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SHORT COMMUNICATION

Genome Size Evaluation in Tetraodontiform Fishes from the Neotropical Region

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Abstract Smooth pufferfish of the family Tetraodontidae had become pure genomic models because of the remarkable compaction of their genome. This trait seems to be the result of DNA loss following its divergence from the sister family Diodontidae, which possess larger genomes. In this study, flow cytometry was used for estimate the genome size of four pufferfish species from the Neotropical region. Cytogenetic data and confocal microscopy were also used attempting to confirm relationships between DNA content and cytological parameters. The haploid genome size was 0.71±0.03 pg for *Sphoeroides greeleyi*, 0.34±0.01 pg for *Sphoeroides spengleri*, 0.82±0.03 pg for *Sphoeroides testudineus* (all Tetraodontidae), and 1.00±0.03 pg for *Chilomycterus spinosus* (Diodontidae). These differences are not related with ploidy level, because 46 chromosomes are considered basal for both families. The value for *S. spengleri* represents the smallest vertebrate genome reported to date. Since erythrocyte cell and nuclear sizes are strongly correlated with genome size, the variation in this last is considered under both adaptive and evolutionary perspectives.

Keywords DNA content · Flow cytometry · Karyotype · Nucleotype · Pufferfish

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Introduction

The Order Tetraodontiformes comprises ten families, and it includes almost 350 species that are mainly distributed in marine tropical waters, although some species can also be found in fresh waters (Nelson 1994). The group shows an exceptional degree of morphological diversity and genetic peculiarities, such as the low nuclear DNA content. The smooth pufferfishes of the Tetraodontidae family have the smallest genome size among all vertebrates measured so far, with a haploid genome size around 400 million base pairs (Mb) (Hinegardner and Rosen 1972; Brenner et al. 1993). Although they have similar gene complements to other vertebrates, the small size is apparently a result of intron losses and interspersed repetitive DNA (Elgar et al. 1999; Loh et al. 2008). Thus, due to this remarkable compaction of the genome, pufferfish had become an attractive tool for comparative genomics, useful to understand the evolution of vertebrate genomes and karyotype.

Eukaryotic genome size varies by more than five orders of magnitude and is correlated with various phenotypic traits of apparent selective significance, mainly positively with the cellular and nuclear sizes (Cavalier-Smith 1982; Gregory and Hebert 1999). In this regard, several hypoth-

eses have been proposed to explain this remarkable variation. The available evidence favors the nucleotypic theory which postulates a causal influence of DNA amount on the cellular phenotype, such as division rate, cell size, and nuclear size (Bennett 1971; Gregory 2001a; Hardie and Hebert 2004).

The determination of the nuclear DNA content constitutes an important consideration to be used in genomics approaches and may provide relevant information for the establishment of a more reliable scenario of the genomic evolution. The relationship between genome size and cellular parameters was previously a subject of the contest in fishes (Chang et al. 1995; Lay and Baldwin 1999). However, in this study, once more these positive correlations were confirmed. The flow cytometry analysis associated with cytogenetic data and confocal microscopy is conducted for the first time in four pufferfish species that occur in the Neotropical region. The implications of these results concerning the adaptive significance of genome size variation as well as genome evolution of the group are discussed.

Materials and Methods

Sample and Cell-Suspension Preparation

Fifty-one animals from four pufferfish species: *Sphoeroides greeleyi* (n=17), *Sphoeroides spengleri* (n=19), *Sphoeroides testudineus* (n=10), and *Chilomycterus spinosus* (n=5), all collected in Paranaguá Bay (25°30′42″ S; 48°25′15″ W), State of Paraná, Brazil constitute the sample of this study. Voucher specimens have been deposited at the Museum of Natural History Capão da Imbuia (Curitiba—PR, Brazil). The procedures used in this work were in accordance with the guidelines of the Committee for Ethics in Animal Experimentation (UFPR 01/03BL) and the current Brazilian legislation (CONCEA 1153/95). Chromosome plates were obtained after cell culture (Fenocchio et al. 1991) and stained with a 5% Giemsa solution. For nuclear DNA content measurements, sample preparation was based on the fixation and cell permeabilization of a well-established method (Darzynkiewicz et al. 2006), with modifications. Briefly, red blood cells from the caudal

