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Erythrocyte nuclear size as a better diagnostic character than cell size in the identification of live cryptic polyploid species

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Abstract

It is well documented in anurans the cryptic condition of many species complexes involving polyploids. In these complexes the character that clearly differentiates them is the number of chromosome complements. The blood cells of amphibians conserve their nucleus, and so the erythrocyte size is correlated with the DNA content. We analyzed two cryptic-polyploid complexes occurring in the center of Argentina: *Odontophrynus cordobae* (2n)/*O. americanus* (4n) and *Pleurodema kriegi* (4n)/*P. cordobae* (8n). Our aim was evaluate the efficiency in the utilization of nuclear area with respect to cellular area of the erythrocytes to define the limits values for the identification of cryptic-polyploid species. We studied 110 individuals of *Pleurodema* and 116 individuals of *Odontoprhynus*. For each individual, we measured the cellular and nuclear length (L) and width (A) of 40 erythrocytes (Area= L*A*π/4) and boundary values were calculated using distribution curves. In both complexes studied, the erythrometric parameters showed significant differences between related species. Moreover, in both complexes the nuclear area was more efficient for identifying the species (*Pleurodema*: 34.39 μm² (probability=99.96%) and *Odontophrynus*: 24.02 μm² (99.075%)) than the cell area (*Pleurodema*: 273.08 μm² (97.55%) y *Odontophrynus*: 197.69 μm² (97.94%)). Greater efficiency found using nuclear area is novel and significant because most studies use only the cell area to differentiate polyploid complexes.

Key words: Erythrometry, Octoploid, Tetraploid, *Pleurodema*, *Odontophrynus*, Polyploidy

Introduction

The occurrence of natural bisexual polyploids in amphibians provides evidence that polyploidization is an extensive mechanism of speciation in several families of anurans (Bogart 1980; King 1990; Tymowska 1991; Otto & Whitton 2000; Stöck *et al*. 2002; Martino & Sinsch 2002; Rosset *et al*. 2006; Valetti *et al*. 2009). It is well documented in anurans the morphological similarity, or cryptic condition, of species within complex involving polyploids (i.e.; Ralin 1968; Bogart & Wasserman 1972; Bogart & Tandy 1976; Mahony & Robinson 1980; Valetti *et al*. 2009). Two or more species are considered 'cryptic' if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable (Bickford *et al*. 2007). Such cryptic species can only be identified by nonmorphological characteristics, such as differences in ecology, behavior, cytogenetics, or biochemistry (Borkin *et al.* 2001). Species delimitations and identification have implications for both estimating species richness and assessing conservation needs (Angulo & Reichle 2008). In cryptic complexes that include species with different ploidy levels, the character that clearly differentiates them is the number of chromosome complements. However, the chromosome studies require the sacrifice of the animal, and a significant processing time in laboratory to know the ploidy level of an individual. Consequently, novel diagnostic characters should be found for a correct, simple and rapid distinction of the species without sacrificing individuals (Grenat *et al*. 2009b). It is well known that the blood cells of amphibians conserve their nucleus, and so the erythrocyte size is correlated with the DNA content (Uzzell 1964; Stöck &

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Grosse 1997; Schröer & Greven 1998). For this reason, the erythrometry is a technique commonly used for the distinction of related species with different ploidy levels (George & Lennartz 1980; Matson 1990; Stöck & Grosse 1997; Schröer & Greven 1998; Stöck *et al.* 1999; Martino & Sinsch 2002; Rosset *et al*. 2006; Grenat *et al*. 2009a, b; Valetti *et al.* 2009). Most of these authors based the erythrocyte size comparisons on the cell area rather than on the nuclear area.

