for each 10 g of weight) of a 0.03% solution of colchicine 8-12 hr before being euthanized in tricaine methanesulfonate (MS-222). The chromosome preparations were obtained from intestinal epithelium macerated following Schmid (1978). Staining was done with Giemsa 10% diluted in buffer-phosphate (pH 6.8) for 10 minutes. Metaphases were examined using Axiophot Microscope (Carl Zeiss) at magnification of 1000× with Canon Powershot G6 Digital Camera and ZoomBrowser EX. Ploidy of individuals was determined by counting metaphase chromosomes. The karyotypes of triploid individuals were arranged using the program Adobe® Photoshop™ CS2.

Erythrometric analysis and estimation of boundaries for ploidy identification

Blood samples were obtained for 101 individuals by angularis vein puncture immediately after being collected (Nöller, 1959; Martino and Sinsch, 2002) and smears of fresh blood were air-dried. Because juveniles could have significantly lower erythrocyte area values, we analyzed only adult animals following the recommendation of Grenat et al. (2009). We assumed that all individuals are adult because the males were captured emitting their advertisement call and the females were found in amplexus. Individuals that were not used for cytogenetic analysis were immediately returned to their respective collection sites.

Photographs of blood cells were obtained by using an Axiophot Microscope (Carl Zeiss) at magnification of 1000× with a Canon Powershot G6 Digital Camera and ZoomBrowser EX, and saved in TIFF files. We measured length (L) and width (W) of 40 randomly chosen erythrocytes and their respective nuclei for all individuals (Grenat et al., 2009) using ImageJ software. Cell and nuclear area were calculated assuming an ellipsoid shape ($L \times W \times \pi/4$). Differences in cell measurements among ploidy levels were tested using ANOVA.

Following Grenat, Salas and Martino (2009) and Otero et al. (2013), we used probability distributions to calculate the boundary value of erythrocyte area and the probability at which, under or above that value, an individual belongs to one or another ploidy level. Because Otero et al. (2013) demonstrated greater accuracy using nuclear areas rather than cell areas to classify *Odontophrynus* individuals within a given ploidy level, we used only nuclear erythrocyte areas in this analysis. To estimate the limit values we used previously karyotyped individuals, in which the ploidy was confirmed by means of chromosome count. In addition, we tested the effectiveness of the obtained ranges for diploid and tetraploid cells using the model proposed by Otero et al. (2013) based on erythrocyte nuclear size of karyotyped allopatric individuals of *O. cordobae* and *O. americanus*. No overlap was found between diploid, triploid and tetraploid mean erythrocyte nuclear areas. On the basis of these results, we classified the remaining syntopic individuals.

Furthermore, we corroborated the classification obtained using probability distributions by performing a discriminant function analysis based on the four cell and nuclear measurements (length and width) of exclusively karyotyped individuals. We used the classification functions obtained to

evaluate the relative probabilities of the remaining individuals of belonging to a particular ploidy level.

Snout-vent length (SVL) of individual frogs was taken in vivo with a digital caliper Mahr 16ES, and its relation with cell size was analyzed. All calculations were performed using the program package STATGRAPHICS for Windows, version 5.0.

Results

Cytogenetic analysis

The cytogenetic analysis detected three ploidy levels in the sample: $2n = 22$, $3n = 33$ and $4n = 44$ chromosomes (fig. 2). Triploid individuals were discovered at two sites (S2 and S3), coexisting with both diploids and tetraploids (S2: $2n = 10$, $3n = 3$, $4n = 14$; S3: $2n = 3$, $3n = 4$, $4n = 1$), while at the remaining site only two ploidy levels were found (S1: $2n = 2$, $4n = 5$).

The studied karyotypes of triploid individuals showed the same basic structure as the diploid *Odontophrynus cordobae* and tetraploid *O. americanus*, with three major groups of chromosomes being distinguished: three large (triplets 1-3), four medium (4-7), and four small (8-11) sets of elements (fig. 3). Six triplets of metacentric and five triplets of submetacentric chromosomes were observed: Groups 1-2, 5, 7 and 10-11 are metacentric; groups 3-4, 6 and 8-9 are submetacentric (fig. 3). Secondary constrictions were not observed in any of the triploid karyotypes.

