Genetica (2007) 131:195–200
DOI 10.1007/s10709-006-9131-4

ORIGINAL PAPER

Chromosomal mapping of the major and minor ribosomal genes, $(GATA)_n$ and $(TTAGGG)_n$ by one-color and double-color FISH in the toadfish *Halobatrachus didactylus* (Teleostei: Batrachoididae)

Alejandro Merlo · Ismael Cross · José Luis Palazón · Carmen Sarasquete · Laureana Rebordinos

Received: 28 July 2006 / Accepted: 6 December 2006 / Published online: 28 December 2006

Abstract The karyotype of *Halobatrachus didactylus* presents 46 chromosomes, composed of eight metacentric, 18 submetacentric, four subtelocentric, and 16 acrocentric chromosomes. The results of FISH showed that the major ribosomal genes were located in the terminal position of the short arm of a large submetacentric chromosome. They also showed a high variation in the hybridization signals. The products of amplification of 5S rDNA produced bands of about 420 pb. The PCR labeled products showed hybridization signals in the subcentromeric position of the long arm of a submetacentric chromosome of medium size. Double-color FISH indicated that the two ribosomal families are not co-located since they hybridizated in different chromosomal pairs. Telomeres of all the chromosomes hybridized with the $(TTAGGG)_n$ probe. The GATA probe displayed a strong signal in the long arm of a submetacentric chromosome of medium size, in the subcentromeric position. The double-color FISH showed that the microsatellite GATA and the 5S rDNA gene are located in different chromosomal pairs. The majority presence of GATA probes in one pair of chromosomes is unusual and considering its distribution through different taxa it could be due to evolutionary mechanisms of heterochromatine accumulation, leading to the formation of differentiated sex chromosomes.

Keywords FISH · $(GATA)_n$ · *Halobatrachus didactylus* · Major ribosomal genes · Minor ribosomal genes · Toadfish · $(TTAGGG)_n$

A. Merlo · I. Cross · L. Rebordinos (✉)
Laboratorio de Genética, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro, Puerto Real, Cadiz 11510, Spain
e-mail: laureana.rebordinos@uca.es

J. L. Palazón · C. Sarasquete
Instituto de Ciencias Marinas, CSIC, Polígono Río San Pedro, Puerto Real, Cadiz 11510, Spain

Introduction

Halobatrachus didactylus (Schneider, 1801), known as the Lusitanian toadfish, is a teleostean of the Batrachoididae family (Batrachoidiformes Order), whose natural habitats are soft sand as well as rocky, sea beds, and reefs. The Batrachoides are composed of 71 species belonging to three subfamilies (Froese and Pauly 2005) and are widely distributed by the Atlantic, Pacific, and Indian Oceans. *H. didactylus* is the only species of this family in the Iberian Peninsula (Palazón et al. 2003) and populations are distributed along the coasts from of the Bay of Biscay to Ghana as well as the western Mediterranean (Roux 1986).

H. didactylus is an important member of the fish community and is considered to be of commercial interest. Furthermore, it has been used traditionally as a model animal in toxicology experiments (Sarasquete et al. 1982), and in haematology, reproduction and histophysiology (Sarasquete 1983; Palazón et al. 2001; Desantis et al. 2007). It was one of the first animals to be used as a model for research in outer space (Boyle et al. 2001). In spite of this, genetic knowledge of Batrachoididae family is scarce and cytogenetical studies have been limited to the analysis of the karyotypes of some species, such as *Porichthys notatus* (Gold et al.

1980), *Batrachoides pacifici* (Nirchio et al. 2001), *Amphichthys cryptocentrus, Batrachoides manglae, Thalassophryne maculosa, Porichthys plectrodon* (Nirchio et al. 2002, 2004a, b), and *Porichthys porosissimus* (Brum et al. 2001).