Two pairs of cryptic species including polyploids are present in central Argentina: *Odontophrynus cordobae* (diploid)/*O. americanus* (tetraploid) and *Pleurodema kriegi* (tetraploid)/*P. cordobae* (octoploid). The species within of each complex are exomorphologically indistinguishable and their advertisement calls are very similar. Differences in erythrocyte cell size between diploid and tetraploid *Odontophrynus* have been observed (Rosset *et al*. 2006; Martino & Sinsch 2002; Grenat *et al*. 2009a, b). Martino and Sinsch (2002) defined a range of erythrocyte cell area for *O. cordobae* and *O. americanus* but individuals were not cytogenetically studied. Rosset *et al*. (2006), showed cell areas from only two preserved museum specimens of *O. cordobae*. Grenat *et al*. (2009b) reported boundary values of cell area for the identification between *O. cordobae* and *O. americanus* using cytogenetically studied individuals. On the other side, Valetti *et al*. (2009) found differences in erythrocyte cell size between *P. kriegi* and *P. cordobae* but nuclear measurements were not analyzed. Grenat *et al*. (2009a) analyzed erythrocyte nuclear areas of *O. cordobae* and *O. americanus*, and suggested that this character would be used as diagnostic character between these species when juvenile individuals were involved. However, at present, the power of nuclear area of erythrocyte as diagnostic character between adult individuals of related polyploid species had not been evaluated.

In this paper, we analyzed a larger sample size to evaluate the accuracy of using erythrocyte nuclear size in comparison with cell size for the identification of cryptic polyploid related species. We define boundary values of nuclear area for the differentiation of *Odontophrynus* and *Pleurodema* cryptic species.

Materials and methods

We analyzed a total of 110 individuals of *Pleurodema* (*P. kriegi*: $n = 34$/*P. cordobae*: $n = 76$), from 10 sites of Córdoba, and 116 individuals of *Odontophrynus* (*O. cordobae*: $n = 54$/*O. americanus*: $n = 62$) from 17 localities of Córdoba and San Luis provinces, in central Argentina. Syntopic individuals of *O. cordobae* and *O. americanus* occur in the localities of La Escondida and El Cano (Grenat *et al*. 2009b). We analyzed adult animals following the recommendation of Grenat *et al*. (2009a). We assumed that all individuals are adult because the males were captured emitting their advertisement call and the females were found in amplexus.

A blood sample of each specimen was obtained by angularis vein puncture (Nöller 1959) and blood smears were prepared. The smears were analyzed with Zeiss Axiophot microscope-Axiolab, photographed using a camera Sony™ DXC-950P Power HAD 3CCD Color, and saved in TIFF files. We measured length (L) and Width (W) of 40 randomly chosen erythrocytes and their respective nuclei for each individual (Grenat *et al*. 2009a), from the photographic archives obtained by means of ImageJ software. Erythrocyte area was calculated assuming an ellipsoid shape ($L*W*\pi/4$). Differences in all erythrocyte measurements among the analyzed species were tested using ANOVA. We used probability distributions to calculate the boundary value for separate species following to George and Lennartz (1980) and Grenat *et al*. (2009b). To estimate these boundary values we used previously katyotyped individuals of *Odontophrynus* (*O. cordobae*: $n = 26$/*O. americanus*: $n = 20$), and individuals from two allopatric populations of *Pleurodema* (*P. kriegi*, La Posta: $n = 24$/*P. cordobae*, Tabaquillo: $n = 33$) (Table 1), in which the ploidy was confirmed by means of cytogenetic analyzes (Valetti *et al*. 2009). The remaining individuals were used to test the models obtained for each genus.

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

TABLE 1. Mean and standard deviation of cell and nuclear measurements (40 erythrocytes per individual) from the different populations analyzed of *Pleurodema* and *Odontophrynus*. These individuals were included into the analysis to determine the boundary values for the identification of species.

