

Classical and molecular cytogenetic characterization of *Agonostomus monticola*, a primitive species of Mugilidae (Mugiliformes)

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Abstract This study reports the first description of the karyotype of *Agonostomus monticola*, a species belonging to a genus which is considered to be the most primitive among living mugilid fish. Specimens from Panama and Venezuela were cytogenetically analysed by conventional chromosome banding (Ag and base-specific-fluorochrome staining, C-banding) and by fluorescent in situ hybridization (FISH). *Agonostomus monticola* showed a chromosome complement of $2n = 48$, composed of 23 acrocentric and one subtelocentric chromosome pairs and a pericentromeric distribution of the C-positive heterochromatin in all chromosomes. Major ribosomal genes were found to be located on the short arms of the subtelocentric chromosome pair number 24 and minor ribosomal genes in a paracentromeric position of a single medium-sized chromosome pair. All these observed cytogenetic features are similar to those previously described in four representatives of two genera, *Liza* and *Chelon*, which are considered to be among the most advanced in the family. Thus, this karyotypic form might represent the plesiomorphic condition for the mullets. This hypothesis regarding the plesiomorphic condition, if confirmed, would shed new light on the previously inferred cytotaxonomic

relationships for the studied species of Mugilidae, because the karyotype with 48 acrocentric chromosomes, which has been so far regarded as primitive for the family, would have to be considered as derived.

Keywords Constitutive heterochromatin · FISH · Karyotype · 18S and 5S rDNA · NORs

Introduction

The family Mugilidae contains approximately 70 fish species (Nelson 2006), distributed in all the tropical and temperate coastal marine and brackish waters of the world. Both the assignment of the family (to a distinct order or to a suborder of Perciformes) and the number of valid genera and species within it have been widely revised over the years (see Sola et al. 2007, for a review). Mugilidae have also been the subject of many cytogenetic investigations. Earlier studies indicated that the family is mainly characterized by the conservative 48 all-uniarmed karyotype originally proposed by Ohno (1974) as the primitive teleostean karyotype, which is typically shared by many marine euteleostean families. More recent studies (reviewed in Sola et al. 2007) have shown that the application of suitable chromosome markers revealed finer chromosome rearrangements, suggesting that the chromosomal evolution in Mugilidae is more complex than was originally assumed.

To summarize, the available information, regarding approximately 25% of the mugilid species, indicates three main cytotypes: cytotype A, composed of 48 exclusively acrocentric chromosomes ($NF = 48$), which is the most common (ten species belonging to four genera); cytotype B, composed of 46 acrocentric plus two subtelocentric chromosomes ($NF = 48$), displayed by five species

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belonging to three genera; and cytotype(s) C, mainly, or even exclusively, composed of biarmed chromosomes. The latter cytotypes have been found in specimens of *Mugil curema* from Louisiana and Brasil ($2n = 28$, $NF = 48$; LeGrande and Fitzsimons 1976; Nirchio et al. 2005) and from Venezuela ($2n = 24$, $NF = 48$; Nirchio and Cequea 1998; Rossi et al. 2005; Nirchio et al. 2007, among others). They were originally interpreted (LeGrande and Fitzsimons 1976) to be due to extensive Robertsonian fusions which occurred in an ancestral group with an all-acrocentric chromosome complement (cytotype A) similar to that of *M. cephalus*. The cytotype A has been regarded to date as plesiomorphic in any cytotaxonomic consideration in the family (Sola et al. 2007). In fact, it is shared by all the *Mugil* species investigated (with the exception of *M. curema*) and this genus is phylogenetically more basal (Thomson 1997) compared to the other genera (*Rhinomugil*, *Valamugil*, *Liza*, *Chelon*, *Oedalechilus*) so far karyologically studied.

According to Thomson (1997), all the morphological features suggest that *Agonostomus* is the most primitive among living Mugilidae. Moreover, the mountain mullet, *Agonostomus monticola* (Bancroft 1834), is the only one that ascends far inland and spends all its adult life in freshwater, although it possibly spawns catadromously (Phillip 1993). Distributed from North Carolina, in North America, to Colombia and Venezuela, including the West Indies, in South America, *A. monticola* is the only representative of the genus in the area. The other two species, *A. catalai* and *A. telfairii*, in fact, are found in the South West Indian Ocean. In spite of its peculiar systematic position and bio-ecological traits, no phylogenetic studies using more modern approaches, already applied in other Mugilidae (Caldara et al. 1996; Papatotiropoulos et al. 2002; Rossi et al. 2004; Turan et al. 2005; Sola et al. 2007), have been carried out in this genus.

In this study, specimens of *A. monticola* from Panama and Venezuela were cytogenetically characterized by classical (Ag and base-specific-fluorochrome staining, C-banding) and molecular techniques (fluorescent in situ hybridisation—FISH), in order to verify whether this primitive species shows the chromosome constitution which has, to date, been considered plesiomorphic.