Ploidy level identification by mean erythrocyte nuclear area

Mean values and ranges of erythrocyte nuclear areas of karyotyped individuals included in the estimation of boundary values were: *O. cordobae* ($n = 15$): 20.73 μm^2 (15.62-22.54 μm^2); triploid specimens ($n = 7$): 25.34 μm^2 (24.07-26.66 μm^2); *O. americanus* ($n = 20$): 32.69 μm^2 (29.64-38.21 μm^2). Significant differences in nuclear areas among ploidy levels were found (ANOVA, $F_{2,39} = 165.44$; $p < 0.0001$).

The limit of the model obtained to separate diploid and triploid individuals using nuclear area was 23.62 μm^2 (probability to be assigned to a respective ploidy level = 94.78%) while the boundary to distinguish between triploid and tetraploid individuals was 27.67 μm^2 (98.62%;

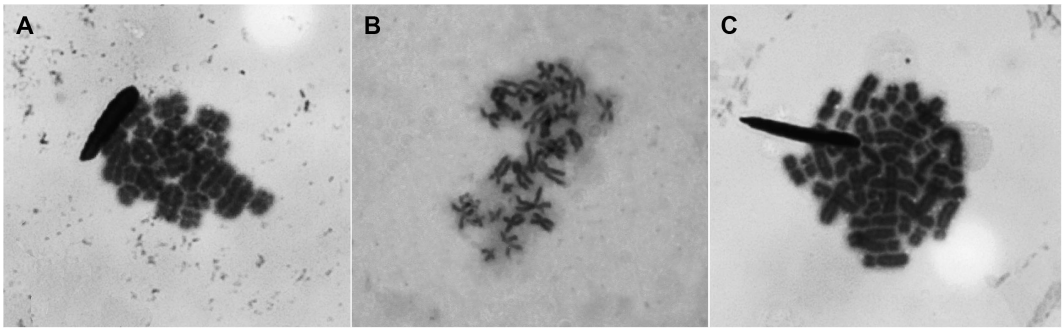

Figure 2. Metaphase of (A) a diploid *Odontophrynus cordobae* from S2; (B) a triploid individual from S3; and (C) a tetraploid *O. americanus* from S2.

Figure 3. Karyotypes of triploid individuals from the sites S2 and S3, Córdoba, Argentina.

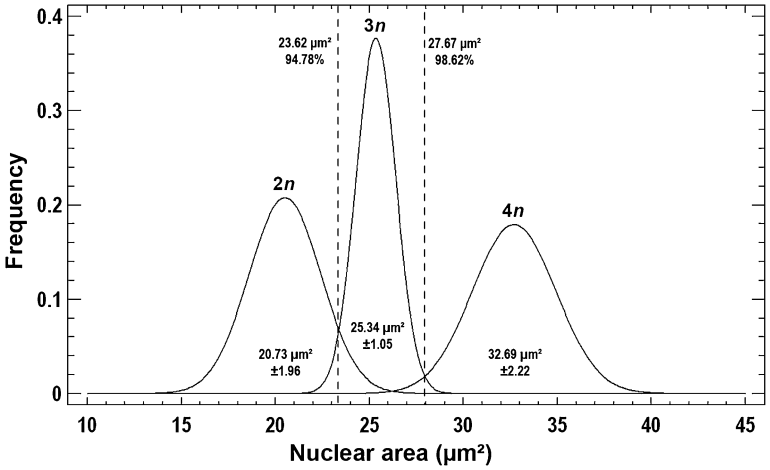

Figure 4. Distribution curves of nuclear areas (μm^2) from diploid, triploid and tetraploid individuals. Mean and standard deviation are shown within each curve. Dotted lines represent the values limits to distinguish between each ploidy level.

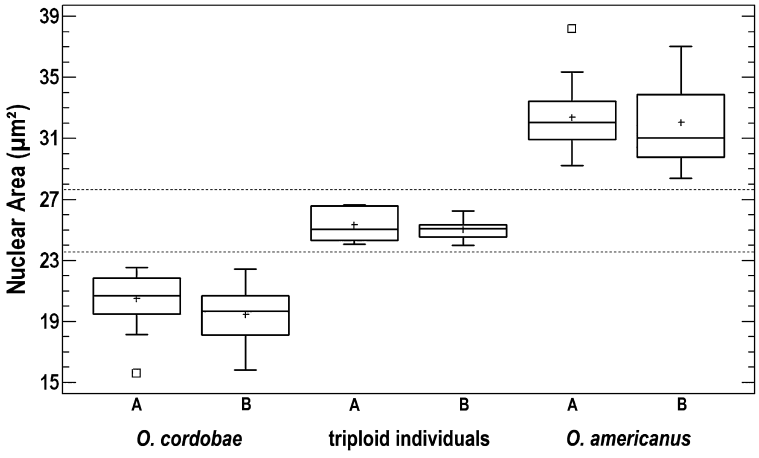

Figure 5. Comparison of nuclear areas of karyotyped (A) and non-karyotyped individuals (B) of *Odontophrynus cordobae*, triploid individuals and *O. americanus*. The dotted line represents the limit nuclear area to separate ploidy levels. Upper and lower ends of boxes represent 75th and 25th percentiles. Whiskers represent the minimum and the maximum values. The line within each box shows the locations of the sample median and the plus sign indicates the location of the sample mean. Outliers are shown as empty squares.

