KARYOTYPE AND NUCLEOLUS ORGANIZER REGIONS OF *Ophisternon aenigmaticum* (Teleostei: Synbranchiformes: Synbranchidae) FROM VENEZUELA

Mauro Nirchio, Tatiane Casagrande Mariguela, Irani Alves Ferreira, Fausto Foresti and Claudio Oliveira

SUMMARY

The first cytogenetic analysis by conventional Giemsa staining*, silver nitrate impregnation of nucleolus organizer regions (Ag-NOR), and fluorescence in situ hybridization (FISH) carried out on* Ophisternon aenigmaticum *from Margarita Island, Venezuela, is reported. Two cytotypes were found: cytotype C1, 2n=46, composed of 6 medium size metacentrics, 2 subtelocentrics and 38 acrocentrics; and cytotype C2, 2n=45, composed of one large metacentric plus 5 medium size metacentrics, 1 subtelocentric and 38 acrocentric chromosomes. In cytotype C1, major ribosomal DNA (rDNA) cistrons are located in the terminal position of the short arms of the first metacentric pair as determined by Ag-NORs and FISH with the 18S rDNA probe. In cytotype C2, silver staining revealed only one positive signal in a single chromosome of the pair 1 whereas FISH with the 18S rDNA probe hybridized in two different chromosomes: one coincident with the Ag-NOR (pair 1) and other in one acrocentric chromosome, suggesting the occurrence of a peri-centric inversion. In both cytotypes, FISH with 5S rDNA probe produced hybridization signals in subtelomeric position of the short arm of the chromosome pair N° 3. Chromosome evolution in Synbranchidae is discussed.*

Introduction

Synbranchidae is a freshwater eel-like percomorph fish family usually found in brackish water, widely distributed throughout Central and South America, Caribbean, tropical west Africa, and the Indian subcontinent throughout Asia and the Indo-Australian archipelago to Australia (Nelson, 2006).

The family comprises 15 species contained in four genera (*Macrotrema*, *Ophisternon*, *Synbranchus* and *Monopterus*), of which only two, *Ophisternon* and *Synbranchus*, with two species in the former genus (Kullander, 2003) and three in the later (Favorito *et al*., 2005), are currently recognized in the Neotropical region. *Synbranchus marmoratus*, commonly known as the swamp eel or water snake, is widely distributed from Mexico to northern Argentina (Kullander, 2003) while *Ophisternon aenigmaticum* is distributed in Central America: throughout the Atlantic Slope of Guatemala and Mexico to Cuba (Froese and Pauly, 2009).

Cytogenetic studies so far performed in Synbranchidae from South America are restricted to *S. marmoratus* from Brazil and Argentina (Foresti *et al*., 1992; Melillo *et al*., 1996; Sánchez and Fenocchio, 1996; Torres *et al*., 2005; Table I). These studies have revealed an extensive level of karyotypic variation with diploid numbers ranging from 2n=42 to 2n=46 and at least nine cytotypes (Torres *et al*., 2005), suggesting that *S. marmoratus* from South America could represent a species complex (Foresti *et al*., 1992; Torres *et al*., 2005).

It is the aim of the present study to present the first description of the karyotype of *O. aenigmaticus* from Margarita Island, Venezuela, and the locations of the 18S rRNA and the 5S rRNA genes obtained by conventional (Giemsa staining, Ag-NORs) and molecular techniques (fluorescence *in situ* hybridization; FISH).

Materials and Methods

The sample consisted of nine specimens of *O. aenigmaticum* collected in Margarita Island at two different localities, eight at El Valle (10°58'47.6', 63°52'27.58'W) and one at La Vega (11º01'34.5'N, 63º50'49.1'W). Voucher specimens were deposited in the fish collection of the Escuela de Ciencias Aplicadas del Mar, Universidad de Oriente, Nueva Esparta, Venezuela and Laboratório de Biologia e Genética de Peixes, Universidade Estadual Paulista, São Paulo, Brazil.

Chromosome preparations were obtained from a suspension of kidney cells (Foresti *et al*., 1993). For the conventional karyotype, preparations were stained during 20min with 10% Giemsa in phos-

KEYWORDS / Chromosome Evolution / Chromosome Fusion / 18S DNA / Fish Chromosome / Pericentric Inversion / 5S rDNA /

Received: 01/11/2010. Accepted: 02/21/2011.

