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Conventional karyotype and nucleolar organiser regions of the toadfish *Halobatrachus didactylus* (Schneider, 1801) (Pisces: Batrachoididae)*

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SUMMARY: For this report, we studied the conventional karyotype and the NOR-bearing chromosomes in the toadfish *Halobatrachus didactylus.* We found a karyotype of 46 chromosomes made up of 8 metacentric, 12 submetacentric, and 26 acrocentric elements (FN = 66). No heteromorphic sex chromosomes were observed in the species. Metacentric chromosomes were easily classified as homologous pairs according to their morphology and L/S ratio. The rest of the chromosomes could not be accurately classified as homologous pairs because differences in chromosome size and L/S ratio were too slight between adjacent pairs within a size-ranged series. A single pair of NOR-bearing chromosomes was found. Active NORs were terminally located in a submetacentric pair of chromosomes.

Key words: karyotype, NOR, cytogenetic, toadfish, *Halobatrachus didactylus.*

RESUMEN: CARIOTIPO Y REGIONES ORGANIZADORAS DEL NUCLEOLO DEL PEZ SAPO MARINO *HALOBATRACHUS DIDACTYLUS* (SCHNEIDER, 1801) (PISCES: BATRACHOIDIDAE). – Se estudió el cariotipo convencional (Giemsa) y las Regiones Organizadoras del Nucleolo (NOR) en el pez sapo marino *Halobatrachus didactylus.* El número diploide de cromosomas 2n = 46 estuvo compuesto de 8 elementos metacéntricos, 12 submetacéntricos y 26 acrocéntricos (NF = 66). No fueron observados cromosomas sexuales heteromórficos en esta especie. Los cromosomas metacéntricos fueron fácilmente clasificados como pares homólogos según su morfología y la relación longitud del brazo largo/longitud del brazo corto (L/S). El resto de los cromosomas no pudo ser clasificado con precisión como pares homólogos debido a que las diferencias en el tamaño de los cromosomas y la razón L/S fueron muy pequeñas entre pares adyacentes dentro de la misma serie de tamaños. Fue detectado un par de cromosomas portadores de NOR. Las NOR están localizadas en la región terminal de un par de cromosomas submetacéntricos.

Palabras clave: cariotipo, NOR, citogenética, pez sapo marino, *Halobatrachus didactylus.*

INTRODUCTION

The Family Batrachoididae (Order Batrachoidiformes) is divided into 3 subfamilies with approximately 19 genera and 69 species (Collette, 1995) distributed worldwide in the Atlantic, Pacific and Indian oceans. The species are mostly marine (coastal and benthic) and are rarely found in brackish or fresh water (Nelson, 1994).

Cytogenetic information on Batrachoididae is limited. Before 2001, only two reports were known, one for *Porichthys notatus* (Chen 1967, cited by Gold *et al*., 1980) and one for *Halobatrachus didactylus* (Gutiérrez *et al*. 1984), but they

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provide no description of chromosome morphology, modal counts or fundamental number. Three recent papers describe the chromosome complement and karyotype for *Porichthys porosissimus* from Brazil (Brum *et al.*, 2001), *Batrachoides pacifici* from Panama (Nirchio *et al.,* 2001), and *Amphychthys cryptocentrus, Batrachoides manglae* and *Thalassophryne maculosa* from Venezuela (Nirchio *et al.,* 2002).

Here we present the chromosome complement, karyotype formula and nucleolar organiser regions (NOR) of *Halobatrachus didactylus* as a contribution to the biology of this species and in order to extend the existing cytogenetic data in the batrachoids. *Halobatrachus didactylus* is the only species of Batrachoididae found in the Iberian Peninsula. It is distributed from the Bay of Biscay to Ghana and the western Mediterranean (Roux, 1986). This species represents an important component for fishing communities in the Bay of Cádiz (Arias, 1976) and has received special attention in recent years because of its use as a laboratory animal in toxicological and cardiological experiments (see Palazón *et al*., 2001, for references).

MATERIALS AND METHODS

Eleven adult specimens (5 males and 6 females) of *Halobatrachus didactylus* were captured from shallow waters in the Bay of Cádiz (southwest Spain). Chromosome preparations were made from the cephalic portion of the kidney as described by Nirchio and Cequea (1998) but modifying the concentration of colchicine and the exposure time: each specimen was injected with 0.5% colchicine (i.p. 1 ml/100 g fish weight). The fish were maintained in a well aerated aquarium and after 4 h they were sacrificed. The kidneys were removed and placed in a hypotonic solution of 0.4% KCl. Each kidney was minced with fine forceps and a fine cellular suspension was obtained by repeated aspiration and forced release with a glass syringe. After 30 min in the hypotonic solution, the cellular suspension was centrifuged at 1,000 rpm for 3 min. The hypotonic solution was discarded and the cellular pellet was suspended and washed 3 times in a methanol-acetic acid mixture (3:1 V:V). One droplet of the cellular suspension was dropped on a clean microscope slide, previously chilled in a freezer, from a height of 60 cm. The slides were then allowed to air-dry.