fig. 4). Non-karyotyped individuals were classified based on these limit values. Mean values and ranges for non-karyotyped individuals were: diploid *O. cordobae* ($n = 24$): 19.48 μm^2 (15.85-22.43 μm^2); triploid specimens ($n = 16$): 25.05 μm^2 (24-26.25 μm^2); tetraploid *O. americanus* ($n = 19$): 32.08 μm^2 (28.39-37.05 μm^2). We found no significant differences within each ploidy level in nuclear area between individuals included in the analysis of limit values and the remaining individuals: *O. cordobae* (ANOVA, $F_{1,37} = 2.65$; $P = 0.119$); triploid specimens (ANOVA, $F_{1,21} = 0.72$; $P = 0.4054$); *O. americanus* (ANOVA, $F_{1,37} = 0.14$; $P = 0.7065$) (fig. 4). In all cases, both maximum range values of lower ploidy individuals (*O. cordobae*) and minimum nuclear areas of higher ploidy individuals (*O. americanus*) did not exceed the limit calculated for a correct identification of ploidy levels (fig. 5).

Discriminant function analysis (DFA) based on karyotyped individuals ($n = 42$) yielded two

discriminant functions. Function 1 was highly significant (variance explained = 95.5%; Wilks' lambda = 0.0634504; canonical correlation = 0.9525; $p < 0.0001$) and was strongly related with nuclear width (standardized coefficient = 0.5905) and length (standardized coefficient = 0.5323). The obtained functions correctly classified 100% of individuals to the respective ploidy level. Then, we used these functions to classify non-karyotyped individuals ($n = 59$). All individuals (100%) were reclassified within the same group to which they were previously assigned using the limit values of nuclear areas (fig. 6).

Mean erythrocyte cell and nuclear area for each ploidy level and sampling site, considering all individuals, are shown in table 1. Cellular area ($F_{2,76} = 63.69$), length ($F_{2,76} = 48.28$) and width ($F_{2,76} = 49.26$) also showed highly significant differences among ploidy levels (ANOVA, $P < 0.0001$). Cell area was not correlated with SVL (*O. cordobae*: $r =$

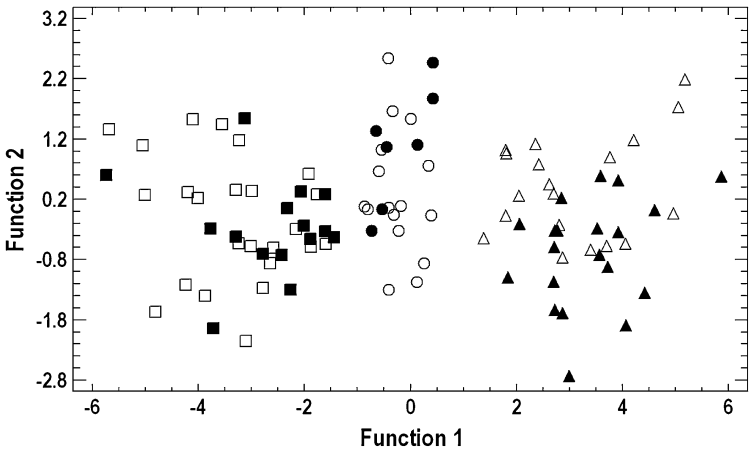

Figure 6. Discriminant function analysis based on erythrocyte measurements differentiating diploid (square), triploid (circles) and tetraploid (triangles) karyotyped (fill) and non-karyotyped (empty) individuals.

Table 1. Mean and standard deviation of body length (SVL) and cell and nuclear measurements (40 erythrocytes per individual) from all *Odontophrynus* individuals of different ploidy.