In *H. didactylus*, only the karyotype has been described (Gutiérrez et al. 1984) and localization of the nucleolar organizer regions has been obtained by means of silver staining (Ag-NORs) (Palazón et al. 2003). Within fish species the localization of sequences of DNA in the chromosomes has often been obtained by means of Fluorescence in situ Hybridization (FISH), this becoming an essential tool in their citogenetic analysis (Schwarzacher 2003). The FISH analysis has only been applied to the toadfish *Thalassophryne maculosa* for location of the ribosomic genes 18S-5.8S-28S (Nirchio et al. 2004a). Sequences $(TTAGGG)_n$, are present in the telomeres of the chromosomes of all vertebrates and their study allows one to establish the presence of chromosomal rearrangements, such as Robertsonian fusions or inversions, which are involved in the evolution of the chromosomes. The locations of GATA repetitions have rarely been studied in marine organisms using FISH (Vitturi et al. 2002; Cross et al. 2005) and there only exists one previous work within the fish species (Cross et al. 2006). It has been proposed that GATA sequences may play an important role in evolution of the sexual chromosomes and in the organization of genomes (Singh and Jones 1982; Subramanian et al. 2003). The locations of the major and minor chromosomal genes are very useful as chromosomal markers in evolutionary and phylogenetic studies in fish (Phillips and Reed 1996). In order to characterize the karyotype of *H. didactylus* we carried out an exhaustive analysis using several probes including 5.8S + 18S + 28S rDNA, 5S rDNA, $(GATA)_n$ and $(TTAGGG)_n$ by one-color and two-color FISH.

Materials and methods

Sampling and slide preparation

The sampling of specimens of *H. didactylus* (six males and two females) was carried out in shallow water regions of the Bay of Cádiz. The chromosome preparations were made from cephalic kidney as described by Palazón et al. (2003). Slides were pretreated with RNAse, pepsin and formaldehyde according to Weigant et al. (1991). Finally, the samples were dehydrated in successive steps using ethanol and stored at –80°C up to the moment of hybridization.

Probes and FISH techniques

The 5.8S-18S-28S rDNA probe was obtained with polymerase chain reaction (PCR)-based cloning and labeled according to the methods described by Cross et al. (2003, 2005). The $(GATA)_n$ and $(TTAGGG)_n$ probes were obtained by PCR as was described by Ijdo et al. (1991), using the primers $(GATA)_7$ and $(TATC)_7$, respectively. The major ribosomic, the GATA and the telomeric probes were labeled by Nick Translation procedure according to the manufacturer instructions.

The 5S rDNA probe was obtained using the primers reported by Pendás et al. (1994) and the products of amplification consisted of bands of about 420 pb. The 5S rDNA probe was also labeled with tetramethylrhodamine-5-dUTP (Roche Molecular Biochemicals) for two-color FISH treatment. The PCR reaction mix was composed of a 4 μl of DNA template, 3 μM of $MgCl_2$, 200 μM of each dNTP, 6 μM of tetramethylrhodamine-5-dUTP, 400 pM of each primer, 2.5 U of Taq polymerase (Euroclone®), and the appropriate polymerase buffer. PCR conditions were 35 amplification cycles at an annealing temperature of 59°C.

Labeled telomeric and GATA probes, were mixed with hybridization solution, and added to the chromosome preparations and denatured for 7 min at 72°C. Ribosomal probes and slide preparations were denatured separately at 75 and 83°C, respectively. The slides were left to hybridize overnight at 37°C. Post-hybridization washes and immunocytochemical incubations were carried out as described by Cross et al. (2003, 2005). For the double-FISH protocol, the chromosomes were counterstained with DAPI (1.3 μg/ml) in antifade solution (Vector Labs.). Finally, better images were obtained with an epifluorescence microscope (*Axioskop 2 Plus, Zeiss*), equipped with a cooled camera (*CoolSnap, Photometrics© Inc.*).

