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Karyotype diversification in fishes of the Balistidae, Diodontidae and Tetraodontidae (Tetraodontiformes)

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Abstract — Among the great marine fish diversity, the Order Tetraodontiformes is remarkable by presenting post-Perciformes modern features, representing one of the major branches of the teleosteans radiation. Patterns of chromosomal evolution in this group are not fully understood. In the present work, cytogenetical analyses were carried out in the species *Balistes vetula* (2n=44; FN=44) and *Melichthys niger* (2n=40 and FN=40) (Balistidae), *Chilomycterus antennatus* (2n=52; 6M+46st/a, FN=58) (Diodontidae) and *Sphoeroides testudineus* (2n=46; 18m+4sm+6st+18a, FN=74) (Tetraodontidae), collected along the Brazilian coast and Saint Pauls Rocks. All species presented Ag-NORs sites on a single chromosomal pair. Heterochromatic regions in this group are reduced and located at centromeric position over most of chromosomal pairs. The evolutionary patterns of chromosomal changes were diverse in the distinct Tetraodontiformes families. In Balistidae, the evolution process seems to be determined by in tandem or centric fusions, followed by pericentric inversions. The higher chromosomal number in Diodontidae indicates that centric fissions and pericentric inversions played an important role in the karyotypical definition of this group. The Tetraodontidae *S. testudineus* displayed small chromosomes with a modal number shared with other species previously analyzed from this family. Such great karyotypical diversity is compatible with a scenario of several modifications established by the adaptative irradiation of this group.

Key words: C-bands, fish cytogenetics, karyotypical diversity, pufferfish, Tetraodontiformes.

INTRODUCTION

The Tetraodontiformes order has about 428 species, distributed in nine families (Triacanthodidae, Triacanthidae, Balistidae, Monacanthidae, Ostraciidae, Triodontidae, Tetraodontidae, Diodontidae and Molidae) widely distributed circuntropically in tropical and temperate freshwater and marine environments (NELSON 1994).

Balistes vetula and *Melichthys niger* (Balistidae) of the Western Atlantic are outstanding because they adapt easily to artificial environments and because they are used as a food source in Northeastern Brazil (HAIMOVICI and KLIPPEL 2000). Furthermore, they are distributed along the coast of isolated ocean islands, forming an effective model for the detection of genetic populational patterns.

Most of the Diodontids, such as *Chilomycterus antennatus,* are distributed in the Atlantic, Pacific and Indian oceans, presenting characteristically the capacity to inflate their bodies with water or air, permitting the erection of a great number of spines, if they have them. The Tetraodontids have the same geographical distribution as the other Tetraodontiformes, present toxins (tetrodoxin) in their viscera, spines on the body, and the four frontal teeth fused (SANTOS 1992). Particularly abundant on sheltered shores, such as bays and estuaries, *Sphoeroides testudineus* is one of the most frequent species on the Brazilian coast. These fish are considered toxic, with cases of fatal accidents caused by ingestion recorded in Bahia state (ALMEIDA and ROCHA 1989; SANTOS 1992).

This study aimed to identify the karyotypic diversity existing in the Tetraodontiformes by cytogenetic analyses carried out on *B. vetula* and *M. niger* (Balistidae), *C. antennatus* (Diodontidae) and *S. testudineus* (Tetraodontidae) using conventional staining, C banding, Ag-NORs and treatment with the restriction enzyme (RE) *Eco*RI.

MATERIALS AND METHODS

The specimens studied came from different locations, from north to south of the Brazilian coast.

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The samples consisted of four specimens of *Balistes vetula* from coastal reefs in Bahia (Salvador - 12°58'S, 38°31'W), 28 specimens of *Melichthys niger* collected in Saint Pauls Rocks (0°55'N, 29°21'W), four individuals of *Chilomycterus antennatus* from Rio de Janeiro coast (Angra dos Reis - 23°00'S, 44°18'W and Niterói - 22°55'S, 43°50'W), and 15 specimens of *Sphoeroides testudineus* obtained in the Rio Grande do Norte coast (Natal - 5°46'S, 35°12'W). The fishes were submitted to mitotic stimulation (Lee and Elder 1980) for 24 hours before mitotic chromosomes were obtained by the *in vitro* method (Gold *et al.* 1990). The Ag-NORs were detected according to the technique by Howell and Black (1980) and the heterochromatic segments visualization, according to Sumner (1972).