Sampling site	*n*	Ploidy	SVL (mm)	Cell			Nucleus		
				Length (μm)	Width (μm)	Area (μm^2)	Length (μm)	Width (μm)	Area (μm^2)
S1	5	2*n*	50.26 (±3.22)	17.30 (±0.55)	12.43 (±0.22)	169.02 (±7.29)	6.20 (±0.41)	4.06 (±0.15)	19.78 (±1.83)
	17	4*n*	45.31 (±2.79)	20.28 (±0.87)	13.90 (±0.80)	221.55 (±17.11)	8.06 (±0.34)	5.12 (±0.28)	32.39 (±2.28)
S2	13	2*n*	47.13 (±2.42)	17.06 (±1.26)	11.64 (±0.93)	156.84 (±22.36)	6.60 (±0.47)	3.90 (±0.32)	20.19 (±2.29)
	4	3*n*	48.54 (±2.22)	16.85 (±0.96)	11.70 (±0.70)	154.77 (±9.37)	6.80 (±0.26)	4.67 (±0.13)	24.91 (±0.40)
	15	4*n*	46.87 (±2.99)	20.27 (±0.78)	14.82 (±0.65)	236.21 (±15.64)	8.17 (±0.36)	5.09 (±0.23)	32.69 (±2.22)
S3	21	2*n*	46.75 (±2.95)	16.91 (±1.22)	11.41 (±0.98)	152.28 (±22.36)	6.34 (±0.34)	3.95 (±0.22)	19.71 (±1.83)
	19	3*n*	47.69 (±2.85)	18.04 (±0.92)	12.38 (±0.68)	175.67 (±14.89)	7.17 (±0.37)	4.48 (±0.26)	25.19 (±0.82)
	7	4*n*	46.41 (±1.91)	19.00 (±0.44)	12.65 (±0.63)	189.00 (±10.81)	8.01 (±0.39)	4.90 (±0.34)	30.85 (±2.86)

-0.009; $P < 0.9554$/triploid specimens: $r = 0.024$; $P < 0.9118$/*O. americanus*: $r = -0.128$; $P < 0.4355$).

After the ploidy classification and considering all individuals collected at the three sites, our results show the presence of triploids at sites S2 and S3. All triploid individuals were males except two females collected at site S3. The percentage of individuals for each ploidy level was at site S1: $2n = 22.7\%$ ($n = 5$), $4n = 77.3\%$ (17); S2: $2n = 40.6\%$ (13), $3n = 12.5\%$ (4), $4n = 46.9\%$ (15), and at site S3: $2n = 44.7\%$ (21), $3n = 40.4\%$ (19), $4n = 14.9\%$ (7).

Discussion

Cytogenetic and erythrometric analyses on individuals from contact zones between diploid *O. cordobae* and tetraploid *O. americanus* revealed three ploidy levels. Importantly, at two syntopic sites we discovered a number of triploid individuals.

Triploidy is rare in natural populations of anurans because polyploidization events are generated mainly by two ways, from one species (autopolyploidy) or from interspecific hybridization events (allopolyploidy; Bogart and Bi, 2013). An autopolyploid individual may result from the fertilization of an unreduced diploid gamete or alternatively, from the fertilization of a haploid egg by more than one sperm (Otto and Whitton, 2000; Choleva and Janko, 2013). In anurans, spontaneous autotriploids have already been reported for diploid populations of genera *Lithobates* (Richards and Nace, 1977; Wiley and Braswell, 1986), *Leiopelma* (Green, Kezer and Nussbaum, 1984), *Eupsophus* (Formas, 1994), *Bufotes* (Odierna et al., 2004; Borkin et al., 2007; Fakharzadeh et al., 2015), *Holoaden* (Campos et al., 2012), *Strauchbufo* (Litvinchuk et al., 2012), *Xenopus* (Schmid, Evans and Bogart, 2015), *Pelophylax* (Litvinchuk, Skorinov and Rosanov, 2015), and *Bombina* (Litvinchuk and Rosanov, 2016). However, in all cases only single or very few triploid specimens in diploid populations were found, suggesting that triploidy in these species is an unusual condition. In the genus *Odontophrynus*, Rosset et al. (2006) identified by means of cytogenetics a naturally triploid individual occurring in a diploid population of *O. americanus* from Misiones province, Argentina. They assumed that it could have arisen by autotriploidy but add that the existence of sympatric diploid and tetraploid populations in the area does not allow discarding a potential hybrid origin. However, new records on triploid specimens in this area have not been reported.

