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RESEARCH NOTE

FISH mapping of 18S rDNA and $(TTAGGG)_n$ sequences in two pipefish species (Gasteroisteiformes: Syngnathidae)

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Introduction

Chromosomal location of major ribosomal genes (18S–5.8S–28S rDNA) and $(TTAGGG)_n$ telomeric sequences has been investigated for the first time in pipefish (Syngnathidae) by application of fluorescence *in situ* hybridization (FISH). Two species were studied: *Nerophis ophidion* (L.) (2*n* karyotype = 58 metacentric, submetacentric and subtelocentric chromosomes) and *Syngnathus abaster* Risso (2*n* karyotype = 44 acrocentric chromosomes) (Vitturi *et al*. 1998). The results indicate that in both pipefishes intraspecific numerical variation of ribosomal sites is more conspicuous than previously detected (Vitturi *et al*. 1998). $(TTAGGG)_n$ FISH showed a regular hybridization pattern in *S. abaster*, while in *N. ophidion* it gave unexpected enlarged hybridization signals in the terminal region of either two or five chromosomes, where telomeric sequences were interspersed throughout nucleolus organizer regions NORs (interstitial telomeric sites, ITS). Diversification of ribosomal and telomeric repeats in the karyotype of the two species confirms the hypothesis that the genera *Nerophis* and *Syngnathus* have undergone two different pathways of karyotypic evolution.

The teleostean family Syngnathidae (pipefish and seahorses) includes 277 species, of which 11 inhabit the Mediterranean Sea and belong to the genera *Nerophis* (two species), *Hippocampus* (two species), *Minyichthys* (one species) and *Syngnathus* (six species) (FishBase 2005). Owing to the difficulty in obtaining high-quality chromosome preparations, cytogenetic data on syngnathids are scanty, referring in literature only to five Mediterranean species (two seahorses and three pipefishes), and are restricted to only a few aspects, including diploid chromosome number, karyotypic macrostructure, location of active NORs by silver staining, and genome size (Vitturi *et al*. 1998). With regard to NOR location, staining with silver nitrate and the fluorochrome chromomycin A_3 (CMA_3) has been widely applied to metaphase plates of both vertebrates and invertebrates to detect number and position of active NORs and GC-rich NORs, respectively (Sumner 1990, and references therein).

However, both methods have proven to be inadequate in unequivocally characterizing the ribosomal pattern (active and inactive rDNA genes) in the karyotype of several species. For example, in the salmonids *Salmo trutta* L. and *S. salar* L., inadequacy was due to the presence of GC-rich heterochromatin, either NOR-associated or unrelated to ribosomal sites (Pendás *et al*. 1993). In some scarab beetles, inadequacy was due to extensive silver stainability of heterochromatin, both constitutive and associated with NOR regions (Colomba *et al*. 2004, and references therein).

It has been shown that molecular techniques provide additional opportunities for animal cytogenetics. In teleosts, in particular, although FISH experiments with ribosomal (18S–5.8S–28S rDNA and 5S rDNA) and/or telomeric $(TTAGGG)_n$ sequences as probes were carried out on approximately 30 species (Gornung *et al*. 2004), several orders and families have been neglected, as in the case of the order

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Gasteroisteiformes and its family Syngnathidae. In this note we report the first attempt to apply FISH to pipefish chromosomes.

Materials and methods

Specimens of the straight-nosed pipefish *N. ophidion* ($n = 3$) and the black-striped pipefish *S. abaster* ($n = 4$) were collected by beach seine from shallow *Zostera* sp. meadows in the Lagoon of Venice (northern Adriatic Sea). Chromosome spreads were obtained from primary cultures of dorsal fin cells; cell culture and harvesting were performed according to the method described by Alvarez *et al.* (1991).

FISH was carried out on mitotic chromosomes using two different probes: a sea urchin (*Paracentrotus lividus* (Lamarck)) rDNA probe consisting of sequences of the 18S rDNA, and a telomeric hexanucleotide $(TTAGGG)_n$ generated by PCR (PCR DIG-Probe Synthesis kit, Roche, Basel, Switzerland) in the absence of template (Ijdo *et al.* 1991) using $(TTAGGG)_5$ and $(CCCTAA)_5$ as primers. Nick translation labelling with digoxigenin of 18S rDNA was performed according to the manufacturer's (Roche) instructions, while the $(TTAGGG)_n$ probe was DIG-labelled following the random-priming Roche protocol. In FISH experiments with the $(TTAGGG)_n$ probe, posthybridization washes were carried out either at 37°C or at 42°C.

Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under a Leica (Wetzlar, Germany) I3 filter set. Slides were also processed for Ag-NOR banding and chromomycin A_3 (CMA_3) staining according to the methods reported by Sumner (1990). Some slides of *N. ophidion* were sequentially treated with $(TTAGGG)_n$ FISH and silver impregnation. Chromosomes were observed with a Leica microscope and photographed with 800 ASA film.

Results and discussion

In a previous study (Vitturi *et al.* 1998), silver staining showed that *N. ophidion* and *S. abaster* were characterized by numerical interindividual variation in the number of Ag-NORs, ranging from one to three, and from one to four per cell, respectively.

In the present study a novel NOR cytotype was detected in both species. In particular, in *N. ophidion*, a maximum number of five terminal NORs was found after both FISH with the 18S rDNA probe (figure 1,a) and silver staining (figure 1,b). NORs were also marked by CMA_3 (figure 1,c) owing to the GC richness in rDNA base composition. In two specimens of *S. abaster*, silver staining localized five NORs in the terminal or subterminal region of five chromosomes per spread (figure 2,a). Ag-NORs were large in size in four elements and minute in the fifth one. Both the number and dimension of NORs were confirmed by rDNA FISH (figure 2,b). In two other specimens, two NOR-bearing chromosomes per spread were observed. Altogether, these results indicate that interindividual NOR polymorphism in both species is more marked than previously observed.

Multichromosomal location of NORs is considered an apomorphic condition in fish, derived from the ancestral single chromosome pair with terminal NORs (Amemiya and Gold 1988). Nevertheless, the mechanism generating multiple NORs still remains unclear: transposition of ribosomal cistrons either by nonhomologous exchange or by NOR-associated transposons could be the most probable (Woznicki *et al.* 2000).

In situ hybridization with the telomeric probe was performed on metaphase spreads from *S. abaster* and — as reported for most vertebrates, including fish (Meyne *et al.* 1990) —hybridization signals marked small telomeric zones of all chromosomes (figure 2,c).

In *N. ophidion*, $(TTAGGG)_n$ probe labelled the termini of all chromosomes. Notably, these signals were generally small-sized with the exception of two (in one individual) or five elements (two individuals) displaying enlarged terminal hybridized regions (figure 1,d). Under more stringent conditions, with posthybridization washes at 42 °C, enlarged signals were still clearly observed while some other smaller signals were washed away or faded (figure 1,e). Large hybridized areas were not homogeneously stained owing to the presence of grains which were more or less closely joined to one another. To find out the relationship between telomeric sequences and terminal rDNA repeats, silver staining was carried out on previously $(TTAGGG)_n$-hybridized metaphases showing five enlarged telomeric signals (figure 1,f). Ag-NOR pattern revealed silver dots coinciding with enlarged telomeric blocks.

From these results it is possible to draw two main inferences: (i) interindividual variation in the number of telomeric sequences suggests that additional telomeric sites might have been recently fixed in the genome and are inherited among the individuals of the Venetian population of *N. ophidion*; and (ii) each enlarged hybridized region may consist of multiple ITS scattered along NORs, as shown by the saltatory distribution of fluorescing grains giving the signal an inhomogeneous appearance.

ITS have been reported in many vertebrate taxa, such as mammals, birds, reptiles, amphibians and a few fish species including lake trout *Salvelinus namaycush* (Walbaum), brook trout *Salvelinus fontinalis* (Mitchill), tilapia *Oreochromis niloticus* (L.) and sea scorpion *Scorpaena notata* Rafinesque (Caputo *et al.* 1998; Ocalewicz *et al.* 2004, and references therein). Moreover, ITS were found to be generally associated or overlapping with constitutive heterochromatic regions (Multani *et al.* 2001).