The RE *Eco*RI (GAATTC), used to digest genome DNA was dissolved in buffer solution as recommended by the manufacturer (Amersham Pharmacia), at a final concentration of 0.5 U/µl (Cau et al. 1988). A 40 µl volume was added to a previously prepared slide, covered with a slide cover and incubated in a chamber at 37°C for 10 hours and stained by 5% Giemsa solution for 25 minutes.

RESULTS

The diploid number observed in *B. vetula* was 2n=44, acrocentrics, FN=44 with Ag-NORs localized on the 2nd chromosome pair in telomeric position (Figure 1 (A). *M. niger* showed 2n=40, acrocentrics, FN=40 with Ag-NORs localized on the 5th pair, in telomeric position. A distribution preferentially centromeric of heterochromatic regions was found in these two species (Figures 1 (B) and 2 (B)).

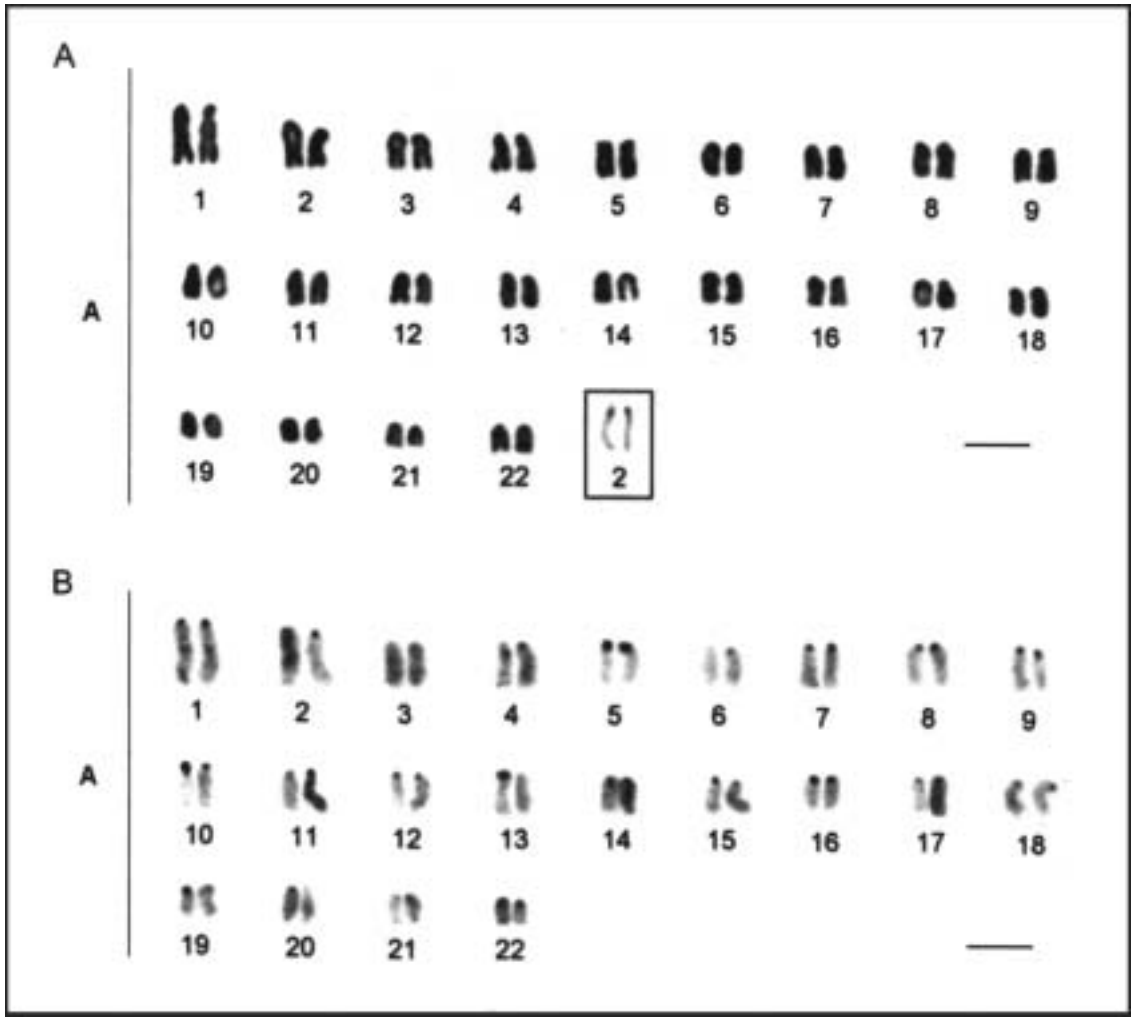

Fig. 1 — *Balistes vetula* karyotype. Ag-NORs sites in telomeric position on the 2nd chromosome pair (A). C banding (B). Bar = 5 µ.