Triploid origin by hybridization is less frequent. Natural allotriploid populations are only known from genera *Pelophylax* and *Bufotes*. The *Pelophylax esculentus* complex consists of two parental species, *P. lessonae* (LL genome) and *P. ridibundus* (RR), and their natural hybridogenetic form, *P. esculentus* (LR). Hybridogenetic lineages include diploid (RL) as well as triploid hybrids, with a double dosage of *P. ridibundus* or *P. lessonae* genome (RRL, RLL), which often form all-hybrid populations (*see* Graf and Polls Pelaz, 1989; Christiansen, 2009). In the second case, the *Bufotes viridis* complex represents the only described anuran system with three bisexually reproducing ploidy levels ($2n$, $3n$, $4n$), all of which occur in Central Asia (Stöck et al., 2002, 2005). In this species complex, triploid *B. baturae*, potentially originated from hybridization between diploids *B. shaartusiensis* and *B. latastii* (Betto-Colliard et al., 2015; Ficetola and Stöck, 2016), consists exclusively of all-triploid populations which reproduces bisexually by a mechanism called "pre-equalizing hybrid meiosis" (Stöck et al., 2002).

Nevertheless, allotriploids originating from natural hybridization between close related species with different ploidy levels are unusual and include a low number of hybrids found (*Bufotes*, Stöck et al., 2010; *Phyllomedusa*, Haddad, Pombal and Batistic, 1994; *Hyla*, Gerhardt et al., 1994). In the genus *Odontophrynus*, natural hybridization between tetraploid *O. americanus* and diploid *O. cultripes* from Brazil has been reported (Ruiz, Bonaldo and Beçak, 1980).

Furthermore, artificial triploid hybrids were obtained by the mating of 4*n* *O. americanus* females with 2*n* males of *O. americanus*, *O. cultripes* and *O. carvalhoi* (Beçak, Beçak and de Langlada, 1968; Beçak and Beçak, 1970). These examples show that reproductive isolation in the genus could be based on premating barriers and does not result from a reduced viability of hybrids.

In this context, the current discovery of natural triploids in the genus *Odontophrynus* represents an extraordinary finding for several reasons. First, the occurrence of triploidy and tetraploidy exemplifies the high level of genomic plasticity in this species-group. Second, the high number of triploid individuals found demonstrates their high viability. Furthermore, the age-size relationship in *O. cordobae* and *O. americanus* (Martino and Sinsch, 2002) suggests that all triploid individuals (SVL range = 43-52 mm) were adults of at least two or three years of age. This is an important point because polyploids with odd numbers of chromosome sets often exhibit low viability and fertility (Husband, 2004; Choleva and Janko, 2013). Advertising triploid males in the two sites and triploid females in amplexus at site S3 were observed but we have currently no evidence of 3*n* individuals' fertility.

The occurrence of triploids at more than one site demonstrates that the process leading to their origin is not an isolated and rare event. Furthermore, we cannot rule out the presence of triploids in the site S1 due to the low number of individuals collected and analyzed by cytogenetics. For site S3 the number of karyotyped individuals is similarly low, but even so the percentage of triploid individuals found was high. In both cases, the erythrocyte size analysis showed similar results for the whole sample, in which no triploids were identified at the site S1 and a high percentage of triploids were found at the site S3. Erythrometry, contrary to cytogenetics, is a method that does not require sacrificing individuals and has proved to be a powerful tool with a high level of accuracy for resolve the problem of distinguishing living individuals with different ploidy levels (George and Lennartz, 1980; Grenat et al., 2009; Otero et al., 2013; our study).

Presently, we can only speculate about the triploid origin and the interaction among syntopic individuals with different ploidy level. The occurrence of triploid individuals exclusively in contact zones, but not in pure diploid populations of *O. cordobae* (Grenat et al., 2009; Grenat, Salas and Martino, 2009, 2012; Grenat and Martino, 2013; Grenat, Valetti and Martino, 2013) would be consistent with the hypothesis of a hybrid origin. The patchy distribution of diploid and tetraploid populations across the study area and the availability of common potential breeding sites may increase the possibility of hybridization. Although the location of secondary constrictions (SC) in different chromosome groups (4 and 11) could have evidenced a possible hybrid origin, SCs were not observed in any of the triploid karyotypes. Consequently, further studies using silver-staining procedure (Ruiz, Soma and Beçak, 1981) could reveal the localization of nucleolar organizers (NOR) associated to secondary constrictions on triploid karyotypes.