Site	n	Ploidy	Cell			Nucleus		
			Length (µm)	Width (µm)	Area (µm?)	Length (µm)	Width (µm)	Area (µm?)
Pleurodema								
La Posta	24	4X	21.32 (± 1.07)	13.84 (± 0.70)	233.06 (± 20.31)	7.93 (± 0.34)	4.5 (± 0.19)	28.05 (± 1.90)
Los Tabaquillos	33	8X	26.82 (± 2.00)	16.41 (± 0.98)	346.45 (± 37.3)	10.59 (± 0.62)	5.56 (± 0.33)	46.29 (± 3.57)
Odontophrynus								
Berrotarán	3	2X	17.02 (± 0.76)	11.13 (± 0.43)	148.93 (± 12.38)	6.05 (± 0.28)	4.09 (± 0.16)	19.35 (± 1.26)
Gigena-El Cano S1	3	2X	17.01 (± 1.01)	11.74 (± 0.50)	156.86 (± 5.7)	5.97 (± 0.35)	3.83 (± 0.13)	17.96 (± 1.49)
Río de los Sauces	2	2X	18.18 (± 0.44)	12.63 (± 0.74)	180.53 (± 14.95)	5.98 (± 0.01)	3.99 (± 0.15)	18.72 (± 0.87)
Gigena-	1	2X	15.53	11.6	141.63	6.58	3.85	19.94
A° San Francisco	3	2X	16.89 (± 0.27)	11.55 (± 0.27)	153.36 (± 5.69)	6.35 (± 0.27)	3.37 (± 0.25)	16.80 (± 1.46)
Camino Oeste	8	2X	16.8 (± 0.34)	12.01 (± 0.5)	158.61 (± 9.13)	6.51 (± 0.39)	3.76 (± 0.28)	19.18 (± 1.90)
El Cano	1	2X	16.71	12.22	160.42	5.86	4.06	18.62
La Escondida	5	2X	17.29 (± 0.32)	12.26 (± 0.09)	166.6 (± 2.78)	6.69 (± 0.32)	4.20 (± 0.19)	21.97 (± 0.52)
Los Membrillos	3	4X	20.3 (± 0.61)	13.75 (± 0.26)	219.5 (± 10.35)	8.65 (± 0.33)	5.34 (± 0.26)	36.27 (± 3.21)
Sampacho	2	4X	21.1 (± 0.36)	14.32 (± 0.87)	237.22 (± 10.21)	8.29 (± 1.23)	14.32 (± 0.43)	34.34 (± 8.21)
Baigorria	3	4X	20.29 (± 0.62)	14.33 (± 0.35)	228.41 (± 10.91)	7.92 (± 0.28)	4.57 (± 0.33)	28.32 (± 1.32)
Gigena	3	4X	20.49 (± 0.22)	13.99 (± 0.9)	225.17 (± 17.07)	8.13 (± 0.20)	5.10 (± 0.34)	32.58 (± 3.02)
El Cano	3	4X	20.86 (± 1.28)	14.37 (± 0.4)	235.91 (± 20.38)	7.98 (± 0.47)	5.03 (± 0.29)	31.48 (± 2.35)
La Escondida	6	4X	20.33 (± 0.43)	14.76 (± 0.51)	235.82 (± 9.9)	8.04 (± 0.32)	5.14 (± 0.23)	32.41 (± 1.87)

Results

Estimation of boundary values for the identification between *Pleurodema kriegi* and *P. cordobae*

The average cell area of tetraploid *P. kriegi* from La Posta was 233.06 µm² (range = 193.62–265.24 µm²) while in octoploid *P. cordobae* from Los Tabaquillos it was 346.45 µm² (270.77–464.22 µm²). The average length and width were 21.32 and 13.84 µm for *P. kriegi* and 26.82 and 16.41 µm for *P. cordobae*, respectively. Differences between species in cell area ($F_{1,55}$ = 181.91), length ($F_{1,55}$ = 149.08) and width ($F_{1,55}$ = 119.94) were highly significant (P<0.0001). In *P. kriegi,* erythrocyte area was not correlated with SVL (r=-0.235; P<0.2793), as well as in *P. cordobae* (r=-0.012; P<0.9434).

The average nuclear area of *P. kriegi* was 28.05 µm² (24.58–31.4 µm²) while in *P. cordobae* it was 46.29 µm²

(40.84–58.73 µm²) (Fig. 1A). The average length and width were 7.93 and 4.5 µm for *P. kriegi* and 10.59 and 5.56 µm for *P. cordobae*, respectively. Differences between species in nuclear area ($F_{1,55}$ = 516.02), length ($F_{1,55}$ = 365.19) and width ($F_{1,55}$ = 195.97) were highly significant (P<0.0001). In *P. kriegi,* nuclear area was not correlated with SVL (r=0.039; P<0.8569), as well as in *P. cordobae* (r=0.073; P<0.6857).

According with probability distributions, the values used for separate species would be 273.08 µm² (probability=97.55%), 23.23 µm (96.33%) and 14.89 µm (93.75%) for erythrocyte area, length and width respectively. On the other hand, analyzing the nuclear data, we found higher probabilities of correct identification than the obtained using cell parameters. The values of nuclear measures used for separate species would be 34.39 µm² (probability=99.96%), 8.86 µm (99.76%) and 4.88 µm (97.92%) for area, length and width respectively.