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CARIOTIPO Y REGIONES DE ORGANIZADORES NUCLEOLARES DE *Ophisternon aenigmaticum* (Teleostei: Synbranchiformes: Synbranchidae) DE VENEZUELA

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RESUMEN

Se registran los primeros análisis por tinción convencional de Giemsa, impregnación con nitrato de plata de las regiones de organizadores nucleolares (Ag-NOR) e hibridización fluorescente in situ (FISH) llevados a cabo en Ophisternon aenigmaticum *proveniente de la Isla de Margarita, Venezuela. Fueron encontrados dos cariotipos: C1, 2n=46, compuesto de seis cromosomas metaméricos de tamaño medio, dos subtelocéntricos y 38 acrocéntricos; y C2, 2n=45, compuesto de un cromosoma metacéntrico grande y cinco metacéntricos medianos, un subtelocéntrico y 38 acrocéntricos. En el cariotipo C1 los cistrones ribosomales mayores (rADN) se localizan en la posición terminal de los brazos cortos del primer par metacéntrico, determinado por Ag-NOR y FISH con la sonda 18S rADN. En el cariotipo C2, la tinción argéntica solo mostró una señal positiva en un cromosoma sencillo del par 1, mientras que FISH con la sonda 18S r ADN hibridizó en dos cromosomas diferentes: uno coincidente con el Ag-NOR (par 1) y otro en un cromosoma acrocéntrico, sugiriendo la ocurrencia de una inversión pericéntrica. En ambos cariotipos, FISH con sonda 5S rADN produjo señales de hibridación en posición subtelomérica del brazo corto del par de cromosomas Nº 3. Se discute la evolución cromosómica en Synbrachidae.*

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RESUMO

Registram-se as primeiras análises por coloração convencional de Giemsa, impregnação com nitrato de prata das regiões de organizadores nucleolares (Ag-NOR) e hibridização fluorescente in situ (FISH) realizados em Ophisternon aenigmaticum *proveniente da Ilha de Margarita, Venezuela. Foram encontrados dois cariotipos: C1, 2n=46, composto de seis cromossomas metaméricos de tamanho médio, dois subtelocêntricos e 38 acrocêntricos; e C2, 2n=45, composto de um cromossoma metacêntrico grande e cinco metacêntricos médios, um subtelocêntrico e 38 acrocêntricos. No cariotipo C1 os cistrons ribossomais maiores (rADN) se localizam na posição terminal dos braços curtos do primeiro par metacêntrico, determinado por Ag-NOR e FISH com a sonda 18S rADN. No cariotipo C2, a coloração prateada somente mostrou um sinal positivo em um cromossoma simples do par 1, enquanto que FISH com a sonda 18S r ADN hibridizou em dois cromossomas diferentes: um coincidente com o Ag-NOR (par 1) e outro em um cromossoma acrocêntrico, sugerindo a ocurrência de uma inversão pericêntrica. Em ambos cariotipos, FISH com sonda 5S rADN produziu sinais de hibridação em posição subtelomêrica do braço curto do par de cromossomas Nº 3. Discute-se a evolução cromossômica em Synbrachidae.*

phate buffer pH 6.88. Active nucleolus organizer regions (NORs) were revealed by impregnation with silver nitrate (Ag-NORs; Howell and Black, 1980). The 5S and 18S rDNA sites were identified by FISH according to the method of Pinkel *et al*. (1986). A sequence of 1800 base pairs of the 18S rRNA gene of *Oreochromis niloticus* (Nile tilapia), cloned in pGEM-T plasmid, was used as a probe to localize sites for 45S rDNA. PCR products containing 5S rDNA repeats from each species were used as probes for the chromosome mapping of 5S rDNA. DNA was extracted from muscle (Sambrook and Russell, 2001) and the 5S rDNA repeats were generated by polymerase chain reaction (PCR) with the primers 5SA (5'TAC GCC CGA TCT CGT CCG ATC3') and 5SB (5'CAG GCT GGT ATG GCC GTA AGC3') according to (Pendás *et al*., 1994).