Materials and methods

A total of 27 specimens of *A. monticola* were caught with seine nets in the Changuinola River, Bocas del Toro, Panama (six specimens) and in La Trilla River, Aragua State, Venezuela (21 specimens). Voucher specimens were deposited at the Ichthyology Collection of the Escuela de Ciencias Aplicadas del Mar, Universidad de Oriente.

Chromosome preparations were obtained from cephalic kidney cells using conventional air-drying techniques. Silver-stained nucleolus organizer regions (Ag-NORs) were obtained as described by Howell and Black (1980). Fluorescence staining with the GC-specific stain chromomycin A₃ (CMA₃) and the AT-specific stain 4',6-diamidino-2-phenylindole (DAPI) was carried out according to Sola et al. (1992). C-banding was performed following the method of Sumner (1972).

The 5S and 18S ribosomal RNA gene (rDNA) loci were identified by FISH according to the method of Pinkel et al. (1986). A 1.8 kb sequence of the 18S rDNA of *Oreochromis niloticus* (Nile tilapia), cloned in pGEM-T plasmid, was used as a probe to localize the major rDNA sites. Repeats used as probes for mapping the minor 5S rDNA 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 Martins and Galetti (1999), employing DNA extracted from muscle of one mountain mullet (Sambrook and Russell 2001).

The probes were labelled by nick translation with biotin-14-dATP, following the manufacturer's instructions (Bionick™ Labelling System-Gibco.BRL). Signals were detected after amplification by a three-round application of Avidin-FITC/biotinylated Anti-avidin (Roche). Chromosomes were counter-stained with propidium iodide (50 µg/ml) diluted in an antifade.

Mitotic chromosomes were photographed using a Motic B400 microscope equipped with a Moticam 5000C digital camera and the images were digitally processed with Adobe Photoshop CS3 (Windows). FISH metaphases were examined with a Zeiss Axiophot photomicroscope, using Kodak Gold Ultra 400 ASA film.

Results

In all of 27 mountain mullet individuals examined, the karyotype (Fig. 1) was found to be made of 48 chromosomes, 46 acrocentrics and 2 subtelocentrics, uniformly decreasing in size. Only the subtelocentric chromosome pair 24, the smallest in the complement, can be identified unequivocally, due to its size and the presence of short arms with prominent terminal achromatic regions, often heteromorphic in size (Fig. 1). No differences in the karyotype were observed among specimens of different sex or from the two geographically distant sampling localities.

As expected from their Giemsa-staining features, Ag-NORs were found to be terminally located on the short arms of chromosomes 24 after silver staining (Fig. 2a). C-banding revealed heterochromatic regions at the centromere of all chromosomes (Fig. 2b).

Fig. 1 Giemsa-stained karyotype of *Agonostomus monticola*. Scale bar = 10 μ m



After CMA₃-staining (Fig. 2c), fluorescent signals corresponding both in number and morphology to Ag-NORs were detected on the short arms of chromosome pair number 24. Apart from NORs, CMA₃ produced a uniform staining pattern along chromosomal arms. The AT-specific DAPI also produced a uniform staining pattern of all chromosomes, with a fainter staining of the NOR-bearing short arms of chromosome pair 24 (data not shown).

FISH with 18S rDNA probe produced bright signals, corresponding to the ones obtained after Ag- and CMA₃-staining, on the short arms of chromosome pair number 24 (Fig. 3a), which indicates that the species possesses no additional NOR-sites. FISH with 5S rDNA probe produced paracentromeric signals in a medium-sized acrocentric chromosome pair (Fig. 3b).

Discussion

By adding the chromosome complement of *A. monticola* reported in this study to the Mugilidae database, the genera of the family so far cytogenetically analysed rises to seven, covering a total of at least 17 species. The number could be higher, given that karyological data have probably disclosed the existence of a *M. curema* species complex (Nirchio et al. 2005). The obtained data confirms that the 48 all-uniarmed karyotype is the most common in the family, as it is shared by all the investigated species, with the exception of *M. curema*.

As far as the *A. monticola* cytogenetic features are concerned, the C-positive heterochromatin distribution did not reveal any chromosome-specific heterochromatic block nor differentially AT or GC-enriched DNA, with the exception of the CMA₃-positive short arms of chromosome

pair number 24, where NORs are located. The GC-richness of NORs is quite common in fish, although evidence of GC-poor NORs (Souza et al. 2001, Rossi and Gornung 2005), sometimes in combination with GC-rich regions other than NORs (Rab et al. 2002; Gromicho et al. 2005; Kavalco et al. 2005) have also been reported. The major and minor rDNA clusters were found located on distinct chromosome pairs, as in the other Mugilidae so far analysed (Sola et al. 2007). A similar situation is also found in fish, in general, with very few exceptions (Moran et al. 1996; Fujiwara et al. 1998, 2007; Almeida-Toledo et al. 2002). Although the major rDNA location is unambiguous in *A. monticola* and in most of the Mugilidae analysed (Sola et al. 2007), the paracentromeric and interstitial location of minor rDNA clusters on a medium-sized chromosome pair does not permit its unequivocal classification. However, pursuing a parsimonious criterion, the 5S rDNA bearing chromosomes pair observed in *A. monticola* might be homeologous to the chromosome pair of similar size, classified as number 8, which shows a similar minor rDNA location observed in the other mugilid species with this type of chromosome complement (see Sola et al. 2007, for references).