Alternatively, triploids in mixed populations with diploid and tetraploid species could act as intermediaries in the establishment of auto- or allotetraploids by providing an additional pathway of polyploid formation (Ramsey and Schemske, 1998; Husband, 2004; Barker et al., 2016). In this process, tetraploids may be produced in two steps, via a "triploid bridge", a process which involves the production of unreduced gametes in two successive generations (Ramsey and Schemske, 1998; Barker et al., 2016). Nevertheless, this process is mainly known in plants with very few cases detected in animals. An example in Amphibia could be the allotetraploid *Bufo pewzowi* for which a potential rise through an intermediate allotriploid state cannot be discarded (Stöck et al., 2010).

In summary, our results do not allow for a distinction between an auto- and allopolyploid origin of *Odontophrynus* triploids. Further molecular (allozymes and DNA-based markers) and cytogenetic analyses are needed to provide a reliable hypothesis on the origin of triploids. Furthermore, the finding of naturally occurring triploids only in mixed populations of *Odontophrynus* raises many questions about interactions among different ploidy levels, reproductive strategies, fitness, dispersal and ecological features of triploids that should be considered at different spatial and temporal scales.

Acknowledgements. We thank to the anonymous reviewers and editors for their valuable comments and corrections. This article is dedicated to the memory of Juan Carlos Boarini for his invaluable help facilitating our field work. Financial support was provided by SECyT-UNRC (Grant PPI 18/C416) and FONCyT (Grant PICT 0932-2012 and PICT 2533-2014). P.R.G., F.P. M.O. and M.B. thank CONICET-Argentina (National Scientific and Technical Research Council) for postgraduate fellowship granted. Our study was authorized by Environmental Secretary of Córdoba Government (A01/2013).

References

Barale, G., Maria, G.A., di Tada, I.E., Lisanti, J.A. (1981): Presencia de *Odontophrynus americanus* (Anura, Leptodactylidae) tetraploide en la provincia de Córdoba. Revista UNRC **1**: 121-125.

Barker, M.S., Arrigo, N., Baniaga, A.E., Li, Z., Levin, D.A. (2015): On the relative abundance of autopolyploids and allopolyploids. New Phytologist **210**: 391-398.

Barrio, A., Pistol de Rubel, D. (1972): Encuesta cariotípica de poblaciones argentino-uruguayas de *Odontophrynus americanus*; (Anura, Leptodactylidae) relacionada con otros rasgos taxonómicos. Physis **31**: 281-291.

Beçak, M.L., Beçak, W. (1970): Further studies on polyploid amphibians (Ceratophrydidae). III. Meiotic aspects of the interspecific triploid hybrid: *Odontophrynus cultripes* ($2n = 22$) $\times$ *O. americanus* ($4n = 44$). Chromosoma **31**: 377-385.

Beçak, M.L., Beçak, W., Rabello, M.N. (1966): Cytological evidence of constant tetraploidy in the bisexual South American frog *Odontophrynus americanus*. Chromosoma **19**: 188-193.

Beçak, M.L., Beçak, W., Vizotto, L.D. (1970): A diploid population of the polyploid amphibian *Odontophrynus americanus* and the artificial intraespecific triploid hybrid. Experientia **26**: 545-546.

Beçak, W., Beçak, M.L., de Langlada, F.G. (1968): Artificial triploid hybrids by interspecific mating of *Odontophrynus* (Amphibia, Anura). Experientia **24**: 1162-1164.

Betto-Colliard, C., Sermier, R., Litvinchuk, S., Perrin, N., Stöck, M. (2015): Origin and genome evolution of polyploid green toads in Central Asia: evidence from microsatellite markers. Heredity **114**: 300-308.

Bogart, J.P. (1980): Evolutionary implications of polyploidy in amphibians and reptiles. In: Polyploidy: Biological Relevance, p. 341-378. Lewis, W.H., Ed., Plenum Press, New York.

Bogart, J.P., Wasserman, A.O. (1972): Diploid-polyploid species pairs: a possible clue to evolution by polyploidization in anuran amphibians. Cytogenetics **11**: 7-24.

Bogart, J.P., Bi, K. (2013): Genetic and genomic interactions of animals with different ploidy levels. Cytogenet. Genome Res. **140**: 117-136.

Borkin, L.J., Shabanov, D.A., Brandler, O.V., Kukushkin, O.V., Litvinchuk, S.N., Mazepa, G.A., Rozanov, J.M. (2007): A case of natural triploidy in European diploid green toad (*Bufo viridis*), with some distributional records of diploid and tetraploid toads. Russ. J. Herpetol. **14**: 121-132.