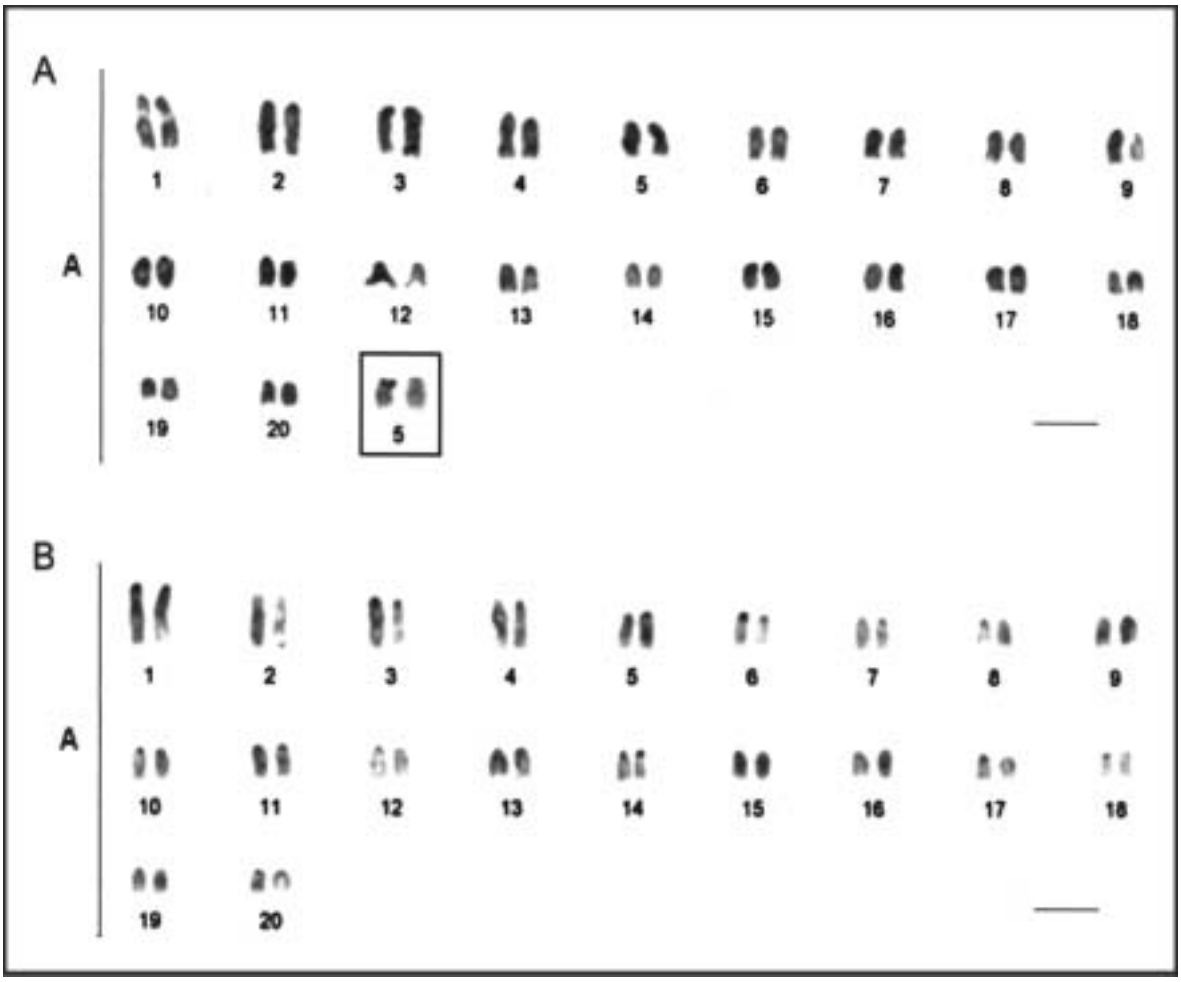

Fig. 2 — *Melichthys niger* karyotype with Ag-NORs localized on telomeric position of the 5th pair, in (A). C banding (B). Bar = 5 μ.