The 18S rDNA and 5S rDNA probes were labeled by nick translation with biotin-14-dATP, or 11-digoxigenine dUTP following the manufacturer's (Bionick TM Labelling System-Gibco. BRL) instructions. The preparations with metaphase chromosomes were incubated with RNAse ($40\mu g \cdot ml^{-1}$) at 37ºC for 1h. After DNA denaturation in formamide 70%/2xSSC at 70°C for 2min, the chromosome preparations were dehydrated in an ethanol series (70, 85, 100%). After drying, the hybridization mix (100ηg probes, $10mg \cdot ml^{-1}$ dextran sulfate, 2xSSC, and 50% formamide in a 30µl final volume) was pipetted onto the samples, which were then incubated overnight at 37°C in a humid chamber. After hybridization, the slides were washed 2× in 15% formamide solution/0.2xSSC pH 7.0 at 42°C by shaking for 10min each, followed by 3× wash in 0.1xSSC at 60°C for 5min each, shaking; the mix was then incubated in 5% NFDM/4xSSC buffer for 15min and washed with Tween 0.5%/4xSSC for 5min at room temperature, shaking. Probe detection was done with 0.07% avidin-FITC (Sigma) conjugated with NFDM/4xSSC buffer for 30min, followed by signal amplification using 2.5% anti-avidin-biotin conjugated with NFDM/4xSSC buffer for 30min. Treatments with avidin-FITC and antividin-biotin were accomplished at 37°C in a humid chamber. After each step for signal detection, preparations were washed 3× with 0.5%/4xSSC Tween for 5min at room temperature. Chromosomes were counterstained with 0.2% propide iodide (PI) diluted in antifade (Vector) for marking with biotin, and stained with antifade/DAPI for marking with digoxigenine. The mitotic figures were photographed using a Motic B400 microscope equipped with a Moticam 5000C digital camera. From digitalized photographs, long arm (L), short arm (S) and whole chromosome lengths were measured for each chromosome to the nearest 0.01mm, using the measuring

TABLE I
SUMMARY OF THE AVAILABLE KARYOTYPICAL DATA FOR SYNBRANCHIDAE

Species	Locality	2n	Karyotype	References
S. marmoratus	Coxim, MS, BR	42	4M,SM+38ST,A	Foresti *et al.* (1992)
S. marmoratus	São Simão, GO, BR	42	4M,SM+38ST,A	Foresti *et al.* (1992)
S. marmoratus	Nova Granada, SP, BR	42	4M,SM+38ST,A	Foresti *et al.* (1992)
S. marmoratus	Botucatu, SP, BR	42	4M,SM+38ST,A	Melillo *et al.* (1996)
S. marmoratus	Birigui, SP, BR	42	4M,SM+38ST,A	Melillo *et al.* (1996)
S. marmoratus	Paraguaçu Paulista, SP, BR	42	4M,SM+38ST,A	Melillo *et al.* (1996)
S. marmoratus	Pirassununga, SP, BR	42	6M,SM+36ST,A	Melillo *et al.* (1996)
S. marmoratus	Ribeirão Preto, SP, BR	42	6M,SM+36ST,A	Melillo *et al.* (1996)
S. marmoratus	Bataguaçu, MS, BR	42	6M,SM+36ST,A	Melillo *et al.* (1996)
S. marmoratus	Pereiras, SP, BR	42	4M+6SM+8ST+24A	Torres *et al.* (2005)
S. marmoratus	Presidente Epitácio, SP, BR	42	4M+6SM+8ST+24A	Torres *et al.* (2005)
S. marmoratus	Londrina, PR, BR	42	4M+2SM+8ST+28A	Torres *et al* (2005)
S. marmoratus	Guairá, PR, BR	42	4M+2SM+8ST+28A	Torres *et al.* (2005)
S. marmoratus	Miranda, MS, BR	42	4M+2SM+8ST+28A	Torres *et al.* (2005)
S. marmoratus	Rio Claro, SP, BR	44	4M,SM+40ST,A	Foresti *et al.* (1992)
S. marmoratus	Pentecostes, CE, BR	44	4M,SM+40ST,A	Foresti *et al.* (1992)
S. marmoratus	Botucatu, SP, BR	44	4M,SM+40ST,A	Melillo *et al.* (1996)
S. marmoratus	Birigui, SP, BR	44	4M,SM+40ST,A	Melillo *et al.* (1996)
S. marmoratus	Bataguaçu, MS, BR	44	4M,SM+40ST,A	Melillo *et al.* (1996)
S. marmoratus	Ituzaingó, Corrientes, AR	44	4M,SM+40ST,A	Sánchez and Fenocchio (1996)
S. marmoratus	Reconquista, Santa Fé, AR	44	4M,SM+40ST,A	Sánchez and Fenocchio (1996)
S. marmoratus	Garabato, Santa Fé, AR	44	4M,SM+40ST,A	Sánchez and Fenocchio (1996)
S. marmoratus	Pirassununga, SP, BR SP	44	4M+2SM+8ST+30A	Torres *et al.* (2005)
S. marmoratus	Pirassununga, SP, BR	46	4M,SM+42ST,A	Melillo *et al.* (1996)
S. marmoratus	Ribeirão Preto, SP, BR	46	4M,SM+42ST,A	Melillo *et al.* (1996)
S. marmoratus	Bandeirantes, PR, BR PR	46	4M+2SM+8ST+32A	Torres *et al.* (2005)
S. marmoratus	Miranda, MS, BR	46	6M+2SM+6ST+32A	Torres *et al.* (2005)
O. aenigmaticum	La Vega, García, MI, VE	45	6M+1ST+38A	The present study
	El Valle, García, MI, VE	46	6M+2ST+38A	

