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Micron 36 (2005) 243–246

micron

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Chromosomal location polymorphism of major rDNA sites in two Mediterranean populations of the killifish *Aphanius fasciatus* (Pisces: Cyprinodontidae)

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Received 27 September 2004; revised 26 October 2004; accepted 30 November 2004

Abstract

The chromosomes of the Mediterranean killifish, *Aphanius fasciatus* from two populations, the Lagoon of Venice (LV, 15 specimens) and the Lagoon 'Stagnone di Marsala' (Sicily) (SM, 48 specimens), have been investigated using conventional Ag-staining and fluorescent in situ hybridization (FISH) with 18S rDNA probe. The two methods revealed variation in the number of major rDNA sites ranging from 8 to 14 (LV) and from 1 to 4 (SM) per individual. The fact that each individual possessed its own number of sites implies that observed variation was structural. Moreover, overlapping of silver staining and FISH patterns demonstrated that all ribosomal genes were transcriptionally active in each specimen.

Keywords: Karyotype polymorphism; rDNA; In situ hybridization; *Aphanius fasciatus*; Mediterranean sea

1. Introduction

Aphanius fasciatus (Valenciennes, 1821) is a cyprinodontid, euryhaline fish that lives preferably in coastal brackish-water of estuaries and lagoons, throughout the Mediterranean Sea (Tortonese, 1975; Villwock, 1982). *A. fasciatus* has also been found in salt ponds and inland waters, such as Sicilian rivers (Parenti and Tigano, 1993) and Tunisian oases (Kraiem, 1983).

Since distribution of *A. fasciatus* is naturally fragmented (Maltagliati, 1998a,b, 1999), this species represents an appropriate model for studies of genetic variation induced by natural events and/or habitat constraints. Consequently, several papers were focused on these subjects including allozymes + DNA (Comparini et al., 1985; Maltagliati, 1998a,b, 1999; Cimmaruta et al., 2003) and cytogenetic investigations (Vitturi et al., 1995; Ferrito et al., 2000). The cytogenetic research was carried out using silver impregnation (Ag-NOR banding) on nucleolus organizer region (NOR) variability in individuals representatives of five *A. fasciatus* populations from Sicily revealing that considerable variation occurred in the activity of major ribosomal genes (18S–28S rDNA) within and among populations.

However, since traditional silver staining reveals only transcriptionally active NORs (Jordan, 1987), this technique is not adequate to allow all NORs (active + inactive) to be conclusively stated.

The present study examined previously observed variation of NORs in specimens of two populations from the Lagoon of Venice (LV) and the Lagoon 'Stagnone di Marsala' (SM) by means of silver impregnation (Ag-NOR banding) and fluorescent in situ hybridization (FISH) to

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doi:10.1016/j.micron.2004.11.006

reveal either functional and structural nature of this cytogenetic marker.

2. Materials and methods

Analyzed specimens of *Aphanius fasciatus* were collected at two geographical locations (distance: about 1200 km) namely, the Lagoon of Venice (LV, 15 specimens, 5 males included) and the Lagoon 'Stagnone di Marsala' (SM, 48 specimens, 18 males included). Fishes were identified according to Tortonese (1975) and sexed by examination of both colour pattern and gonad state. Somatic chromosomes were obtained from pooled gills, spleen and kidney cells. Spermatocyte chromosomes were prepared from mature testes. Slides were processed for conventional Giemsa staining (Vitturi et al., 2000), silver impregnation of nucleolus organizer regions (Ag-NORs) (Howell and Black, 1980) and FISH (Vitturi et al., 2000). Chromosomes were classified according to the criteria of Levan et al. (1964).

In FISH experiments, a sea urchin (*Paracentrotus lividus*, Echinodermata) rDNA probe, consisting of the 18S rDNA was used. Nick translation labeling with digoxigenin was performed according to the manufacturer's instructions (Roche). Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under a Leica I3 filter set. Chromosomes were observed with a Leica microscope and photographed with a 800 ASA film.

3. Results

Diploid chromosome number $2n=48$ and karyotype entirely composed of subtelocentric to acrocentric (st-a) chromosomes gradually decreasing in size were observed in the specimens from both locations confirming previous finding (Vitturi et al., 1995) about the karyotype of *A. fasciatus* (Fig. 1A). Correspondingly, spermatocytes contained 24 metaphase-I bivalents (Fig. 1B).

Major ribosomal sites were detected invariably at the telomeric regions of the respective chromosomes by using rDNA FISH and silver staining. The number of observed rDNA sites ranged from 8 (Fig. 2A) to 14 (Fig. 2B) in LV specimens and from 1 (Fig. 2C) to 4 (Fig. 2D) in SM. Each specimen possessed a particular number of major ribosomal cistrons and the frequencies of various cytotypes were 5 (8 sites) and 3 (14 sites) (LV), 5 (1 site), 6 (2 sites), 8 (3 sites) and 16 (4 sites) (SM). Because of the similarity in both morphology and size, homologous NOR-bearing chromosomes could not be distinguished, therefore their frequencies for establishing Hardy-Weinberg equilibrium were not evaluated. However, a constant number of two hybridized bivalents occurred in specimens with four NORs (Fig. 2E) suggesting that a maximum number of 2 chromosome pairs were involved in nucleolus organization in SM, while a constant number of 7 hybridized bivalents in specimens with 14 NORs (Fig. 2F) suggesting a maximum number of 7 NOR-bearing chromosome pairs in LV. In Fig. 2G,H the maximum number of 14 (LV) and 4 (SM) silver stained NORs are represented.