Results

The karyotype of *H. didactylus* presents a diploid number of 46 chromosomes and it is composed of eight metacentric, 18 submetacentric, four subtelocentric, and 16 acrocentric chromosomes (Fig. 1). In the present study four probes were used to make the one-color FISH: 5.8-18-28S rDNA, 5S rDNA, $(GATA)_n$ and $(TTAGGG)_n$. The probe for the major ribosomal gene hybridized in the terminal position of the short arm of a large submetacentric chromosomal pair of *H. didactylus* (Fig. 2a), with notable size variation of signals between chromosomes and among metaphases.

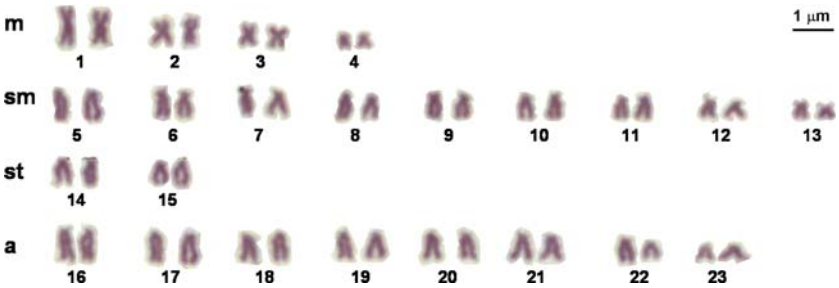

Fig. 1 Karyotype of toad fish *H. didactylus* after conventional Giemsa staining

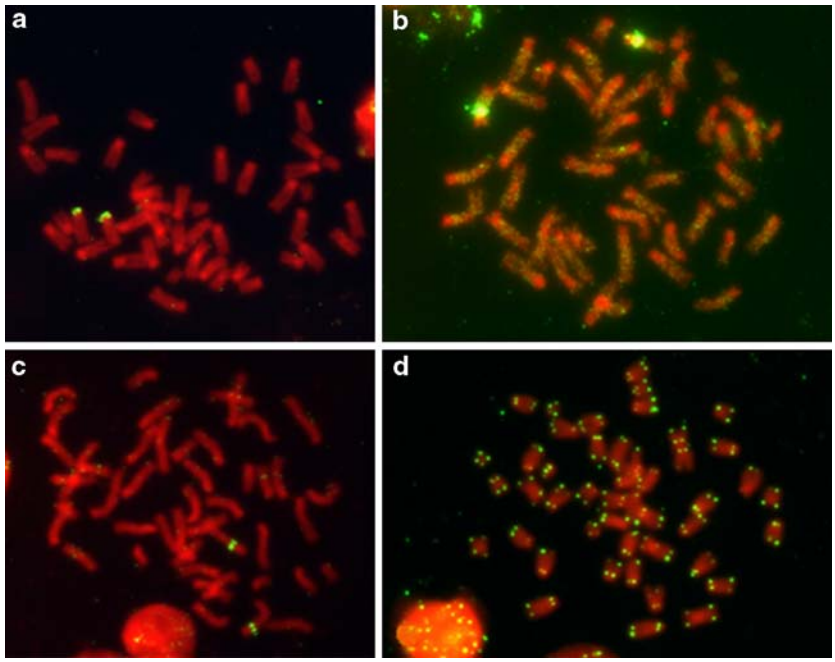

Fig. 2 *H. didactylus* metaphases showing FISH with 18S-5.8S-28S rDNA (**a**), 5S rDNA (**b**), $(GATA)_n$ (**c**), and $(TTAGGG)_n$ (**d**) probes

On the other hand, the 5S rDNA probe showed hybridization signals in the subcentromeric position of the large arm of a submetacentric chromosome of medium size (Fig. 2b). The GATA probe displayed a strong signal in the long arm of a submetacentric chromosome of medium size, in its subcentromeric position (Fig. 2c). The $(TTAGGG)_n$ probe, hybridized to both ends of each of the arms of the chromosomes of *H. didactylus* (Fig 2d). Inner signals were not detected, but size polymorphisms referring to the signals between chromatids and arm chromosomes were detected.