Locations of the samples taken by municipality and state in Brazil (BR), Argentina (AR) and Venezuela (VE). SP: State of São Paulo; PR: State of Paraná; MS: State of Mato Grosso do Sul; MT: State of Mato Grosso; GO: State of Goiás; CE: State of Ceará; BO: State of Bolivar; MI: Margarita Island. (2n): diploid numbers.
Karyotypes: M: metacentric; SM: submetacentric; ST: subtelocentric; A: acrocentric.

tool in Adobe Photoshop CS2. The chromosomes were classified according to the arm ratios (Levan *et al*., 1964). The fundamental number (NF) of arms was determined considering acrocentrics (A) as having one chromosome arm and metacentrics (M), submetacentrics and subtelocentrics as having two chromosome arms. FISH metaphases were photographed with an Olympus BX61 photomicroscope equipped with a DP70 digital camera.

Results and Discussion

The analysis of cells from nine individuals showed the presence of, at least, two cytotypes which we named C1 and C2. Karyotypes of cytotypes C1 and C2 obtained by arranging the chromosomes in order of decreasing size are shown in Figure 1. Cytotype C1, 2n=46, found in six individuals, was composed of 6 medium size metacentrics, 2 subtelocentrics and 38 acrocentrics. Elements of the metacentric and subtelocentric series were classified as homologous pairs. Similar morphology and slight differences in chromosome size prevented identification of the homologous in the acrocentrics series. Cytotype C2, 2n=45, with one large metacentric plus 5 medium size metacentrics, one subtelocentric, and 38 acrocentrics chromosome, was found in three individuals.

In the cytotype C1, major ribosomal DNA cistrons are located in the terminal position of the short arms of the first metacentric as determined by silver nitrate impregnation (Figure 1b) and FISH with the 18S rDNA probe (data not shown). In cytotype C2, silver staining revealed only one positive signal in the single chromosome of the pair 1 (Figure 1d) whereas FISH with the 18S rDNA probe hybridized in two different chromosomes: one coincident with the Ag-NOR signal in the single chromosome N° 1 and the other in one acrocentric chromosome (Figure 2a).

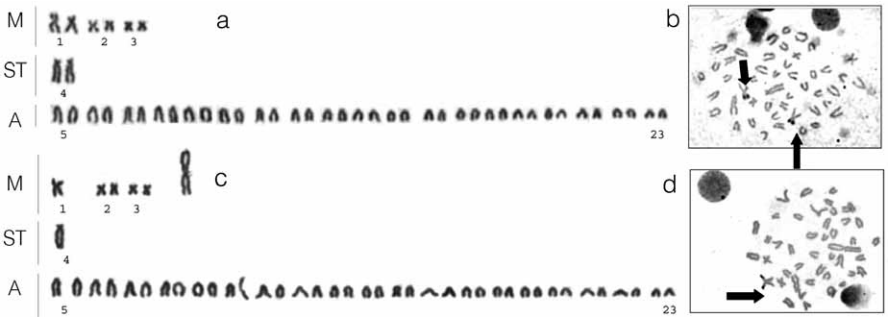

Figure 1. Chromosomes of *O. aenigmaticus*. Cytotypes C1 stained with Giemsa (a) and the Ag-NORs technique (b). Cytotype C2 stained with Giemsa (c) and the Ag-NORs technique (d). Arrows show NOR-bearing chromosomes.