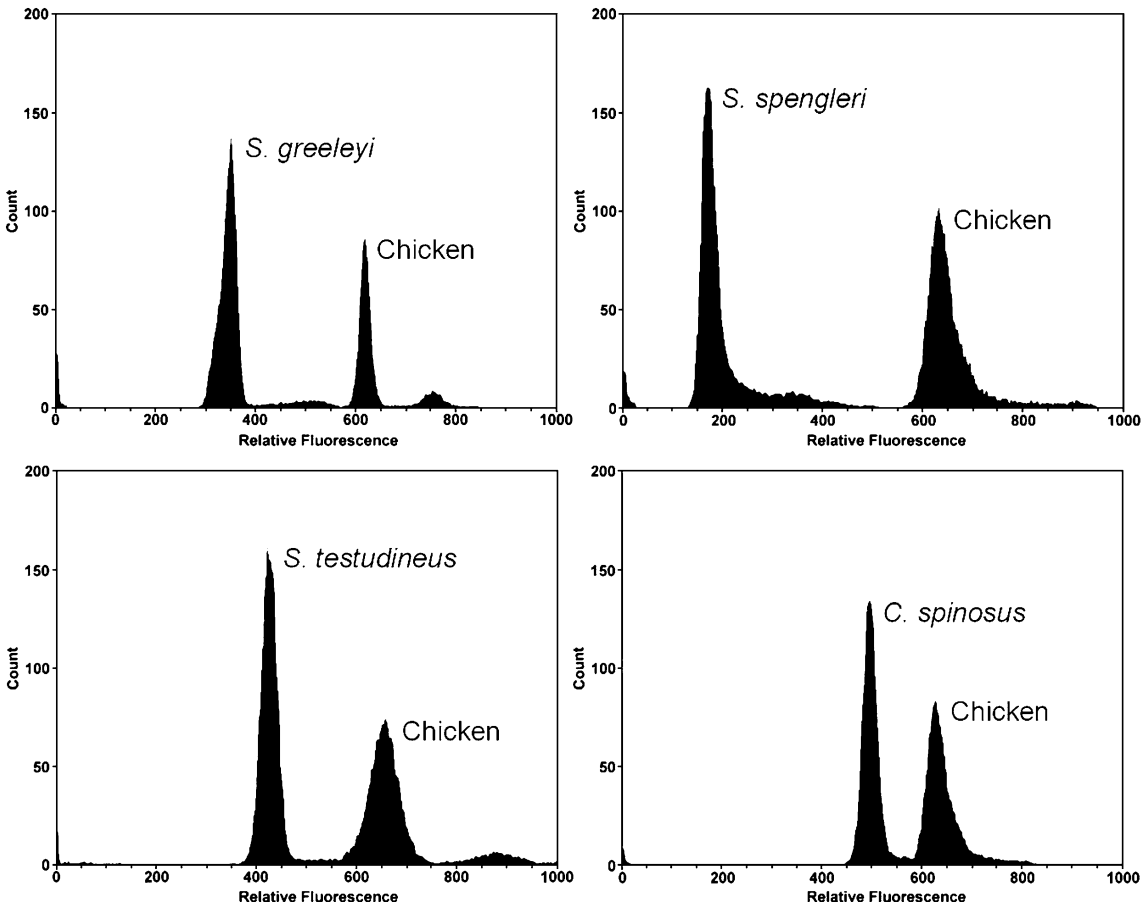

Fig. 1 Relative fluorescence histogram for propidium iodide-stained nuclei from Neotropical pufferfishes, measured by flow cytometry using chicken erythrocytes as a standard. The fish peak is approximately two times higher than the chicken peak because the relative concentration of fish to chicken cells in the sample was set at 2:1