Campos, J.R.C., Martins, I.A., Haddad, C.F.B., Kasahara, S. (2012): The karyotype of *Holoaden luederwaldti* (Anura, strabomantidae), with report of natural triploidy. Folia Biol. **58**: 144-150.

Choleva, L., Janko, K. (2013): Rise and persistence of animal polyploidy: evolutionary constraints and potential. Cytogenetic and genome research **140**: 151-170.

Christiansen, D.G. (2009): Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). BMC Evol. Biol. **9**: 135.

Evans, B.J., Pyron, R.A., Wiens, J.J. (2012): Polyploidization and sex chromosome evolution in amphibians. In: Polyploidy and Genome Evolution, p. 385-410. Soltis, P.S., Soltis, D.E., Eds, Springer, Berlin.

Fakharzadeh, F., Darvish, J., Kami, H.G., Ghassemzadeh, F., Rastegar-Pouyani, E., Stöck, M. (2015): Discovery of triploidy in Palearctic green toads (Anura: Bufonidae) from Iran with indications for a reproductive system involving diploids and triploids. Zool. Anz. **255**: 25-31.

Ficetola, G.F., Stöck, M. (2016): Do hybrid-origin polyploid amphibians occupy transgressive or intermediate ecological niches compared to their diploid ancestors? J. Biogeogr. **43**: 703-715.

Formas, J.R. (1994): A triploid individual of the Chilean leptodactylid frog *Eupsophus vertebralis*. J. Herpetol. **28**: 394-395.

George, S.A., Lennartz, M.R. (1980): Methods for determining ploidy in the amphibians: nucleolar number and erythrocyte size. Experientia **36**: 687-688.

Gerhardt, H.C., Ptacek, M.B., Barnett, L., Torke, K.G. (1994): Hybridization in the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor*. Copeia **1994**: 51-59.

Graf, J.D., Polls Pelaz, M. (1989): Evolutionary genetics of the *Rana esculenta* complex. In: Evolution and Ecology of Unisexual Vertebrates, 466, p. 289-302. Dawley, R.M., Bogart, J.P., Eds, New York State Mus. Bull., New York.

Green, D.M., Kezer, J., Nussbaum, R.A. (1984): Triploidy in Hochstetter's frog, *Leiopelma hochstetteri*, from New Zealand. New Zealand J. Zool. **11**: 457-460.

Gregory, T.R., Mable, B.K. (2005): Polyploidy in animals. In: The Evolution of the Genome, p. 427-517. Gregory, T.R., Ed., Elsevier, New York.

Grenat, P.R., Bionda, C., Salas, N.E., Martino, A.L. (2009): Variation in erythrocyte size between juveniles and adults of *Odontophrynus americanus*. Amphibia-Reptilia **30**: 141-145.

Grenat, P.R., Salas, N.E., Martino, A.L. (2009): Erythrocyte size as diagnostic character for the identification of live cryptic *Odontophrynus americanus* and *O. cordobae* (Anura: Cycloramphidae). Zootaxa **2049**: 67-68.

Grenat, P.R., Salas, N.E., Martino, A.L. (2012): Estudio de la variación morfométrica intra e interespecífica en poblaciones de *Odontophrynus* (Anura: Cycloramphidae) del área central de Argentina. Rev. Biol. Trop. **60**: 1589-1601.

Grenat, P.R., Valetti, J.A., Martino, A.L. (2013): Intraspecific variation in advertisement call of *Odontophrynus cordobae* (Anura, Cycloramphidae): a multilevel and multifactor analysis. Amphibia-Reptilia **34**: 471-482.

Haddad, C.F.B., Pombal, J.P., Batistic, R.F. (1994): Natural hybridization between diploid and tetraploid species of leaf-frogs, genus *Phyllomedusa* (Amphibia). J. Herpetol. **28**: 425-430.

Husband, B.C. (2004): The role of triploid hybrids in the evolutionary dynamics of mixed ploidy populations. Biological Journal of the Linnean Society **82**: 537-546.

Litvinchuk, S.N., Rosanov, J.M., Schepina, N.A., Kazakov, V.I., Skorinov, D.V., Borkin, L.J. (2012): The first case of natural triploidy in the Mongolian toad. Russ. J. Herpetol. **19**: 333-336.

Litvinchuk, S.N., Skorinov, D.V., Rosanov, J.M. (2015): Natural spontaneous autotriploidy in the genus *Pelophylax* (Anura: Ranidae). Russ. J. Herpetol. **22**: 318-320.