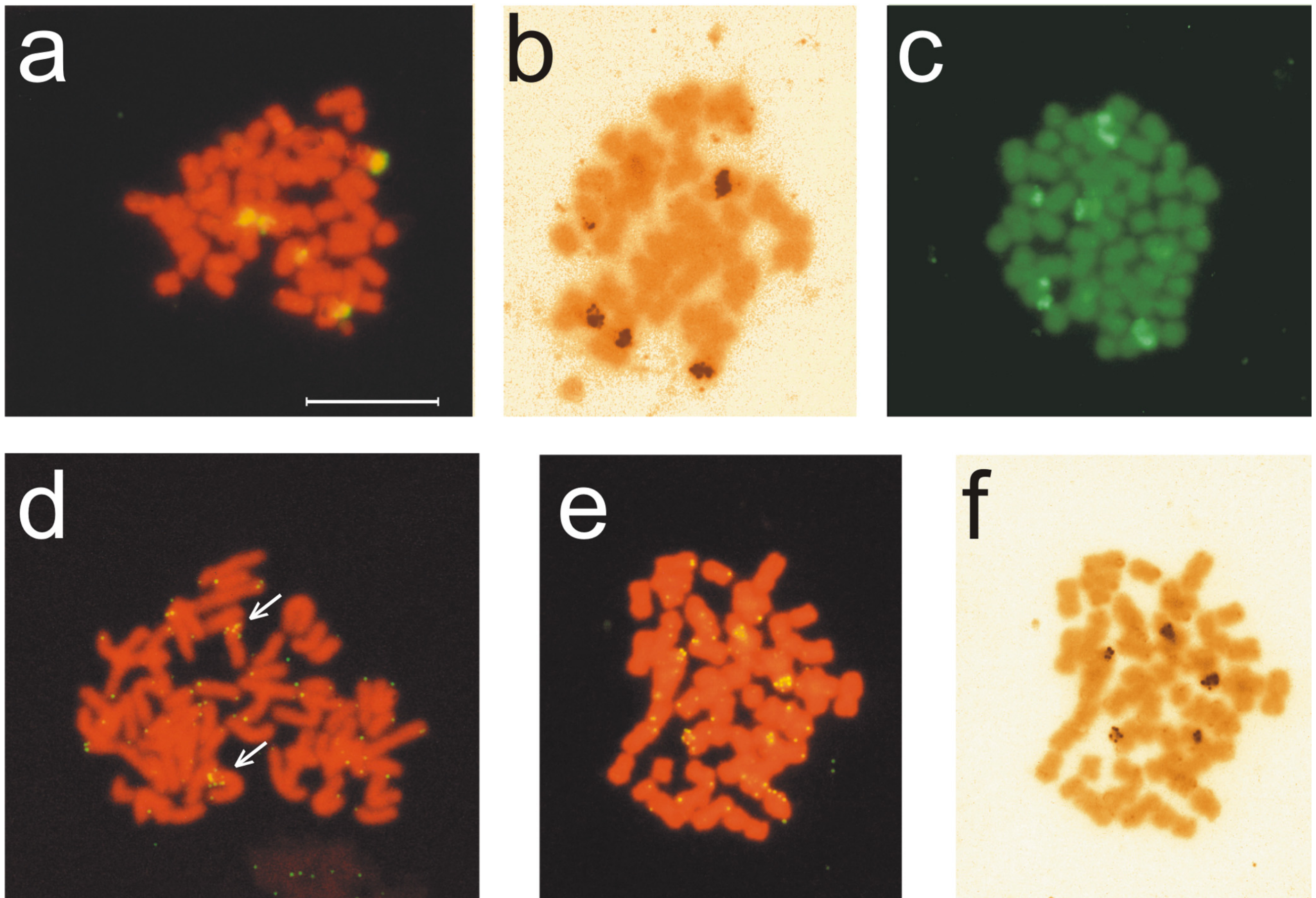

Figure 1. Mitotic metaphase plates of *Nerophis ophidion*: (a) after rDNA FISH treatment with a heterologous 18S rDNA probe, five ribosomal sites (yellow) are detectable; (b) silver staining showing five active NORs; (c) chromomycin A_3 fluorochrome staining enhancing five GC-rich sites, corresponding to NORs; (d) chromosomes after FISH with the telomeric probe $(TTAGGG)_n$ performed at low-stringency conditions, arrows indicate two enlarged telomeric signals; (e) chromosomes after FISH with the telomeric probe $(TTAGGG)_n$ performed at higher-stringency conditions, five enlarged telomeric signals were detected; (f) the same plate as 'e' subsequently stained with silver nitrate showing five NORs lying in the same regions as the large-sized telomeres. (Scale bar = 10 μm)