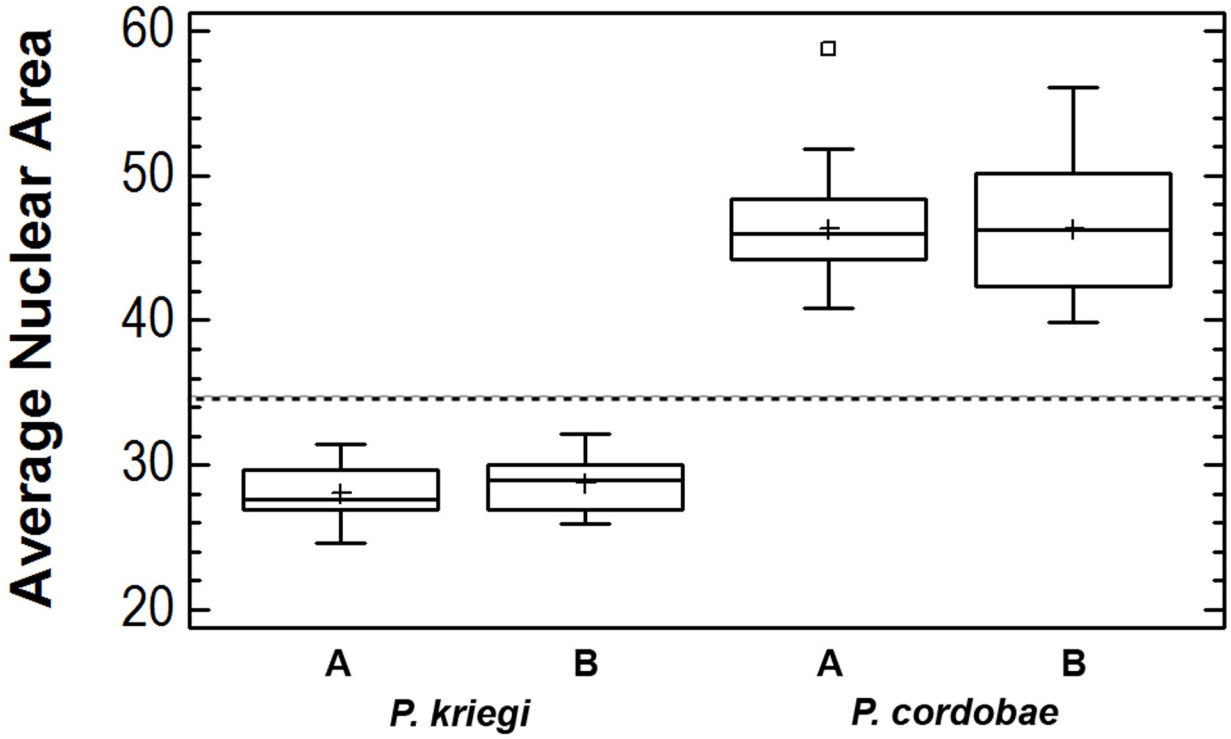

FIGURE 1. Comparison of nuclear areas of the individuals included in the estimation of boundary values (A) and the remaining individuals (B) of *Pleurodema kriegi* and *P. cordobae*. The dotted line represents the limit nuclear area to separate species. Upper and lower ends of boxes represent 75th and 25th percentiles. Whiskers represent the minimum and the maximum values, except for outlier points. The center line within each box shows the locations of the sample median and the plus sign indicates the location of the sample mean.

Estimation of boundary values for the identification between *Odontophrynus cordobae* and *O. americanus*

Diploid *O. cordobae* showed an average cell area of 168.16 µm² (136.84–187.5 µm²) while in tetraploid *O. americanus* it was 236.92 µm² (201.86–281.3 µm²). Values of average cell length and width were 17.54 and 12.17 µm for *O. cordobae* and 20.83 and 14.46 µm for *O. americanus*, respectively. Cellular area ($F_{1,44}$ = 192), length ($F_{1,44}$ = 168,58) and width ($F_{1,44}$ = 120.25) showed highly significant differences between these species (P<0.0001). In both species, erythrocyte area was not correlated with SVL (*O. cordobae*: r=0.007; P<0.9754 / *O. americanus*: r=-0.014; P<0.5502).