Slides were stained for 20 minutes with Giemsa (10% in a phosphate buffer at pH 6.88). Nucleolar Organiser Regions were evidenced by silver nitrate staining (Howell & Black, 1980). A total of 200 mitotic spreads (100 per sex; at least 10 per individual) were scanned to determine the modal chromosome number.

For the analysis of chromosome morphology, high quality spreads with a known modal chromosome number were photographed with a green filter and ASA 50 (ILFORD) film. For each chromosome, the long arm (L), the short arm (S) and the length of the whole chromosome were measured to the nearest 0.01 mm on digitalised and enlarged pictures using the measuring tool of Adobe Photoshop v.0.5 Software. The L/S ratio was calculated from these data (Levan *et al.,* 1964*)*.

Chromosomes with arm ratios < 1.7 were considered to be metacentric (m); those with arm ratios ≥ 3.0 were considered acrocentric (a); those with arm ratios between 1.7 and 3.0 were classified as submetacentric (sm). These criteria reduce subjectivity and result in a conservative estimation of chromosome variation (Elder *et al.,* 1993). The fundamental number of chromosome arms (FN) was obtained considering m and sm chromosomes as biarmed.

RESULTS

Figure 1A shows the chromosome complement of *Halobatrachus didactylus.* Chromosome counting of 200 metaphase plates showed a diploid number ranging from 33 to 46 with a modal diploid number of 2n = 46 (Fig. 2). The karyotype obtained on the basis of chromosome size and centromere position (based on the L/S ratio), consisted of 8 metacentric, 12 submetacentric and 26 acrocentric elements. The fundamental number of chromosome arms (FN) was 66. The distribution of the number of chromosomes was asymmetrical with most 2n values appearing below the modal value. No differences were detected in the distribution of the number of chromosomes between male and female (χ^2 = 10.22, p>0.05).

Silver staining revealed a single pair of NOR-bearing chromosomes. Active NORs were terminally located on the short arms of a submetacentric pair of chromosomes (Fig. 1B). One of the NOR regions always gave a more conspicuous signal than the other after silver impregnation.