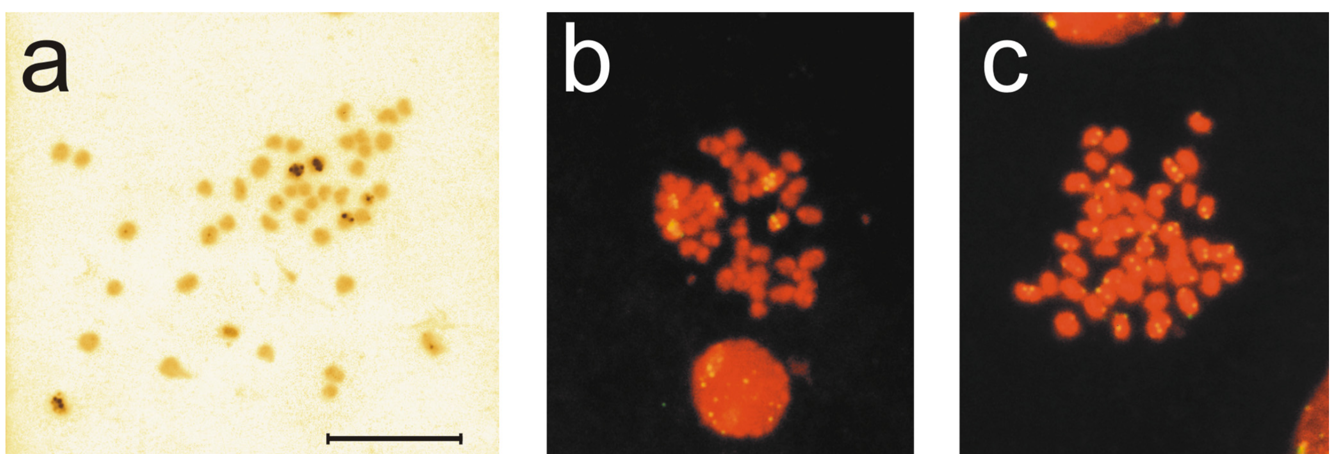

Figure 2. Mitotic metaphase plates of *Syngnathus abaster*: (a) with five active NORs localized by silver staining; (b) with five ribosomal sites showed by rDNA FISH treatment with a heterologous 18S rDNA probe; (c) chromosomes after FISH with the telomeric probe $(TTAGGG)_n$. (Scale bar = 10 μm)

The ribosomal–telomeric-repeat syntenical organization observed in *N. ophidion* is an uncommon condition. Among fish, it has been reported in a few species, such as *Anguilla anguilla* (L.) and *A. rostrata* (Lesueur), *Oncorhincus mykiss* (Walbaum), *Rhodeus ocellatus kurumeus* Kimura and Nagata, and in some Mediterranean mullets (Gornung *et al*. 2004, and references therein). Telomeric intrachromosomal sites can be remnants of chromosome rearrangements as centric and tandem translocations or pericentric inversions (Go *et al*. 2000, and references therein) occurring in the course of genome evolution. However, despite chromosome rearrangements of this type being very common events in fish (Caputo *et al*. 1998), and supposed to have played a role in *N. ophidion* karyotypic evolution (Vitturi *et al*. 1998), they seem to be unrelated to ITS in this species, since no trace of intrachromosomal location of telomeric block near the centromere was found. Alternatively, taking into account that *N. ophidion*, as well as *S. fontinalis* (Ocalewicz *et al*. 2004), showed telomeric–rDNA blocks positive to CMA_3 staining, interspersion of TTAGGG sequences on ribosomal regions might be due to the presence of GC-rich regions besides telomeres. In fact, chromosomal regions enriched in GC base pairs, like NORs, may be involved in jumping-translocation processes followed by ITS formation (Ashley and Ward 1993).

As inferred by morphological and ultrastructural comparison of male brood pouch structure (Herald 1959; Carcupino *et al*. 2002) as well as by mitochondrial DNA sequence analyses (Wilson *et al*. 2001), pipefishes (*Syngnathus*) and seahorses (*Hippocampus*) have been considered as closely related groups sharing advanced characters, while the lineage leading to *Nerophis* was apparently an early offshoot in the Syngnathidae tree, and *N. ophidion* has been thought to represent the unelaborated brooding configuration, more closely representative of the presumed ancestral condition (Herald 1959; Wilson *et al*. 2001). It is worth noting that our results, and some earlier cytogenetic studies on Syngnathidae (Vitturi *et al*. 1998), also suggest that *N. ophidion* underwent a different pathway from the genera *Syngnathus* and *Hippocampus* in evolution at the karyological level, as pointed out by the analysis of karyotype morphology, location and structure of ribosomal and telomeric sequences, and genome size. The present results compared with previous data provide a new insight into interpretation of *N. ophidion* evolution. In fact, the cooccurrence of plesiomorphic characters along with some apomorphic cytogenetic traits—such as a karyotype composed by biarmed chromosomes, multichromosomal NOR location, and multiple ITS interspersed within NORs—suggests that *N. ophidion* experienced a mosaic evolution.