4. Discussion

Major ribosomal clusters (18S–28S rDNA) were mapped physically by FISH in some specimens of the killifish *Aphanius fasciatus* from two Italian locations. The finding of variation in the number of rDNA sites ranging from 8 to 14 per individual in the sample from the Lagoon of Venice, and from 1 to 4 per individual in the sample from Stagnone di Marsala, demonstrates the occurrence of structural NOR polymorphism within and among populations. According to literature, heteromorphism involving the number and dimension of NORs regularly affects fish species with multiple NOR pattern (see references in Vitturi et al. (1999) and Jankun et al. (2001)). We exclude that NOR inconsistency may have arisen from an underestimation of NOR phenotypes in SM specimens, due to the constant presence of 2 hybridized bivalents per cell in specimens

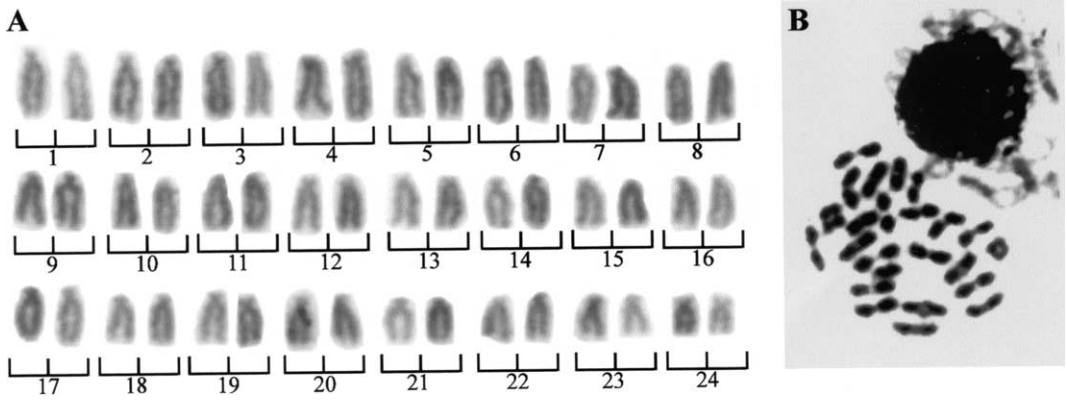

Fig. 1. *Aphanius fasciatus*. (A) Giemsa stained karyotype; (B) Giemsa stained metaphase-I bivalents.