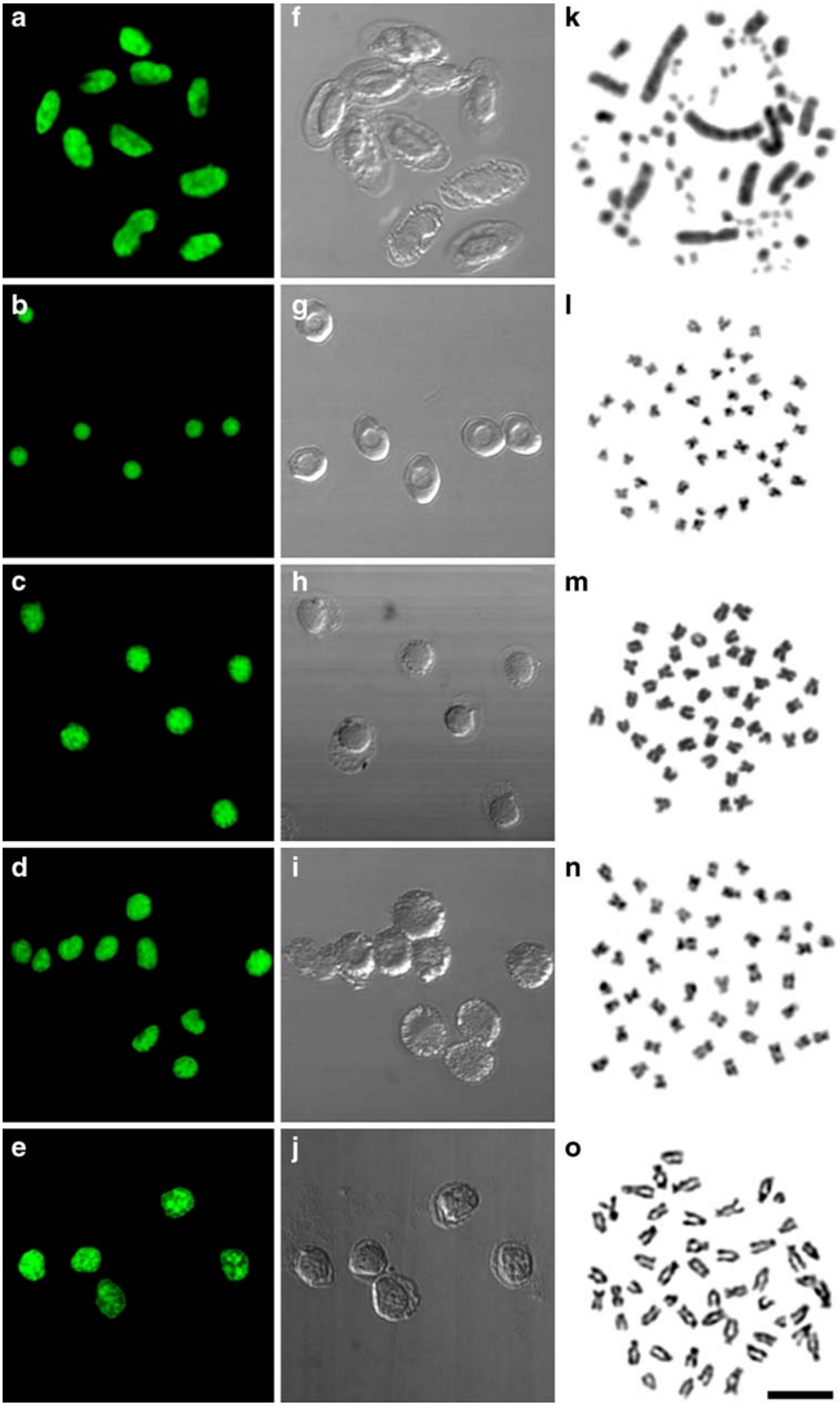

Fig. 2 Causative effects of genome size changes on cell size cellular phenotype and chromosome size. Laser confocal images of red blood cells: nuclear morphology was analyzed after staining with acridine orange (**a–e**) and the cell morphology was visualized by differential interference contrast (DIC) (**f–j**). Giemsa stained metaphases of: *Gallus gallus domesticus* (**k**), *Sphoeroides spengleri* (**l**), *S. greeleyi* (**m**), *S. testudineus* (**n**), and *Chilomycterus spinosus* (**o**). *Bar* =10 μm

artery were collected using a 1-ml heparinized syringe and used immediately. The cell suspension was fixed by 1% formaldehyde solution (Sigma, St. Louis, MO, USA), to prevent extraction of the fragmented DNA, for 1 h at room temperature. After a double washing in phosphate buffer solution (PBS) at pH 7.4, cells were permeabilized in ethanol 70% overnight at −20°C. The samples were washed in PBS and aliquots of the suspension containing approximately 1×10^6 cells/ml were pretreated with RNAse (Sigma, 20 µg/ml) at 37°C for 1 h. Fresh blood of the white leghorn chicken (*Gallus gallus domesticus*), prepared as presented above, was added to each fish sample as an internal size standard to achieve a final concentration of 0.5×10^6 cells/ml. The mixture was stained with a propidium iodide solution (Sigma, 50 µg/ml) and was incubated in darkness for 30 min. For confocal microscopy analysis the dry erythrocytes were stained with acridine orange (Electron Microscopy Sciences).