References

Alvarez M. C., Otis J., Amores A. and Guise K. 1991 Short term cell culture technique for obtaining chromosomes in marine and freshwater fish. *J. Fish Biol*. **39**, 817–824.

Amemiya C. T. and Gold J. R. 1988 Chromosomal NORs as systematic characters in North cyprinid fishes. *Genetica* **76**, 81–90.

Ashley T. and Ward D. C. 1993 A "hot spot" of recombination coincides with an interstitial telomeric sequence in Armenian hamster. *Cytogenet. Cell Genet*. **62**, 169–171.

Caputo V., Sorice M., Vitturi R., Magistrelli R. and Olmo E. 1998 Cytogenetic studies in some species of Scorpaeniformes (Teleostei: Percomorpha). *Chromosome Res*. **6**, 255–262.

Carcupino M., Baldacci A., Mazzini M. and Franzoi P. 2002 Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *J. Fish Biol*. **61**, 1465–1480.

Colomba M., Vitturi R., Volpe N., Lannino A. and Zunino M. 2004 Karyotype, banding and rDNA FISH in the scarab beetle *Anoplotrupes stercorosus* (Coleoptera, Scarabaeoidea: Geotrupidae). Description and comparative analysis. *Micron* **35**, 717–720.

FishBase 2005 (ed. R. Froese and D. Pauly). World Wide Web electronic publication, www.fishbase.org, version 11/2005.

Go Y., Rakotoarisoa G., Kawamoto Y., Randrianjafy A., Koyama N. and Hirai H. 2000 PRINS analysis of the telomeric sequence in seven lemurs. *Chromosome Res*. **8**, 57–65.

Gornung E., Mannarelli M. E., Rossi A. R. and 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.

Herald E. S. 1959 From pipefish to seahorse – a study of phylogenetic relationships. *Proc. Calif. Acad. Sci*. **29**, 465–473.

Ijdo J. W., Wells R. A., Baldini A. and Reeders S. T. 1991 Improved telomere detection using a telomere repeat probe $(TTAGGG)_n$ generated by PCR. *Nucl. Acids Res*. **19**, 4780.

Meyne J., Baker R. J., Hobart H. H., Hsu T. C., Rider O. A., Ward O. G. et al. 1990 Distribution of non-telomeric sites of the $(TTAGGG)_n$ telomeric sequence in vertebrate chromosomes. *Chromosoma* **99**, 3–10.

Multani A. S., Ozen M., Furlong C. L., Zhao Y. J., Hsu T. and Pathak S. 2001 Heterochromatin and interstitial telomeric DNA homology. *Chromosoma* **110**, 214–220.

Ocalewicz K., Sliwinska A. and Jankun M. 2004 Autosomal localization of interstitial telomeric sites (ITS) in brook trout, *Salvelinus fontinalis* (Pisces, Salmonidae). *Cytogenet. Genome Res*. **105**, 79–82.

Pendás A. M., Morán P. and Garcia-Vazquez E. 1993 Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. *Cytogenet. Cell Genet*. **63**, 128–130.

Sumner A. T. 1990 *Chromosome banding*. Unwin Hyman, London.

Vitturi R., Libertini A., Campolmi M., Calderazzo F. and Mazzola A. 1998 Conventional karyotype, nucleolar organizer regions and genome size in five Mediterranean species of Syngnathidae (Pisces, Syngnathiformes). *J. Fish Biol*. **52**, 677–687.

Wilson A. B., Vincent A., Ahnesjö I. and Meyer A. 2001 Male pregnancy in seahorses and pipefishes (Family Syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *J. Hered*. **92**, 159–166.

Woznicki P., Sánchez L., Martínez P., Pardo B. G. and Jankun M. 2000 A population analysis of the structure and variability of NOR in *Salmo trutta* by Ag, CMA_3 and ISH. *Genetica* **108**, 113–118.

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