The two-color FISH analyses were carried out using the major and minor rDNA probes and the GATA probes. As shown in the Fig. 3a–c, the two ribosomal families were not co-located since they were in different chromosomal pairs occupying different positions: in the terminal position for the 18-5.8-28S rDNA and in the subcentromeric position for the 5S rDNA. The results indicated that the GATA and the 5S rDNA were located in the subcentromeric position of different submetacentric pairs (Fig. 3d–f).

Discussion

The present chromosome findings of *H. didactylus* shows some differences when compared with results reported by Palazón et al. (2003), in which they observed more submetacentric chromosomes and less acrocentric chromosomes. Instead the number of subtelocentric chromosomes was higher in the present observation. This discrepancy may be due to factors related to the resolution of the technique, and to the grade of condensation of chromosomes in the technical preparations (Cross et al. 2002) which would lead to a difference in the number of chromosome arms within a same species (Gül et al. 2004). The fundamental number (FN) in the Batrachoididae family is variable (52–72). According to Nirchio et al. (2002), this variation could be due to chromosome re-organizations, such as the fusion of acrocentric chromosomes, which would lead to a reduction of the diploid number of 48–46 (or even to 44 in *Porichthys*) in the Batrachoididae family. These chromosome re-organizations play a very important role in the processes of speciation (Molina

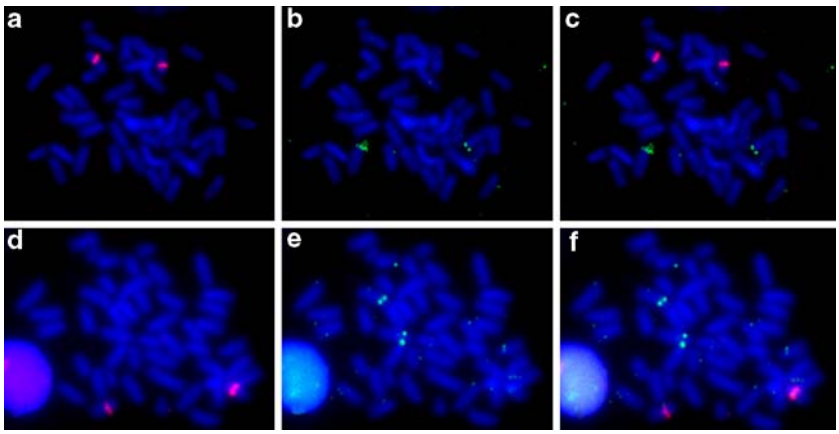

Fig. 3 Double-color FISH with rDNA and GATA probes in *H. didactylus* chromosomes. (**a**–**c**) Rhodamine-labelled 5S rDNA probe (red) (**a**), Digoxigenin-labelled 18S-5.8S-28S probe (green) (**b**), 5S and 18S-5.8S-28S probes (**c**). (**d**–**f**) Rhodamine-labelled 5S rDNA probe (red) (**d**), Digoxigenin-labelled $(GATA)_n$ probe (green) (**e**), 5S and $(GATA)_n$ probes (**f**)

and Galetti Jr 2004). Considering that species with a larger FN are more advanced in evolutionary terms, the most recent species within the Batrachoididae family would be *H. didactylus* (FN = 72) and the more ancient would be *Amphychthys cryptocentrus* (FN = 52) (Nirchio et al. 2002). But heteromorph sex chromosomes, such as suggested by Palazón et al. (2003) have not been found in the members of the Batrachoididae family, including *H. didactylus*.

Hybridization of the $(TTAGGG)_n$ probe at the ends of the chromosomes confirms the presence in *H. didactylus.* The observed variability in the size of the signals may be due to random variations in the conditions of hybridization, or alternatively to a variation in the number of repetitions in telomeres (Guo and Allen 1997). In some fish species, the $(TTAGGG)_n$ family clusters have been found intercalated among the ribosomal family clusters (Sola et al. 2003; Gornung et al. 2004). This association is unusual in vertebrates, and it has been suggested that it functions as a recombination hot point of the NORs (Gornung et al. 2004).