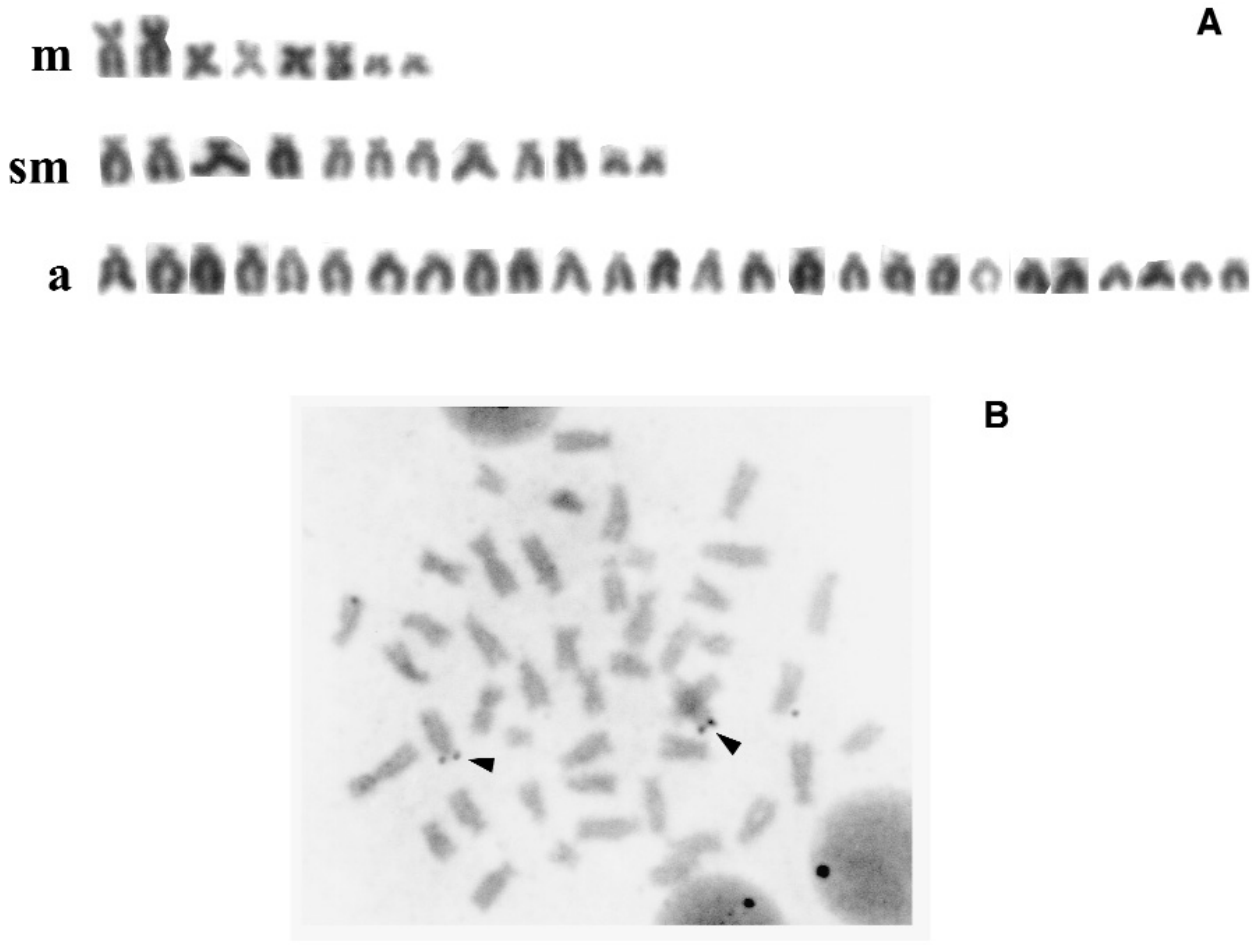

FIG. 1. – Karyotype of *Halobatrachus didactylus* with 2n = 46. (A) Giemsa stained karyotype with 8 pairs of metacentric (m), 12 pairs of submetacentric (sm) and 26 pairs of acrocentric (a) chromosomes. (B) Silver stained metaphase spread. Active NORs are located in a submetacentric pair of chromosomes (arrows).

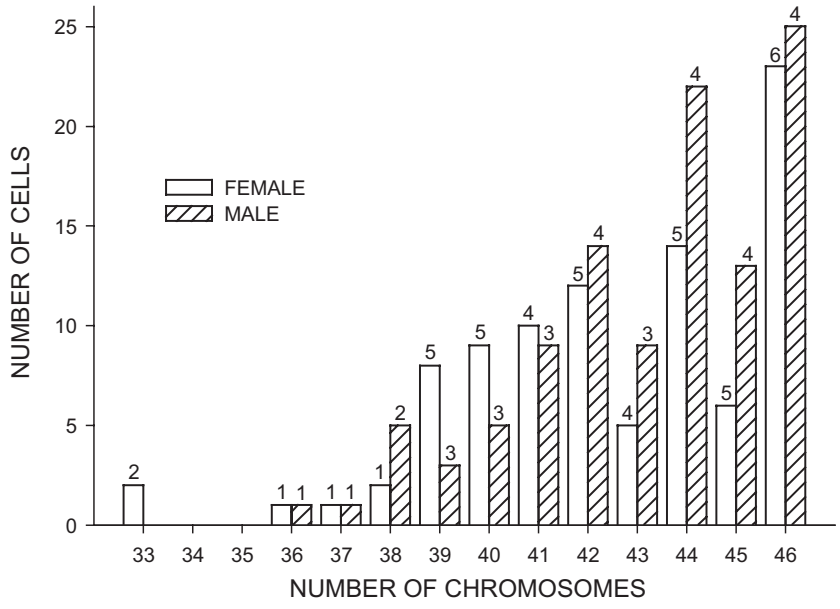

FIG. 2. – Chromosome number distribution of 200 metaphase spreads from kidney preparations from 10 individuals (4 male, 6 female) of *H. didactylus*. The figures on top of the bars represent the number of fish.

DISCUSSION

Table 1 presents cytogenetic data of the batrachoid species already studied. Except for *Porichthys notatus* (2n = 48), the rest of the species have a chromosome diploid number of 2n = 46. Following the opinion of Eastbrook (1977), who indicated that the characteristic that occurs most frequently in a group or taxon can be considered as ancestral, the condition 2n = 46 would probably be the ancestral diploid number for the family. The modal diploid number was observed in 24% of the cells examined. No significant statistical differences in the modal counts between females and males were detected ($p > 0.05$), so the hypomodal counts can be attributed to chromosome loss, overlap, miscounting (a frequent preparation-caused defect observed in fish cytogenetic studies), or the presence of naturally occurring atypical nuclei with incomplete chromosome complements (Nirchio *et al.,* 2001; 2002). Hypermodal counts observed in other species, and explained as representing additional chromosomes from another spread, or attributed to a premature separation of chromatids or to additional chromosomes in atypical nuclei (Nirchio *et al.,* 2002) were not observed in *Halobatrachus didactylus*.