C. antennatus specimens showed a karyotype with 2n=52 (6m+46st/a), FN=58, presenting Ag-NORs sites on the 2nd pair and heterochromatic blocks distributed on most of the pairs in telomeric position (Figures 3 (A) and (B)).

S. testudineus showed a modal value of 2n=46 and its karyotype consisted of diminutive sized chromosomes, with karyotypic formula equal to 18m+4sm+6st+18a and FN=74. In this species, telomeric NORs sites were identified on the first pair of the karyotype and a heterochromatic pattern with segments distributed on the telomeric portions of its chromosomes (Figures 4 A and B).

The treatment with RE *Eco*RI did not shows band patterns in the species analyzed (data not shown).

DISCUSSION

Among the Tetraodontiformes, the karyotypes of the Balistidae, Diodontidae and Tetraodontiformes families have been considered derived, compared to the more basal members of the Triacanthidae family (Brum 1995).

Some previous studies on species of the *Sphoeroides* genus in the Atlantic (Brum *et al.* 1994b; Brum 1995) described the *S. greeleyi* (2n=46; FN=70) and *S. spengleri* (2n=46; FN=64) karyotypes from Rio de Janeiro. Another species, *S. tyleri*, showed a karyotype of 2n=46, and a chromosome formula of 12m/sm/+34st/a (FN=54) (Brum 2000).

The different Tetraodontiformes families underwent an extremely diversified karyotype evolution, considering the numerical and structural aspects of their complements, with 2n varying from 28 to 52 chromosomes, and marked differences in the fundamental numbers that varied from 33 to 72. Analyses performed highlight the combined importance of the different chromosome rearrangements in the evolutionary modeling of their karyotypes, such as centric fissions (Arai and Na-