Flow Cytometry and Confocal Microscopy Analysis

Measurements of cell DNA contents were performed in a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA). For each sample tube, 20,000 cells were acquired using Cell Quest software. Genome size (diploid DNA content per cell) in picograms of each fish sample was calculated from fluorescence intensity (arbitrary units) by multiplying the fish-to-chicken fluorescence ratio by the known value of the chicken genome size (2.54 pg—Rasch et al. 1971). The fish peak was always higher than the chicken peak because the samples contained twofold greater concentration of fish cells. Quality controls performed with calibration beads of BD Calibrite Beads (Becton Dickinson Biosciences) showed stability in the cytometer and potential for consistent results. Confocal microscopy provides cellular and nuclear area measurements. The fluorescent images were acquired using an optical filter appropriate for acridine orange and differential interference contrast (DIC) sequentially. Relative areas of at least 50 cells and nuclei were measured and in all cases. Relationships among DNA content, nucleus, and cell areas were analyzed by least-squares regression of $\log_{10}$-transformed data. Standard procedures were applied for statistical analysis as presented in Results and Discussion.

Results and Discussion

Fishes of the Tetraodontiformes order, in particular the family Tetraodontidae, have become an interesting model for genome studies due to their highly compact genome (approximately 400 Mb) (Brenner et al. 1993). This remarkable compaction, which facilitates gene identification and sequencing projects, is directly related to the reduction in intron lengths, intergenic regions, and the lack of significant amounts of repetitive sequences (Elgar 1996; Elgar et al. 1999; Venkatesh et al. 2000; Loh et al. 2008). Our measurements confirmed these findings and presented one of the smallest known vertebrate genomes yet measured, *S. spengleri*, which might be more compact than the previously reported estimate for *Fugu rubripes* and *Tetraodon nigroviridis* genomes (Jaillon et al. 2004; Aparicio et al. 2002). According to the internal standard, average values for *S. greeleyi*, *S. spengleri*, *S. testudineus*,

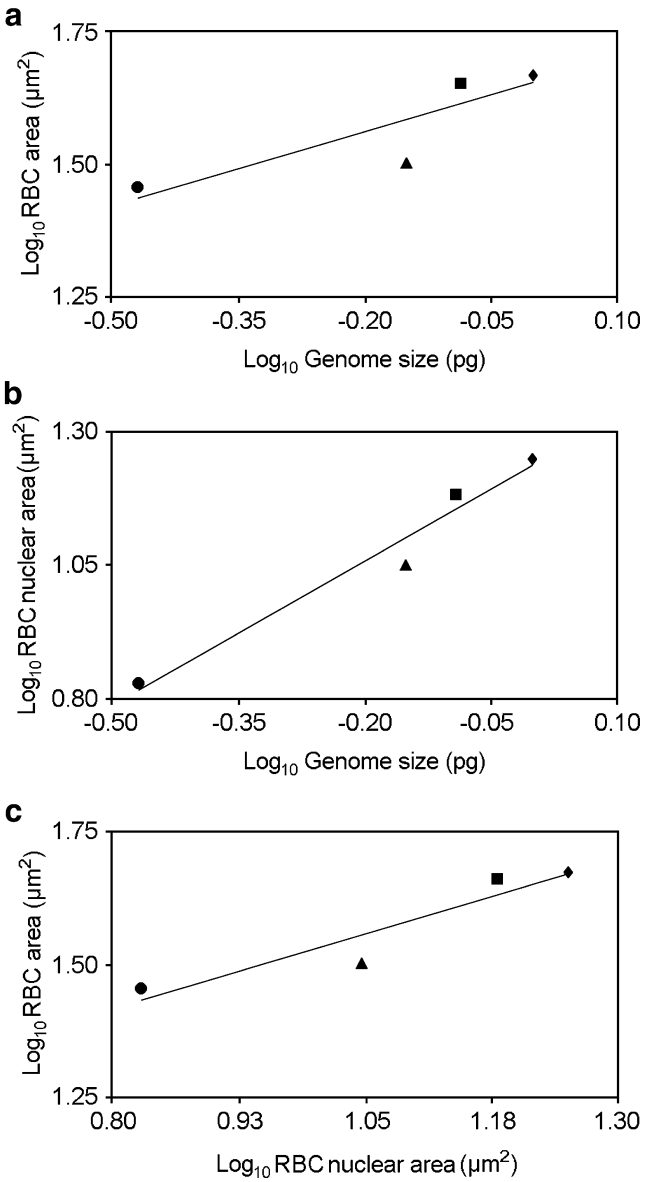

Fig. 3 Relationship between dry erythrocyte area (**a**) and nuclear area (**b**) with genome size. Cellular and nuclear areas also were positively correlated (**c**). Different symbols represent different species: *Sphoeroides spengleri* (●), *S. greeleyi* (▲), *S. testudineus* (■), and *Chilomycterus spinosus* (♦). These relationships are highly significant (all $r^2>0.72$, all $P<0.0001$)