Regarding the nuclear measures, the average area of *O. cordobae* was 19.29 µm² (15.44–22.54) while in *O. americanus* the mean value was 32.45 µm² (26.88–40.14) (Fig. 2A). The average length and width were 6.34 and 3.87 µm for *O. cordobae* and 8.14 and 5.07 µm for *O. americanus*, respectively. Differences between species (P<0.0001) were also found in nuclear area ($F_{1,44}$ = 250.34), length ($F_{1,44}$ = 197.10) and width ($F_{1,44}$ = 140.88). In

both species, nuclear area was not correlated with SVL (*O. cordobae*: r=-0.17; *P*<0.4677 / *O. americanus*: r=0.31; *P*<0.18).

The values obtained for separate species were 197.69 µm² (probability=97.94%), 19.22 µm (97.36%) and 13.16 µm (94.69%) for cell area, length and width respectively. Similar to the results obtained in *Pleurodema*, the values for nuclear area showed higher probabilities for a correct differentiation of species than those estimated using cell size: nuclear area, length and width would be 24.02 µm² (probability=99.075%), 7.19 µm (98.09%) and 4.43 µm (96.01%).

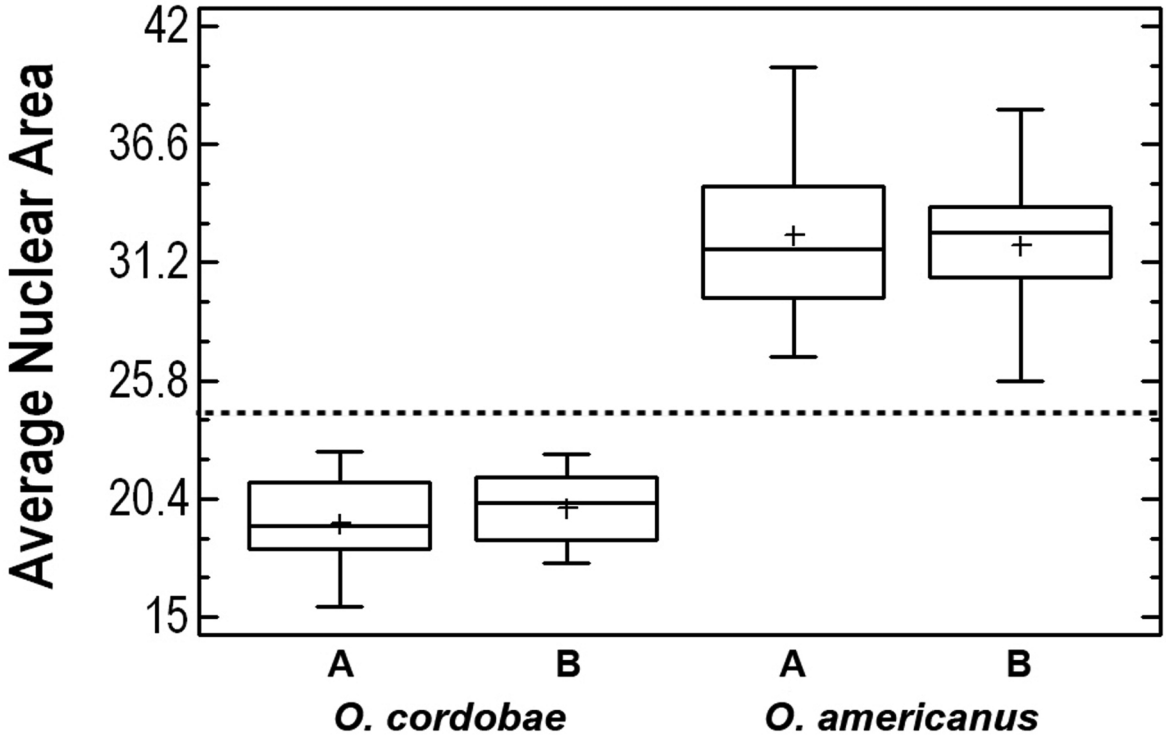

FIGURE 2. Comparison of nuclear areas of the individuals included in the estimation of boundary values (A) and the remaining individuals (B) of *Odontophrynus cordobae* and *O. americanus*. The dotted line represents the limit nuclear area to separate species. Upper and lower ends of boxes represent 75th and 25th percentiles. Whiskers represent the minimum and the maximum values. The center line within each box shows the locations of the sample median and the plus sign indicates the location of the sample mean.

Testing the boundary values of erythrocyte nuclear area

Considering the above analyzes and the higher probability of correct identification found, we tested the limit values obtained using the erythrocyte nuclear area.