FISH localization of the major ribosomal genes in a submetacentric pair of large size is coincident with the Ag-NOR location reported by Palazón et al. (2003). In other toadfish species, Ag-NORs have been located in centromeric position in an acrocentric chromosome in *Porichthys plectrodon* (Nirchio et al. 2004b) and in terminal position of a subtelocentric pair, in *Thalassophryne maculosa* (Nirchio et al. 2004a). The presence of NORs, either in the terminal position or close to the centromeres (but not in the interstitial positions) is considered to be the plesiomorphic character (Vitturi et al. 1995). This has occurred with analyzed species of the Batrachoididae family including *H. didactylus*.

Usually, in fish, the major and minor rDNA are themselves detected in different chromosomes (Martins and Galetti Jr 2001). It has been suggested than the transcription for different RNA polymerases of every ribosomal family would favor evolutionarily the separation of both the major and the minor loci (Amarasinghe and Carlson 1998). Besides, the mechanisms that apply to the processes of evolution (such as gene conversion and unequal cross-over) would be supported if the clusters were found to be separated (Martins and Galetti Jr 1999). Due to the absence of 5S rDNA sequence and its genomic organization in the family Batrachoididae, the present results obtained in *H. didactylus* would suggest that further cytogenetic, evolutionary, and phylogenetic studies be carried out in this and related fish families.

GATA repetitions are the main component of a satellite DNA isolated from the W chromosome of the females of the snake *Elaphe radiata* (so-called Bkm for banded krait minor satellite DNA) (Epplen et al. 1982). The Bkm family sequences are preserved within this group of snakes, but the quantity increases with the differentiation of sex chromosomes, and they tend to accumulate in W or Y chromosomes of some organisms (Jones and Singh 1985). For example, sex-associated specific Bkm has been identified in turtles *Chelonia mydas* and *Lepidochelys kempi* (Demas et al. 1990). In mice, the Bkm sequences have been located in the Y chromosome, associated with the genes that determine the sex (Singh and Jones 1982). However, a specific distribution of the Bkm sequences has not been observed in other organisms even in species with sex chromosomes (Vitturi et al. 2002). In this context, the results obtained in *H. didactylus* show for the first time a concentration of GATA at a specific location in fish.

A recent analysis of the GATA family sequences in the genome of prokaryotes and eukaryotes (Subramanian et al. 2003) has revealed that GATA repetitions are lacking in prokaryotes, but they are gradually accumulated with evolution. In addition a functional role of the GATA sequences in the gene regulation in

human Y has been proposed (Subramanian et al. 2003).Taken together, the results obtained in the present molecular study may provide a direction for future research with toadfish to study their karyotype evolution, sex chromosome differentiation and phylogeny.

Acknowledgments This paper was partially supported by PAI-CVI-219 (Spain) and ICMAN, CSIC (PAI-RNM-181) and Unidad Asociada de Calidad Ambiental & Patologia (UCA & CSIC, Spain). We thank Jon Taylor for reviewing and correcting the manuscript and two anonymous reviewers for constructive criticism and helpful comments.

References

Amarasinghe V, Carlson JE (1998) Physical mapping and characterization of 5S rDNA genes in Douglas-fir. J Hered 89:495–500

Boyle R, Mensinger AF, Yoshida K, Usui S, Intravaia A, Tricas T, Highstein SM (2001) Neural readaptation to Earth's gravity following return from space. J Neurophysiol 86:2118–2122

Brum MJI, Affonso PRAM, Mota LCG, Pauls E, Neto MRCB (2001) Cytogenetic characterization of *Porichthys porosissimus* (Valenciennes, 1857) (Batrachoididae, Batrachoidiformes) from the Rio de Janeiro Coast, Brazil. Chromosome Sci 5:15–18

Cross I, Vega L, Rebordinos L (2002) Análisis cariotípico y variabilidad de los Ag-NOR en *Crassostrea angulata* (Lamarck, 1819). Boletín del Instituto Español de Oceanografía 18:221–227