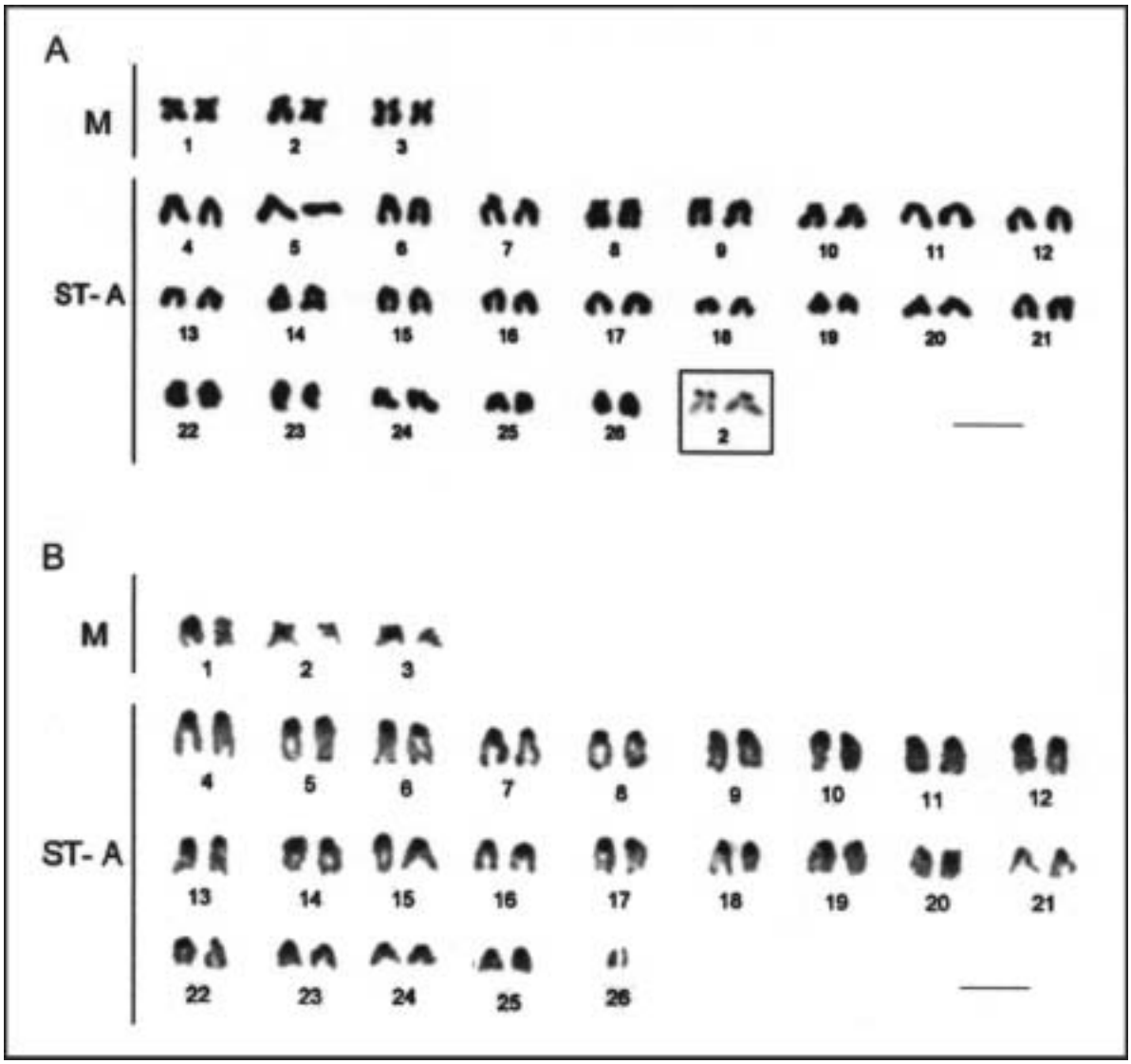

Fig. 3 — *Chilomycterus antennatus* karyotype. In the box the Ag-NORs site localized on the 2nd pair (A). C banding (B). Bar = 5 μ.

GAIWA 1976), fusion and especially pericentric inversions.

The Balistidae have diploid and FN values lower than 2n=48, varying from 34 to 46 (MUROFUSHI and YOSIDA 1979; ARAI and NAGAIWA 1976; TAKAI and OJIMA 1987), with most of their representatives presenting subtelo-acrocentric chromosomes. This karyotypic pattern was also observed in the present study in *B. vetula* (2n=44) and *M. niger* (2n=40). The origin of the reduced diploid numbers in these species seems to be the result of centric fusions or in tandem followed by pericentric inversions, which seems to be common in other species in the family (ARAI and NAGAIWA 1976). In the *Melichthys* genus a diploid value of 2n=40a seems to be conserved, and has been found in *M. vidua* (KITAYAMA and OJIMA 1984) in addition to *M. niger*.

The Diodontidae species, *Diodon bleekeri* (2n=46 and FN=56; ARAI and NAGAIWA 1976), *D. holocanthus* (2n=46, FN=66; SÁ-GABRIEL and MOLINA 2001) and *C. spinosus* (2n=52, with 16sm+36st; BRUM 2000) showed a marked numerical diversity for the group. The diploid number (2n=52) identified in *C. antennatus* (present study) was the largest diploid value detected for the Order up to now. It is probable that its origin has occurred by centric fission, the same evolutionary mechanism involved in species of the Ostraciidae (2n=50) (ARAI and NAGAIWA 1976; ARAI 1983).

The Tetraodontids, especially the *Sphoeroides* genus, have as karyotypic pattern the presence of