Mean values and ranges obtained for the individuals of *Pleurodema* and *Odontophrynus* not included in the estimation of boundary values were: *P. kriegi* (*n* = 10): 28.79 µm² (25.92–32.16 µm²); *P. cordobae* (*n* = 43): 46.31 µm² (39.82–56.07 µm²); *O. cordobae* (*n* = 23): 20.28 µm² (17.71–22.44 µm²); *O. americanus* (*n* = 40): 31.98 µm² (25.75–38.21 µm²) (Table 2). In all cases, both maximum range values of lower ploidy individuals (*P. kriegi* and *O. cordobae*) and minimum nuclear areas of higher ploidy individuals (*P. cordobae* and *O. americanus*) not exceeded the limit calculated for a correct identification of species (Fig. 1B and 2B).

We found no significant differences in both *Pleurodema* and *Odontophrynus* in nuclear area between the individuals included in the analyzes of boundary values and the remaining individuals: *P. kriegi* ($F_{1, 32}$ = 1.00; *P* = 0.325); *P. cordobae* ($F_{1,74}$ = 0.0; *P* = 0.99); *O. cordobae* ($F_{1,47}$ = 3.69; *P* = 0.085); *O. americanus* ($F_{1,60}$ = 0.84; *P* = 0.364) (Fig. 1 and 2).

TABLE 2. Mean and standard deviation of nuclear measurements (40 erythrocytes per individual) from populations of *Pleurodema* and *Odontophrynus*, used to test the boundary values.

Site	n	Ploidy	Nucleus		
			Length (µm)	Width (µm)	Area (µm?)
Pleurodema					
Km. 10.4	2	4X	8.36 (± 0.16)	4.55 (± 0.06)	29.86 (± 0.17)
Km. 15.4	1	4X	8.37	4.90	32.16
Km. 22.8	2	4X	7.75 (± 0.28)	4.83 (± 0.24)	29.38 (± 2.46)
Represa Los Gigantes	2	4X	8.33 (± 0.31)	4.34 (± 0.15)	28.35 (± 2.10)
Refugio Los Gigantes	3	4X	7.73 (± 0.10)	4.42 (± 0.14)	26.83 (± 1.13)
Los Linderos	21	8X	10.61 (± 0.45)	5.53 (± 0.30)	46.13 (± 3.31)
Cerro Negro	6	8X	10.00 (± 0.63)	5.41 (± 0.32)	42.45 (± 2.97)
Mal Paso	16	8X	10.43 (± 0.88)	5.85 (± 0.57)	47.97 (± 5.53)
Odontophrynus					
Río de los Sauces	9	2X	6.22 (± 0.21)	4.08 (± 0.21)	19.93 (± 1.44)
El Cano	4	2X	6.29 (± 0.42)	4.06 (± 0.17)	20.06 (± 1.98)
La Escondida	5	2X	6.88 (± 0.19)	3.79 (± 0.28)	20.43 (± 1.51)
Berrotarán	4	2X	6.58 (± 0.35)	4.02 (± 0.13)	20.72 (± 1.73)
Gigena-El Cano S1	3	4X	8.10 (± 0.35)	5.21 (± 0.17)	33.15 (± 1.62)
Gigena-El Cano S2	3	4X	7.91 (± 0.54)	5.17 (± 0.36)	32.11 (± 3.57)
Gigena-El Cano S3	2	4X	7.84 (± 0.55)	5.31 (± 0.31)	32.60 (± 0.36)
Río Cuarto	4	4X	8.15 (± 0.15)	5.15 (± 0.27)	32.94 (± 1.67)
Sampacho	2	4X	8.12 (± 0.76)	4.36 (±0.23)	27.70 (± 1.06)
Baigorria	1	4X	7.34	4.50	25.97
Gigena	3	4X	7.12 (± 0.33)	4.46 (± 0.17)	24.95 (± 0.75)
El Cano	15	4X	8.04 (± 0.35)	5.10 (± 0.31)	32.22 (± 2.65)
La Escondida	9	4X	8.25 (± 0.37)	5.07 (± 0.25)	32.89 (± 2.52)