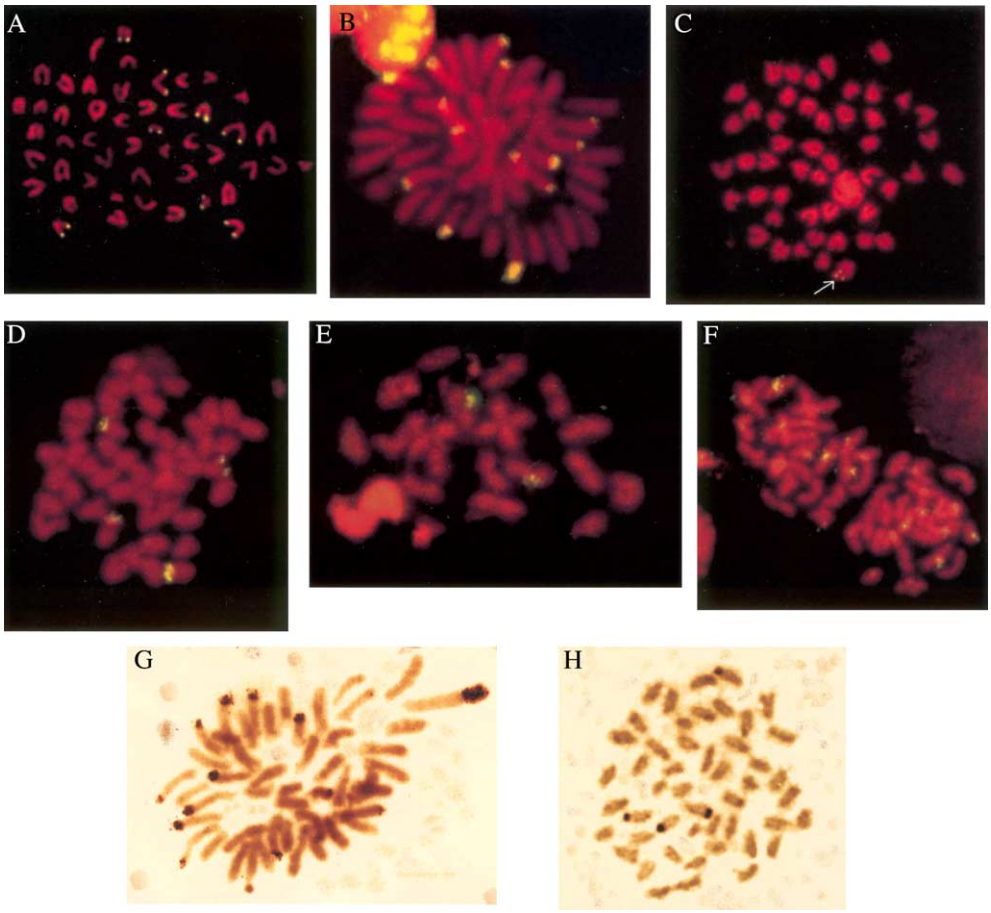

Fig. 2. *Aphanius fasciatus*. Mitotic metaphase chromosomes after rDNA FISH treatment with a heterologous 18S rDNA probe from the sea urchin *Paracentrotus lividus*: (A) with eight ribosomal sites (5 specimens) and (B) with fourteen ribosomal sites (3 specimens) (LV); mitotic metaphase chromosomes after rDNA FISH: (C) with one ribosomal site (see arrow) (5 specimens) and (D) with four ribosomal sites (16 specimens) (SM); FISH-treated metaphase-I bivalents: (E) of a specimen with four ribosomal sites where two bivalents are involved (SM) and (F) of a specimen with fourteen ribosomal sites where seven bivalents are involved (two spreads are represented) (LV); silver stained chromosomes: (G) of a specimen with fourteen ribosomal sites (LV) and (H) of a specimen with four ribosomal sites (MS).

with the maximum number of NORs. This result, in fact, is not consistent with the presence of heterozygosity for NOR sites in these individuals.

Numerical, inter-individual differences in Ag-NOR sites were reported for *A. fasciatus* specimens from other five geographically separated locations of Sicily (Vitturi et al., 1995; Ferrito et al., 2000). This demonstrates that NOR polymorphism is fairly common in the killifish *A. fasciatus*. The naturally fragmented distribution of this species and restricted gene flow among its populations that greatly contribute to population differentiation through genetic drift (Maltagliati, 1998a,b, 1999) may account for that.

Also extreme environmental conditions, in terms of salinity, O_2 concentration and water temperature, to which *A. fasciatus* specimens are frequently exposed, might contribute to promote the observed inter-individual diversity in the number of rDNA genes. In fact, it was demonstrated that adverse environmental conditions would favour chromosome breakage, mainly in correspondence with highly repeated DNA regions including NORs, due to the extreme fragility of these sites (Cowell, 1982). A support for this assumption came from two cytological studies. The first study was carried out by Vitturi et al. (1995) on specimens from a natural, inland population of Sicily subjected to extreme environmental conditions (wide variation of both O_2 concentration and water temperature). The second study was performed on specimens from the Fiumicello river (Sicily) after exposure to toxicants such as diorganotin(IV)chloro and triorganotin(IV)chloro derivatives of pennicillin G (Vitturi et al., 1994). In both cases, paracentromeric inversions of nucleolus organizer regions were observed.

Concerning the mechanism generating diversity in the number of rDNA sites among individuals, literature suggests several possibilities (see Elder and Turner, 1995; Schmid et al., 1995). In *A. fasciatus* inter-individual karyotypic stability ($2n=48$; NF=48) and telomeric position of silver dots support a mechanism not involving major chromosome rearrangements, where ribosomal cistrons may be transferred either by non-homologous exchange or by NOR associated transposons.

Another interesting consideration arises from our results. A maximum number of 7 NOR-bearing chromosome pairs occurs in three *A. fasciatus* specimens (LV). A similar value was never reported in fishes. In fact, NOR data gathered for more than 200 teleosts of diploid origin (Sola et al., 1990; Klinkhardt et al., 1995; Vitturi et al., 1995, 1996) showed that high Ag-NOR numbers were reported in a few species including another cyprinodontid, the Amazon molly *Poecilia latipinna* (Sola et al., 1990) and the sparids *Diplodus vulgaris* ($2n=48$; Ag-NORs up to four pairs), *D. annularis* ($2n=48$; Ag-NORs up to five pairs), *D. sargus* and *D. puntazzo* ($2n=48$; Ag-NORs up to six pairs) (Vitturi et al., 1996), although their effective number of NOR sites has not yet undoubtedly been confirmed by in situ hybridization. This fact makes the killifish *A. fasciatus* a very unusual case of a multiclustered fish species which, undoubtedly, needs to be investigated further through cytological analysis of specimens from additional locations.

Acknowledgements

The authors thank F. Riccato and R. Fiorin (Shallow Water Ecology Group—Dept. of Environmental Sciences—Ca' Foscari University of Venice) for providing the specimens from the Venetian Lagoon. This work was funded by the Ministero Politiche Agricole e Forestali (MiPAF, Italy) and Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Italy).

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