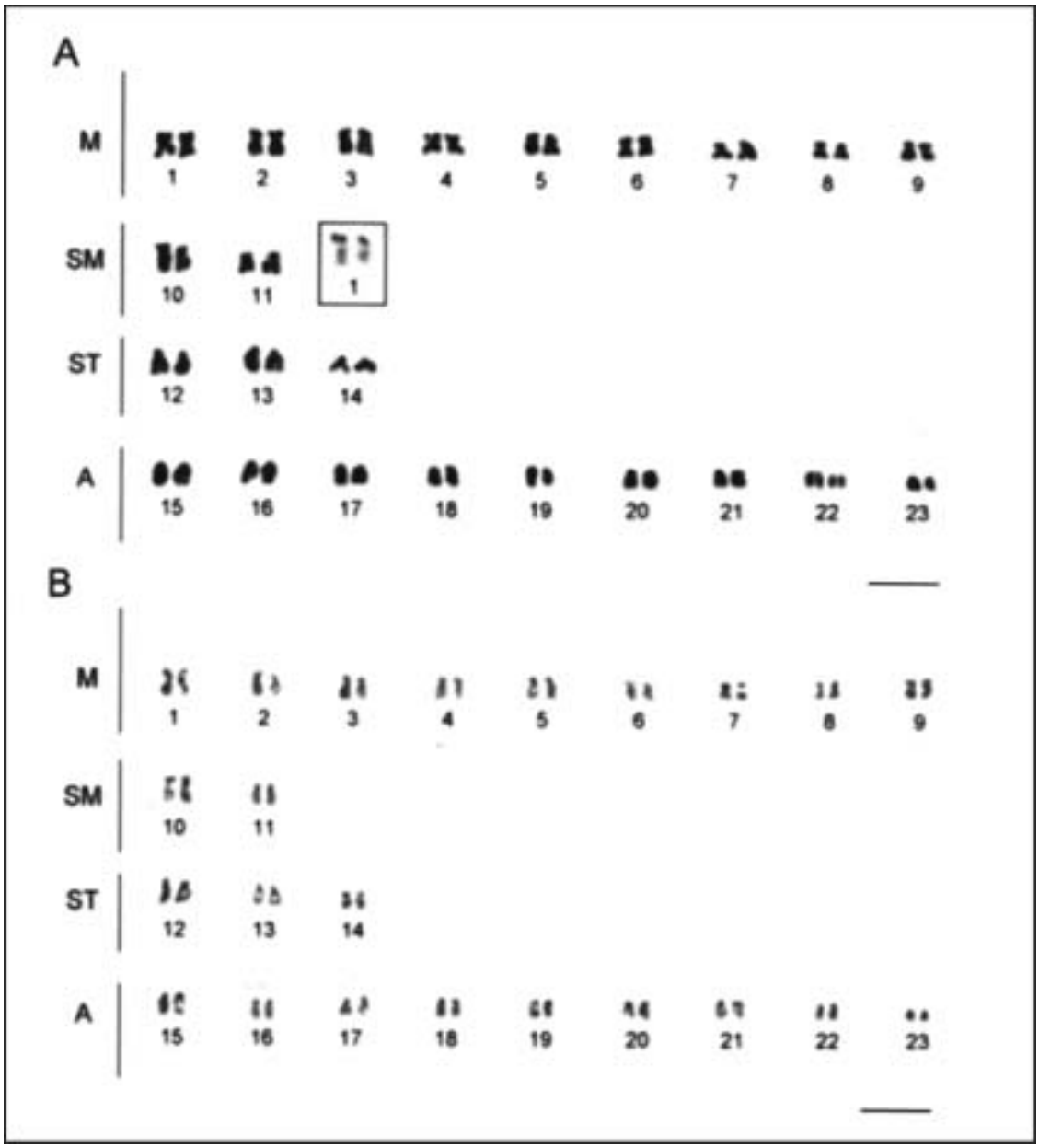

Fig. 4 — *Sphoeroides testudineus* karyotype. Nucleolar organizer pair (1st pair) is highlighted (A). C banding (B). Bar = 5 μ.

small sized chromosomes, indicating its use as a model in studies of chromosome structure and functional genome aspects (BRADFORD *et al.* 1997; BRAINERD *et al.* 2001). The reduction in DNA content seems to be the peculiar mechanism involved in the Tetraodontidae genome evolution (BRAINERD *et al.* 2001).

Among the vertebrates, the largest and also the smallest quantities of DNA, belong to fish species. The low DNA content shown by a species is reflected in a reduced chromosome size and/or reduced chromosome number, as observed in *Tetraodon fluviatilis*, the lowest DNA value known for vertebrates (LAMATSHC *et al.* 2000; BRAINERD *et al.* 2001). It is probable that differences in the genome size are connected with significant heterochromatin losses in some groups (KLOC and ZAGRODZINSKA 2001), as may have occurred in Tetraodontidae. This is particularly evident on comparison of the karyotypes from the different families analyzed that present notable divergences for chromosome size. According to WHITE (1973), a predisposition to certain types of reorganization but not for others, such as the re-

Table 1 — Karyotypic data disponible for Tetraodontiformes species.