Considering the available cytogenetic data, *A. monticola* shows an apparent similarity in the karyotype constitution, C-positive heterochromatin distribution, major and minor rDNA loci numbers and locations, to *Chelon labrosus* and three *Liza* species, *Liza ramada*, *L. aurata*, *L. saliens*, (see Sola et al. 2007, for references), that is, the karyotype of *A. monticola* can be assigned to cytotype B.

The latter cytotype is therefore shared by a genus, *Agonostomus*, which is the most basal in the family, and by two other genera which are, on the other hand, considered to be the most advanced in the family (Thomson 1997).

Fig. 2 Metaphase plates of *Agonostomus monticola* after (a) Ag-staining, (b) C-banding and (c) CMA₃-staining. Arrows indicate NOR-bearing chromosomes. Scale bar = 10 μ m

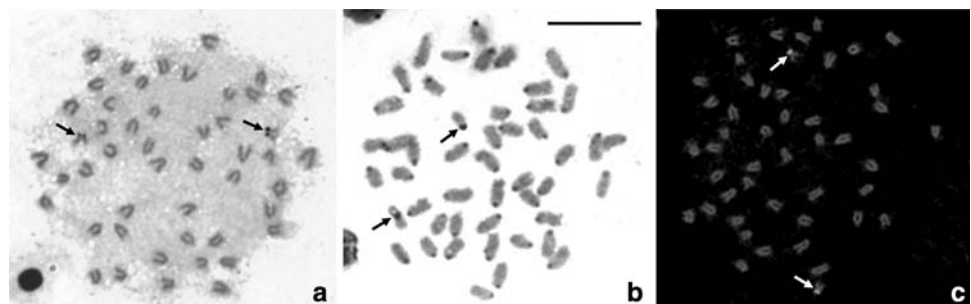
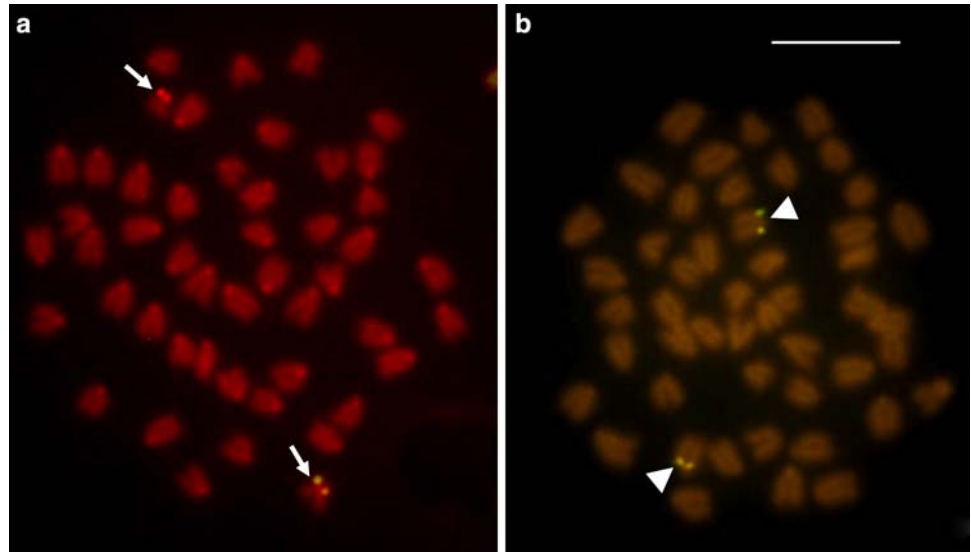


Fig. 3 Metaphase plates of *Agonostomus monticola* after FISH with (a) 18S rDNA, (b) 5S rDNA. Arrows indicate NOR-bearing chromosomes, arrowheads 5S rDNA clusters. Scale bar = 10 μ m



This indicates that the cytotype B might be regarded as the plesiomorphic condition for the karyotype in the family. Consequently, the cytotype A, shared by most of the species of a genus, *Mugil*, in an intermediate systematic position, and which has been so far considered to be the closest to the ancestor' karyotype, should now to be re-considered as derived. This factor should be taken into consideration in any cytotaxonomic reconstruction in the family.

The results obtained in *A. monticola* are therefore of considerable interest and further karyological studies should be carried out on the remaining undescribed species of Mugilidae, in order to provide a more general picture of karyoevolutive trends in the family. This could be useful to gain a more general understanding of chromosomal evolutionary processes in different taxa of fish with the 48-uniarmed-chromosome karyotype.

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