Cross I, Vega L, Rebordinos L (2003) Nucleolar organizing regions in *Crassostrea angulata*: chromosomal location and polymorphism. Genetica 119:65–74

Cross I, Díaz E, Sánchez I, Rebordinos L (2005) Molecular and cytogenetic characterization of *Crassostrea angulata* chromosomes. Aquaculture 247:135–144

Cross I, Merlo A, Manchado M, Infante C, Cañavate P, Rebordinos L (2006) Cytogenetic characterization of the sole *Solea senegalensis*: Ag-NOR, $(GATA)_n$, $(TTAGGG)_n$ and ribosomal genes by one-color and two-color FISH. Genetica 128:253–259

Demas S, Duronslet M, Wachtel S, Caillouet C, Nakamura D (1990) Sex specific DNA in reptiles with temperature sex determination. J Exp Zool 253:319–324

Desantis S, Cirillo F, Deflorio M, Megalofonou P, Palazón JL, Sarasquete C, De Metrio G (2007) Histochemical study of glycoconjugates in the toadfish, *Halobatrachus didactylus* oesophagus epithelium. Histol Histopathol 22:23–35

Epplen JT, McCarrey JR, Sutou S, Ohno S (1982) Base sequence of a cloned snake W-chromosome DNA fragment and identification of a male-specific putative mRNA in the mouse. Proc Natl Acad Sci USA 79:3798–3802

Froese R, Pauly D (2005) Fishbase. World Wide Web electronic publication. www.fishbase.org. version May 2005

Gold JR, Karel WJ, Strand MR (1980) Chromosome formulae of North American Fishes. The Progressice Fish-Culturist 42:10–23

Gornung E, Mannarelli ME, Rossi AR, Sola L (2004) Chromosomal evolution in Mugilidae (Pisces, Mugiliformes): FISH mapping of the $(TTAGGG)_n$ telomeric repeat in the six Mediterranean mullets. Hereditas 140:158–159

Gül S, Çolak A, Sezgin I, Kaloglu B (2004) Karyotype Analysis in *Alburnus heckeli* (Battalgil, 1943) from Lake Hazer. Turk J Vet Anim Sci 28:309–314

Guo X, Allen SKJ (1997) Fluorescence in situ hybridization of vertebrate telomere sequence to chromosome ends of the pacific oyster, *Crassostrea gigas* Thunberg. J Shellfish Res 16:87–89

Gutiérrez M, Sarasquete C, Establier R (1984) Nota sobre los efectos del mercurio inorgánico ($HgCl_2$) en los cromosomas del pez sapo marino, *Halobatrachus didactylus*. Inv Pesq 48:181–185

Ijdo JW, Wells RA, Baldini A, Reeders ST (1991) Improved telomere detection using a telomere repeat probe $(TTAGGG)_n$ generated by PCR. Nucleic Acids Res 19:4780

Jones KW, Singh L (1985) Snakes and evolution of sex chromosomes. Trends Genet 1:55–61

Martins C, Galetti PM Jr (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anastomidae, Characiformes). Chromosome Res 7:363–367

Martins C, Galetti PM Jr (2001) Two 5S arrays in Neotropical fish species: is it a general rule for fishes? Genetica 111:439–446

Molina WF, Galetti PM Jr (2004) Karyotypic changes associated to the dispersive potential on Pomacentridae (Pisces, Perciformes). J Exp Mar Biol Ecol 309:109–119

Nirchio M, Gómez JA, Villalaz J (2001) Cariotipo del pez sapo *Batrachoides pacifici* (Batrachoididae: teleostei) de la costa del Pacífico de Panamá. Saber 13:82–84

Nirchio M, Turner BJ, Pérez JE, Gaviria JI, Cequea H (2002) Karyotypes of three species of toadfish (Batrachoidae: Teleosti) from Venezuela. Sci Mar 66:1–4