and *C. spinosus* were 0.71±0.03, 0.34±0.01, 0.82±0.03, and 1.00±0.04 pg, respectively. Coefficients of variation of fluorescence peaks from nuclei were usually inferior to 5% in all species. Relative fluorescence histograms for propidium iodide-stained erythrocytes from pufferfishes and a chicken are reported in Fig. 1. However, a small genome size is not a typical feature of all tetraodontiform fishes, since spiny puffers (Diodontidae) possess average larger genomes (Neafsey and Palumbi 2003). In addition, in the present study, *C. spinosus* presented the highest value for the family reported (1C=1.00 pg). Several studies had confirmed events whole-genome duplications that occurred in the ray-finned fish lineage (Amores et al. 1998; Robinson-Rechavi et al. 2001; Taylor et al. 2003; Jaillon et al. 2004), and these episodes had certainly contributed to their phenotypic and genomic diversity. In this scenario, the ancestor of Tetraodontidae presented a relatively large genome size and acquired this tendency to lose DNA in the last 50 million years since their divergence from the Diodontidae (Brainerd et al. 2001; Santini and Tyler 2003). In this sense, according to present study size estimations, the process to eliminate the junk DNA was more advanced in *S. spengleri* than their relatives.

Regarding chromosome number, the three species of the genus *Sphoeroides* (Tetraodontidae) showed $2n=46$ and *C. spinosus* (Diodontidae) $2n=50$ chromosomes (Fig. 2k–o). As in most other organisms, the difference in genome size between spiny and smooth puffers is not related to differences in ploidy, but in chromosome size, which is observed for the notable differences in chromosome sizes of species, mainly among the tiny chromosomes of *S. spengleri* and the largest one of *C. spinosus* (Fig. 2l, o respectively). The independence of genome size and chromosome number is reinforced by the fact that, in most teleost species studied, the complement remains remarkably constant around 48 chromosomes, even among species that differ significantly in DNA content (Ohno 1974; Klinkhardt et al. 1995). Since the chromosome number in the order Tetraodontiformes ranges from $2n=28$ to $2n=52$ (Sá-Gabriel and Molina 2005; Galetti et al. 2006), the $2n=46$ observed in the *Sphoeroides* species previously studied (Brum and Mota 2002; Sá-Gabriel and Molina 2005; Noleto et al. 2007; Alves et al. 2008) is considered the basal karyotype for the families Balistidae, Diodontidae, and Tetraodontidae (Arai 1983; Brum 2000).

This study clearly establishes that the DNA content affects erythrocyte cell and nuclear size in fishes. Both cellular and nuclear areas of the red blood cells (RBC) showed a highly significant positive relationship between them and with genome size (Fig. 3). The Laser confocal microscopic analysis confirmed these positive correlations (Fig. 2a–j). Some studies previously reported a lack of association between cellular parameters and genome size in both bony (Lay and Baldwin 1999) and cartilaginous fishes (Chang et al. 1995). It would be surprising not finding the relationship in the fishes, since early observations reported that cell and nuclear sizes varied in concert with DNA content across the protist, plant, and animal kingdoms (Cavalier-Smith 1991; Gregory and Hebert 1999). The constancy of cyto- and nucleogenomic ratios in neopolyploids regarding their diploid progenitors provides further support for the natural cause of these relationships, supporting that the nucleotypic theory is the most parsimonious explanation for adaptive interpretations of genome size diversity (Gregory 2001b).

Associations between genome size and characters such as metabolic and development rates, growth, complexity, and ecological amplitude are present in many groups of organisms, and likely derive from the relationships between DNA content of cells and their size and division rates. DNA content is not correlated with metabolic rate in ray-finned fishes, but it is positively associated with reproductive traits including egg size and parental care, beyond that freshwater fishes have larger genomes than marine fishes (Hardie and Hebert 2004) and cartilaginous than in ray-finned fishes (Stingo and Rocco 2002). The pufferfishes with their *r*-selected traits, including rapid growth, early maturity, no parental care, and high fecundity, may have in this scenario experienced an increased selection for a shorter developmental time (e.g., few DNA to be replicated in consequently a short cell division).

It is unlikely that the genome size evolution might be affected only by selective forces because there are clear indications that mutational mechanisms of addition and loss of DNA such as satellite expansion, heterochromatic shrinkage, or expansion activity of transposable elements also play an important role, as well as random genetic drift. Different evolutionary factors can be important in different organisms and to confer varying fitness in different habitats, especially in fishes, due to their extreme diversity. Therefore, an analysis of complex interaction of factors is needed for a robust understanding of genome size evolution.

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