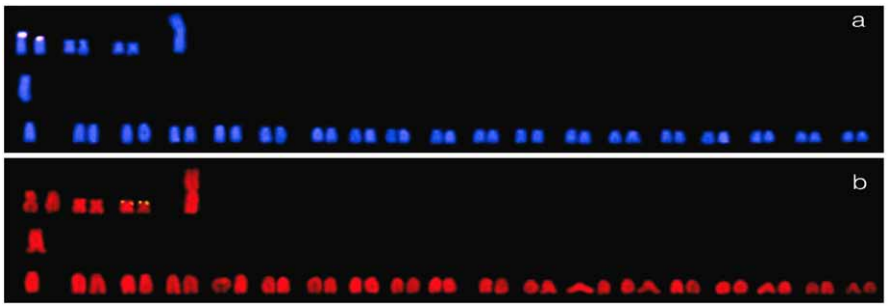

Figure 2. Chromosomes of *O. aenigmaticum* (cytotype C2) after FISH using two rDNA probes showing position of the 18S rDNA in pair N° 1 (a) and 5S rDNA in pair N° 3 (b).

The differences observed in the cytotype C2 in relation to the cytotype C1 (loss of one element of the metacentric pair N° 1, loss of one of the chromosomes of the subtelocentric pair N° 4, and appearance of a large metacentric element) could be explained by: a) a fusion of one of the subtelocentrics of the pair N°

4 with one of the acrocentric elements of pair N° 5 (the largest of the acrocentric series), which would maintain the size relation between those elements and the new large metacentric chromosome (Figure 3); or b) a pericentric inversion of one of the chromosomes of the metacentric pair N° 1 causing the displacement of the centromere from a medium to a terminal location, and maintaining the segment that possesses rDNA cluster at the same position (Figure 4).

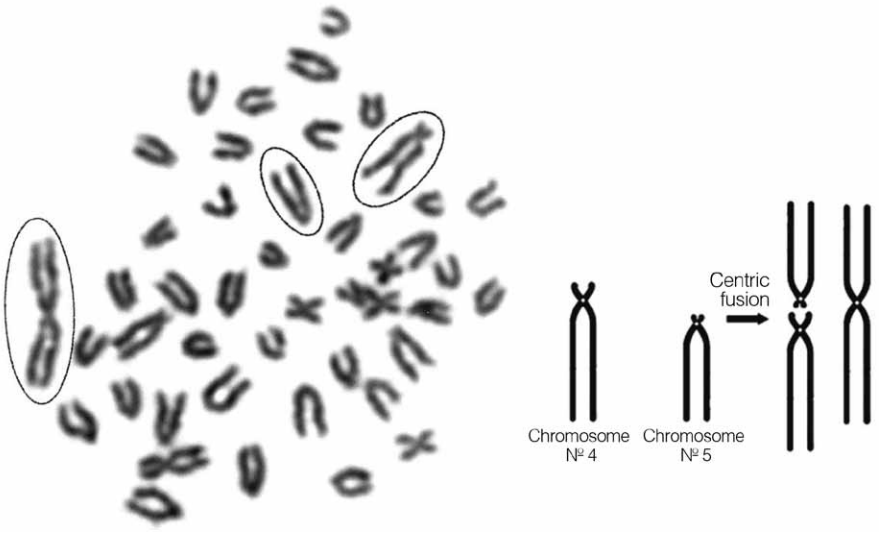

Figure 3. Metaphase plate of *O. aenigmaticum* (cytotype 2) with a scheme of hypothetical centric fusion of chromosomes form pairs 4 and 5 to produce a large metacentric element. Chromosomes into ellipses are the elements involved in this hypothetical fusion event.