Litvinchuk, S.N., Rosanov, J.M. (2016): The first case of natural spontaneous triploidy in the family Bombinatoridae. Amphibia-Reptilia **37**: 243-245.

Mable, B.K., Alexandrou, M.A., Taylor, M.I. (2011): Genome duplication in amphibians and fish: an extended synthesis. J. Zool. **284**: 151-182.

Martino, A.L., Sinsch, U. (2002): Speciation by polyploidy in *Odontophrynus americanus*. J. Zool. **257**: 67-81.

Nöller, H.G. (1959): Eine einfache Technik der Blutentnahme beim Frosch. Pflügers Arch. Physiol. **269**: 98-100.

Odierna, G., Aprea, G., Capriglione, T., Castellano, S., Balletto, E. (2004): Evidence for chromosome and Pst I satellite DNA family evolutionary stasis in the *Bufo viridis* group (Amphibia, Anura). Chromosome Res. **12**: 671-681.

Otero, M.A., Grenat, P.R., Valetti, J.A., Salas, N.E., Martino, A.L. (2013): Erythrocyte nuclear size as a better diagnostic character than cell size in the identification of live cryptic polyploid species. Zootaxa **3694**: 262-270.

Otto, S., Whitton, J. (2000): Polyploid incidence and evolution. Ann. Rev. Genet. **34**: 401-437.

Petit, C., Bretagnolle, F., Felber, F. (1999): Evolutionary consequences of diploid-tetraploid hybrid zones in wild species. Trends Ecol. Evol. **14**: 305-311.

Ramsey, J., Schemske, D.W. (1998): Pathways, mechanisms, and rates of polyploid formation in flowering plants. The Annual Review of Ecology, Evolution, and Systematics **29**: 467-501.

Richards, C., Nace, G.W. (1977): The occurrence of diploid ova in *Rana pipiens*. J. Hered. **68**: 307-312.

Rosset, S., Baldo, D., Lanzone, C., Basso, N.G. (2006): Review of the geographic distribution of diploid and tetraploid populations of the *Odontophrynus americanus* species complex (Anura: Leptodactylidae). J. Herpetol. **40**: 465-477.

Ruiz, I.R.G., Bonaldo, M.F., Beçak, W. (1980): In situ localization of ribosomal genes in a natural triploid of *Odontophrynus*. J. Hered. **71**: 55-57.

Ruiz, I.R.G., Soma, M., Beçak, W. (1981): Nucleolar organizer regions and constitutive heterochromatin in polyploid species of the genus *Odontophrynus* (Amphibia, Anura). Cytogenet. Cell Genet. **29**: 84-98.

Salas, N.E., Martino, A.L. (2007): Cariotipo de *Odontophrynus cordobae* Martino & Sinsch, 2002 (Anura, Leptodactylidae). Journal of basic and applied genetics **18**: 1-5.

Schmid, M. (1978): Chromosome banding in amphibia. 1. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma **66**: 361-388.

Schmid, M., Haaf, T., Schempp, W. (1985): Chromosome banding in Amphibia. IX. The polyploid karyotypes of *Odontophrynus americanus* and *Ceratophrys ornata* (Anura, Leptodactylidae). Chromosoma **91**: 172-182.

Schmid, M., Evans, B.J., Bogart, J.P. (2015): Polyploidy in Amphibia. Cytogenet. Genome Res. **145**: 315-330.

Stöck, M., Lamatsch, D.K., Steinlein, C., Epplen, J.T., Grosse, W.R., Hock, R., Klapperstück, T., Lampert, K.P., Scheer, U., Schmid, M., Schartl, M. (2002): A bisexually reproducing all-triploid vertebrate. Nat. Genet. **30**: 325-328.

Stöck, M., Steinlein, C., Lamatsch, D.K., Schartl, M., Schmid, M. (2005): Multiple origins of tetraploid taxa in the Eurasian *Bufo viridis* subgroup. Genetica **124**: 255-272.

Stöck, M., Ustinova, J., Lamatsch, D.K., Schartl, M., Perrin, N., Moritz, C. (2010): A vertebrate reproductive system involving three ploidy levels: hybrid origin of triploids in a contact zone of diploid and tetraploid Palearctic green toads (*Bufo viridis* subgroup). Evolution **64**: 944-959.

Willey, J.E., Brasswell, A.L. (1986): A triploid male *Rana palustris*. Copeia **1986**: 531-533.

Submitted: April 12, 2017. Final revision received: October 17, 2017. Accepted: October 31, 2017.
Associate Editor: Judit Vörös.