Famíly	Species	2n	Chromosome formula	FN	References
TRIACANTHIDAE	*Triacanthus brevrosins*	48	48a	48	Choudhury *et al.*, 1982
	T. striglifer	48	48a	48	Rish, 1973
BALISTIDAE	*Balistapus undulatus*	42	42st/a	42	Takai and Ojima, 1987
	Balistes vetula	44	44a	44	Sá-Gabriel and Molina, 2004
	Balistoides conspicullum	44	44st/a	44	"
	B. viridescens	44	2m + 2sm +40st/a	48	"
	Cantherhines pardalis	40	40st/a	40	Arai and Nagaiwa, 1976
	Carolinensis gmelin	44	44a	-	Thode, *et al.,* 1994
	Melichthys vidua	40	40st/a	40	Kitayama and Ojima, 1984
	Melichthys niger	40	40a	40	Sá-Gabriel and Molina, 2004
	Novodon modestus	40	40st/a	40	Murofushi and Yosida, 1979
	Odonus niger	42	42st/a	42	Kitayama and Ojima, 1984
	Oxymonacanthus longirostris	36	36st/a	36	Arai and Nagaiwa, 1976
	Paramonacanthus japonicus	34	34st/a	34	Murofushi and Yosida, 1979
	Parika scaber	40	40st/a	40	Murofushi *et al.,*1989
	Pseudobalistes flavimarginatus	44	2sm + 42st/a	46	Arai and Nagaiwa, 1976
	Rhineacanthus aculeatus	44	44st/a	44	Arai and Nagaiwa, 1976
	R. echarpe	44	44st/a	44	Kitayama and Ojima, 1984
	R. verrucosus	44	44st/a	44	Arai and Nagaiwa, 1976
	Rucanus arcodas	36	36 st/a	36	"
	Sufflamen chysopterus	46	46st/a	46	"
	S. traenatus	46	46st/a	46	Takai and Ojima, 1987
MONACANTHIDAE	*Stephanolepis cirrhifer (M)*	33	30st/a	34	Murofushi *et al.,* 1980
	S. cirrhifer (F)	34	34st/a	34	"
	S. hispidus	33	-	-	Pauls, 1993
	S. hispidus (M)	33	32a + 1sm	34	Sá-Gabriel and Molina, 2004
	S. hispidus (F)	34	34a	34	Sá-Gabriel and Molina, 2004
OSTRACIIDAE	*Lactoria diaphana*	36	-	48	Arai, 1983
	Ostracion cubicus	50	4sm + 46st/a	54	Arai and Nagaiwa, 1976
	O. immaculatus	50	4sm + 46st/a	54	Arai, 1983
TETRAODONTIDAE	*Arothron hispidus*	42	36sm + 6st/a	78	Natarajan and Subrahmanjan, 1974
	A. immaculatus	42	14m + 16sm + 12st/a	72	Arai and Nagaiwa, 1976
	A. immaculatus	42	12m + 14sm + 16st/a	68	Choudhury *et al.,*1982
	A. leopardus	40	14m + 14sm + 12st/a	68	"
	A. nigropunctatus	38	14m + 20sm + 4st/a	72	Arai and Nagaiwa, 1976
	A. reticularis	42	12m + 14sm + 16st/a	68	Choudhury *et al.,*1982
	Canthigaster coronata	28	8m/sm + 20st/a	36	Arai, 1983
	C.rivulata	34	4m + 6sm + 10st/a + 14a	54	Arai and Nagaiwa, 1976
	Chelonodon patoca	40	14m + 16sm + 10st/a	70	"
	Fugu chrysops	44	6m + 14sm + 24st/a	64	"
	F. niphobles	44	20m/sm + 24st/a	64	Arai and Katsuyama, 1973
	F. pardalis	44	-	-	Arai, 1983
	F. poecilonotus	44	-	-	Arai, 1983
	Lagocephalus laevigatus	46	-	-	Sá-Gabriel and Molina, 2001
	L. lunaris	44	10m + 14sm + 20st/a	68	Choudhury *et al.,*1982
	Monotetra palambangensis	42	-	-	Hinegardner and Rosen, 1972
	Sphoeroides greeleyi	46	24m/sm + 22st/a	70	Brum *et al.*,1994b
	S. tyleri	46	12m/sm + 34st/a	58	Brum *et al.*,1996
	S. spengleri	46	20m/sm + 26st/a	66	Brum *et al.*,1994b
	S. spengleri	46	-	66	Sá-Gabriel and Molina, 2001
	S. testudineus	46	18m+4sm+6st+18a	74	Sá-Gabriel and Molina, 2004
	Takifugu niphobeles	44	4m/sm + 16sm + 24st/a	64	Miyaki *et al.,*1995
	T. pardalis	44	6m/sm + 16sm + 22st/a	66	"
	T. poecilonotus	44	12m + 10st + 22st/a	66	"
	T. radiatus	44	8m + 14st + 22st/a	66	"
	T. rubripes	44	10m + 12st + 22st/a	66	"
	T. xanthopterus	44	8m + 14st + 22st/a	66	"
	Tetraodon cutcutia	42	16m + 12st + 10a	70	Khuda-Bukhsh and Barat, 1987
	T. fluviatilis	42	2m + 4sm + 2st + 34a	50	Mandrioli, 2000
	T. nigroviridis	42	20m/sm + 22st	62	Fisher, 2000
DIODONTIDAE	*Diodon bleekeri*	46	-	58	Arai and Nagaiwa, 1976
	D. holocanthus	46	20m/sm + 26st/a	66	Sá-Gabriel and Molina, 2001
	Chilomycterus spinosus	52	16m/sm + 36st/a	68	Brum, 2000
	C. antennatus	52	6m+46st/a	58	Sá-Gabriel and Molina, 2004