Discussion

Although morphological differences have traditionally been used to define new species for the most taxonomic groups, there are instances where morphological features alone do not suffice to identify species (Angulo & Reichle 2008). Studies using non-morphological characters to discriminate otherwise indistinguishable species are being published at an increasing rate (see Bickford *et al*. 2007). As the cryptic condition is added ploidy difference between species, the erythrometry appears as a reliable method for the identification of taxa (George & Lennartz 1980; Matson 1990; Stöck & Grosse 1997; Schröer & Greven 1998; Stöck *et al.* 1999; Martino & Sinsch 2002; Rosset *et al*. 2006; Grenat *et al*. 2009a, b; Valetti *et al.* 2009). While several authors have used the erythrocytes size for separation of related cryptic species, few studies (George & Lennartz 1980; Matson 1990; Grenat *et al*. 2009b) have reported boundary values useful to differentiate these species.

Rosset *et al*. (2006) established a limit nuclear volume to separate diploid and tetraploid *O. americanus* but they used only two measurements (large and width) to compute volume. In this case, an elliptic shape not flattened is assumed. For this reason, most authors based the erythrocyte size comparisons on the area rather than on the volume, primarily because the blood cells in anurans are flattened, and three measures (large, width and height) from a three-dimensional plane would be necessary to calculate a correct value of volume (Andrew 1965; Foxon 1964; Hartman & Lessler 1964; Holtfreter 1947; Matson 1990).

In comparison with other authors, cell size in *Odontophrynus* appears to be quite variable. Martino and Sinsch (2002) reported a range with smaller minimal and maximal cell areas (189-233.5μm²) for *O. americanus* while cell area range of *O. cordobae* were included in the range found in our study. Furthermore, mean erythrocyte area obtained by Rosset *et al.* (2006) on 10 *O. americanus* (120.6 μm²) and 2 *O. cordobae* (84.4 μm²) were considerably smaller than those calculated in our study, although this difference is probably related to that these authors used preserved museum specimens. Regarding our previous study (Grenat *et al*. 2009) we observed slight differences in the mean values of cell area and these may be due to the fact that in this work we analyze a significantly higher number of erythrocytes per individual.

Cianciarullo *et al*. (2000) reported lower cell areas in anemic induced individuals of diploid and tetraploid *O. americanus* (105.596 and 146.86 μm² respectively, based on available data). However, mean nuclear areas (17.62 and 30.75 μm² respectively, based on available data) were similar to those reported in our study (19.29 and 32.45 μm²). Consequently, nuclear size seems to be a more stable character than cell size. This fact is reinforced by our results showing a higher probability (> 99%) of correct identification of both species using nuclear areas.

In *Pleurodema* only Valetti *et al.* (2009) reported values of cell area for *P. kriegi* and *P. cordobae*. These authors obtained a range with minimal and maximal cell areas of *P. kriegi* (201.19–284.19) higher than our study, but the mean area does not differ with respect to our results. On the other hand, range and average cell area for *P. cordobae* reported by Valetti *et al.* (2009) (317.46 μm², 296.81–353.12) are considerably smaller than those obtained in our study. These differences could be due to the larger number of erythrocytes per individual considered in the present work.

Although we found significant differences in cell area between *P. kriegi* and *P. cordobae*, the higher values of tetraploid individuals were near to the minimum values of octoploid specimens. Instead, the extreme values obtained for nuclear area of tetraploid and octoploid ranges were very different, thus reducing the potential for overlap or misclassification of individuals. This result was strongly supported by the very high probability (~100%) that we found using nuclear areas of erythrocytes to separate *Pleurodema* related species.

The improved accuracy found in species identification by nuclear area is an important point because most studies carried out on pairs or groups of diploid/polyploid species use cell and not nuclear size to differentiate them (Stöck & Grosse 1997; Stöck *et al*. 1999; Martino & Sinsch 2002). Our results indicate the importance and efficiency of erythrocyte nuclear size in amphibians as diagnostic character for the distinction of anuran cryptic species with different ploidy levels, manly in the cryptic complexes studied here. The advantage of this method is that it is simple, rapid and minimally invasive (Matson 1990; Grenat et al. 2009b). Erythrometry, contrary to cytogenetics, is a method that does not require the sacrifice of the individuals because blood samples can be directly obtained by angularis vein puncture. Therefore, this method, using nuclear areas, represents a powerful tool with a high level of accuracy for resolve the problem of distinguishing living *Pleurodema* and *Odontophrynus* cryptic species.

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