Metacentric chromosomes were easily classified as homologous pairs according to their morphology and L/S ratio. The rest of the chromosomes could not be accurately classified as homologous pairs because differences in chromosome size and L/S ratio were too slight between adjacent pairs within a size-ranged series. As in the other batrachoid species already studied (Brum *et al.,* 2001; Nirchio *et al*., 2002), no heteromorphic sex chromosomes were observed in this species.

Karyological information in fishes is available for only 13% of the 23,000 described species (Klinkhardt *et al.*, 1995). It shows that the most common chromosome number in the group is 2n = 48 telocentric elements, a condition considered as ancestral for teleosts (Gold, 1979; Ohno, 1974; Vitturi *et al.*, 1995). Assuming that the primitive karyotype for actual teleosteans derives from an ancestral diploid number of 48 telocentric chromosomes, the evolution of the karyotype of *Halobatrachus didactylus* can be interpreted as the result of at least the central fusion of two pairs of uniarmed chromosomes to form large metacentric or submetacentric elements, resulting in the reduction of the diploid number from 48 to 46, as well as a series of pericentric inversions, translocations and/or simple heterochromatin additions giving rise to the 20 biarmed chromosomes during the species evolution

It has been argued that any real difference in the FN between closely related species can be explained as the result of pericentric inversions, whereas the differences in the diploid number (2n) presumably represent Robertsonian changes (fusions, fissions) (LeGrande, 1981; López *et al*., 1988; Amores *et al.*, 1993); therefore, the FN could be used as an indicator of the accumulation of chromosome reorganisations. The fundamental number (FN) is a common descriptor of the number of chromosome arms, but the methods for calculating it vary among different reports. Some authors include all visible arms while others do not consider the small arms in small submetacentric or subtelocentric chromosomes. Obviously this means that caution is needed when comparisons are established. Table 1 shows the FN reported for the species of batrachoididae already karyotyped. Owing to the fact that in the present work we used the same criteria (Elder *et al.,* 1993) used by Nirchio *et al.* (2001, 2002) for calculating FN, and considering that an FN over 48 is an apomorphy (Nirchio *et al*., 2002), *Halobatrachus didactylus* seems to possess the most derived karyotype among the batrachoid species already studied.

NORs are the chromosome sites at which 18S and 28S rRNA cistrons are localised, and can be visualised by the silver staining procedure (Howell, 1982) that demonstrates the residues of the Ag

TABLE 1. – Chromosome complement (2n), karyotype formula and fundamental number (FN) in six species of toad fish (Batrachoididae).

Species	2n	Karyotype formula	FN	Reference
Amphychthys cryptocentrus	46	4m:2sm:40a	52	Nirchio *et al.,* 2002
Batrachoides manglae	46	6m:6sm:34a	58	Nirchio *et al.,* 2002
Batrachoides pacifici	46	6m:6sm:34a	58	Nirchio *et al.*, 2001
Halobatrachus didactylus	46	8m:12sm:26a	66	Present work
Porichthys notatus	48	---	--	Chen, 1967
Porichthys porosissimus	44	14m/sm:30st/a	58	Brum *et al.,* 2001
Thalassophryne maculosa	46	8m:6sm:32a	60	Nirchio *et al.*, 2002

Abbreviations: a, acrocentric; m, metacentric; sm, submetacentric; st: subtelocentric.

stainable rRNA-protein complex synthesised only by the active NOR in the preceding interphase (Howell and Black 1980). According to Schmidt (1978: in Vitturi *et al.*, 1998), a single pair of NOR-bearing chromosomes represents a primitive condition in most vertebrate species. It is difficult to make comparisons within Batrachoidid species since no reports including data on NORs for toadfish are available. Brum *et al.* (2001) recently attempted to describe NOR patterns on *Porichthys porosissimus* but they only inferred the occurrence of more than a single NOR-bearing pair based on the presence of one to three nucleoli per nucleus when the silver nitrate technique was applied. Most teleostean fish displaying the ancestral karyotype (2n = 48) exhibit two terminal NORs near the centromere (Vitturi *et al*., 1995), a condition also observed in *Halobatrachus didactylus* that could be considered plesiomorphic for batrachoididae.

More precise conclusions about the karyotype evolution in this group require additional information and there is a need to apply banding techniques in order to establish more accurately the nature of the chromosome rearrangements that have occurred in the family. This would allow a more precise interpretation of the phyletic tendencies in the group. These studies are in progress in our laboratory.

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