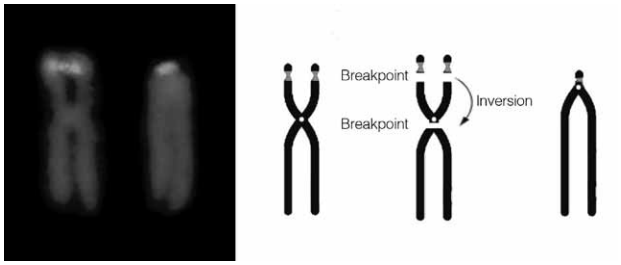

Figure 4. Chromosome pair N° 1 of *O. aenigmaticum* (cytotype 2) with a scheme of hypothetical pericentric inversion of one of the metacentric chromosomes to produce an acrocentric element without movement of NOR clusters.

Silver impregnation stains proteins associated with rDNA cistrons that are actively transcribing rRNA for nucleolus production (NOR site) in the previous interphase. When the NOR-bearing chromosomes condense and enter prophase, these rRNA-associated proteins are entrapped around the NOR and it is these proteins that are still present in the metaphase chromosome that selectively reduce the silver and become heavily stained (Howell, 1977), whereas unstained NORs did not transcribe rRNA during the preceding interphase and there are no rRNA-associated proteins, hence no silver stain (Howell and Black, 1979). Thus, the fact that silver impregnation revealed only one active NOR in Cytotype C2 (Figure 1d) with the certainty that another rDNA cistron is present in the element of the pair N° 1 that probably arose by pericentric inversion, lead to suggest that this NOR cluster could be inactivated after inversion by the loss of sequences involved with the transcriptional control of these genes, thus impeding the activity of those genes during the previous interphase, explaining the presence of a hybridization signal with the probe 18S rDNA on the tip of this chromosome, although without expression as demonstrated by silver nitrate. The results show that the elimination of the whole major rDNA sequences is not the cause of inactivation of NOR expression, but gene silencing due to positional effects seems to be a plausible explanation.

The cytotype C2 in the population of *O. aenigmaticum* could be maintained since only one NOR-bearing chromosome is sufficient to cover the cell rRNA requirement, allowing the presence of this inverted chromosome in a high frequency (33%) in the population studied. On the other hand, the absence of individuals with 2n=44 can be explained by the low number of studied animals or by a putative lethal effect associated with the translocation in homozygous state. A kind of lethal effect related with a chromosome inversion was already identified in the rainbow trout, *Oncorhynchus mykiss* (Porto-Foresti *et al*., 2004).

The 5S rDNA array consists of multiple copies of a highly conserved 120 base pair (bp) coding sequence, separated by a variable nontranscribed spacer (NTS; Long and David, 1980). In *S. marmoratus*, sequencing studies have revealed that the 5S rDNA consist of just one major class of 463bp-tandem repeats (Messias *et al*., 2003). In both cytotypes of *O. aenigmaticum*, FISH with 5S rDNA probe rendered hybridization signals in subtelomeric position of the short arm in the chromosome pair N° 3, the smallest of the metacentric series (Figure 2b), reinforcing the hypothesis of the occurrence of a single 5S rDNA class in Synbranchidae.

Morphological studies showed that *Ophisternon aenigmaticum* is the basal sister species of the genus *Synbranchus* (Favorito-Amorim, 1998). Thus, considering the presence of 2n=46 chromosomes in *O. aenigmaticum* and several samples of *S. marmoratus* (Table I), it is possible to hypothesize that this chromosome number is plesiomorphic for this fish group. Cytotypes with 2n=44 and 2n=42 (Table I) represent apomorphic forms in the genus *Synbranchus*, conceivably originated by chromosome fusions. Additionally, the presence of three metacentric pairs in *O. aenigmaticum* can also represent a plesiomorphic state and the presence of two metacentric pairs in almost all samples of *Synbranchus* (Table I) is also an apomorphic feature. On the other hand, the observed differences among cytotypes of *Ophisternon* and *Synbranchus* show that frequent chromosome rearrangements were fixed during the evolutionary history of these genera.

Karyological data here obtained for *O. aenigmaticum* are the first for the species. Although the results are of interest, further karyological studies should be carried out on other populations of this widely distributed species, as well as on remaining unstudied species of swamp-eels, in order to provide a more conclusive picture of chromosome evolution in Synbranchidae.

ACKNOWLEDGEMENTS

This work was supported by the Consejo de Investigación, Universidad de Oriente, Venezuela, to MN, and by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, to CO.

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