duction in the DNA content, characterizes an orthoselective process. Trend for similar structural changes in the karyotype has been identified in some fish groups, such as the occurrence of Robertsonian translocation in the Chrominae subfamily (MOLINA and GALETTI 2002), multiple pericentric inversions in *Stegastes* genus (W.F. MOLINA, personal communication), belonging to the Pomacentridae family, or the evolution mediated by heterochromatinization events in Anostomidae (MOLINA *et al.* 1998).

Previous analyses carried out on *S. testudineus* and *S. spengleri* (SÁ-GABRIEL and MOLINA 2001) indicated that this genus shows numerical conservatism, as all the species are characterized by presenting 2n=46 and present differentiations for the chromosome formulas, derived from pericentric inversion events. Within some genera, such as in *Takifugu* and *Sphoeroides*, were observed the occurrence of diminutive chromosomes and the presence of diversified karyotypic formulae. These characteristics group the members of this family in a condition of high specialization, not frequently observed in other fish species. Efforts to localize sex-specific sequences in this family using a great number of RAPD markers were fruitless (LI et al. 2002), corroborating the absence of sex chromosomes how shown by cytogenetic data (BRUM 1995).

All the species analyzed presented NORs sites on a single chromosome pair in telomeric position. This is considered a simplesiomorphic condition in fish (ALMEIDA-TOLEDO 1985). The differences among the NOR-bearing pairs did not suggest the existence of interspecific homeology. Another peculiar cytogenetic aspect of Tetraodontiformes is the small quantity of heterochromatic regions, localized in centromeric position on most of the chromosome pairs.

The wide karyotypic diversity present in this Order is compatible with a scenario of intense changes, established by the adaptive irradiation to which the group seems to have been submitted.

The difficulty in obtaining banding patterns in fish has suggested the use of restriction endonucleases as an alternative in the study of their karyotypes. Digestion with RE has contributed to a better understanding of the chromosome structure, identifying different heterochromatin classes and chromosome polymorphisms in fish (VIÑAS *et al.* 1998). This technique has been used in different groups of fish, such as trout and salmon (Salmoniformes, LOZANO *et al.* 1991; PEREZ *et al.* 1999), moray (Anguilliformes, CAU *et al.* 1992), eels (Synbranchiformes, VIÑAS *et al.* 1994), cyprinids (Cypriniformes, PADILLA *et al.* 1993) and cartilaginous fish (ROCCO *et al.* 1996; ROCCO *et al.* 2002). The absence of a banding pattern after treatment with RE *Eco*RI was not unexpected, and can be explained by the inexistence of specific sites of action by the enzyme, as identified in *Astyanax scabripinnis* (MAISTRO *et al.* 1999).

From the cytogenetic data obtained to these Tetraodontiformes groups it was verified that similarly to the derived morphological traits, diverse evolutionary tendencies are also reflected in their karyotypes notably diversified. This condition demonstrates an opposite tendency to the conservatism observed in Perciformes.

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