Nirchio M, Fenocchio AS, Swarça AC, Dias AL, Giuliano-Caetano L, Ron E, Gaviria JI, Pérez JE (2004a). Cytogenetic characterization of *Thalassophryne maculosa* Günther, 1861 (Pisces, Batrachoidae) from Margarita Island, Venezuela. Caribb J Sci 40:218–222

Nirchio M, Fenocchio AS, Swarça AC, Pérez JE, (2004b) Karyology of the toadfish *Porichthys plectrodon* (Jordan and Gilbert, 1882) (Batrachoididae) from Margarita Island, Venezuela. Mar Biol 146:161–165

Palazón JL, Arias AM, Sarasquete C (2001) Aspects of the reproductive biology of the toadfish, *Halobatrachus didactylus* (Schneider, 1801) (Pisces: Batrachoididae). Sci Mar 65:131–138

Palazón JL, Nirchio M, Sarasquete C (2003) Conventional and nucleolar organiser regions of the toadfish *Halobatrachus didactylus* (Schneider, 1801) (Pisces: Batrachoididae). Sci Mar 67:445–449

Pendás AM, Morán P, Freije JP, García-Vásquez E (1994) Chromosomal location and nucleotide sequence of two tandem repeats of the Atlantic salmon 5S rDNA. Cytogenet Cell Genet 67:31–36

Phillips RB, Reed KM (1996) Application of fluorescence in situ hybridization (FISH) techniques to fish genetics: a review. Aquaculture 140:197–216

Roux C (1986) Batrachoididae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen JL, Tortonese E (eds) Fishes of Northeastern Atlantic and Mediterranean. UNESCO, Paris, pp 1360–1361

Sarasquete C, Gutiérrez M, Establier R (1982) Efecto del mercurio inorgánico ($HgCl_2$) sobre la sangre y riñón del pez sapo marino, *Halobatrachus didactylus* (Schneider, 1801). Inv Pesq 46:323–330

Sarasquete C (1983) Estudios de las series eritrocítica, leucocítica y trombocítica de la sangre periférica del pez sapo marino, *Halobatrachus didactylus*. Inv Pesq 47:403–412

Schwarzacher T (2003) DNA, chromosomes, and in situ hybridization. Genome 46:953–962

Singh L, Jones KW (1982) Sex reversal in the mouse (*Mus musculus*) is caused by a recurrent nonreciprocal crossover involving the X and an aberrant Y-chromosome. Cell 28:205–216

Sola L, Gornung E, Naoi H, Gunji R, Sato C, Kawamura K, Arai R, Ueda T (2003) FISH-mapping of 18S ribosomal RNA genes and telomeric sequences in the Japanese biterlings *Rhodeus ocellatus kurumeus* and *Tanakia limbata* (Pisces, Cyprinidae) reveals significant cytogenetic differences in morphologically similar karyotypes. Genetica 119:99–106

Subramanian S, Mishra RK, Singh L (2003) Genome-wide analysis of Bkm sequences (GATA repeats): predominant association with sex chromosomes and potential role in higher order chromatin organization and function. Bioinformatics 19:681–685

Vitturi R, Catalano E, Colomba M, Montagnino L, Pellerito L (1995) Karyotype analysis of *Aphanius fasciatus* (Pisces, Cyprinodontiformes) Ag-NORs and C-band polymorphism in four populations from Sicily. Biol Zent bl 114:392–402

Vitturi R, Libertini A, Armetta F, Sparacino L, Colomba MS (2002) Chromosome analysis and FISH mapping of ribosomal DNA (rDNA), telomeric /TTAGGG)$_n$ and (GATA)$_n$ repeats in the leech *Haemopis sanguisaga* (L.) (Annelida: Hirudinea). Genetica 115:189–194

Weigant J, Ried T, Nederlof PM, van der Ploeg M, Tanke HJ, Raap AK (1991) In situ hybridization with fluoresceinated DNA. Nucleic Acids Res 19:3237–3241