

## Identification of two new repetitive elements and chromosomal mapping of repetitive DNA sequences in the fish *Gymnothorax unicolor* (Anguilliformes: Muraenidae)

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### Abstract

Muraenidae is a species-rich family, with relationships among genera and species and taxonomy that have not been completely clarified. Few cytogenetic studies have been conducted on this family, and all of them showed the same diploid chromosome number ( $2n=42$ ) but with conspicuous karyotypic variation among species. The Mediterranean moray eel *Gymnothorax unicolor* was previously cytogenetically studied using classical techniques that allowed the characterization of its karyotype structure and the constitutive heterochromatin and argyrophilic nucleolar organizer regions (Ag-NORs) distribution pattern. In the present study, we describe two new repetitive elements (called *GuMboI* and *GuDdeI*) obtained from restricted genomic DNA of *G. unicolor* that were characterized by Southern blot and physically localized by *in situ* hybridization on metaphase chromosomes. As they are highly repetitive DNA sequences, they map in heterochromatic regions. However, while *GuDdeI* was localized in the centromeric regions, the *GuMboI* fraction was distributed on some centromeres and was co-localized with the nucleolus organizer region (NOR). Comparative analysis with other Mediterranean species such as *Muraena helena* pointed out that these DNA fractions are species-specific and could potentially be used for species discrimination. As a new contribution to the karyotype of this species, we found that the major ribosomal genes are localized on acrocentric chromosome 9 and that the telomeres of each chromosome are composed of a tandem repeat derived from a poly-

TTAGGG DNA sequence, as it occurs in most vertebrate species. The results obtained add new information useful in comparative genomics at the chromosomal level and contribute to the cytogenetic knowledge regarding this fish family, which has not been extensively studied.

### Introduction

A large portion of eukaryotic genomes is composed of repetitive DNA sequences, that include tandem repeats such as satellites, minisatellites, microsatellites, and interspersed repeats as transposable elements.<sup>1,2</sup> In particular, satellite DNA consists of the same sequence, ranging in length from a few base pairs (bp) to thousands of bp, repeated thousands or millions of times in tandem.<sup>3,4</sup> Repetitive DNA makes up the predominant part of heterochromatin and is located mainly in pericentromeric and/or telomeric chromosome regions.<sup>3,5</sup> This fraction of the genome seems to escape the selective pressure acting on the non-repetitive segments, representing good evolutionary marker in studies of species evolution, chromosome structure and function, and in the detection of chromosomal rearrangements, supernumerary and sex chromosomes.<sup>6-8</sup> The molecular organization and chromosome location of repetitive DNAs have been analyzed in a large number of fish species.<sup>9-13</sup> These studies have demonstrated the enormous potential that the investigation of repetitive DNAs offers in extending our knowledge of karyotype differentiation in fish.

Muraenidae is a species-rich family (185 species) of tropical and subtropical fish, distributed worldwide and especially common on coral reefs.<sup>14</sup> The current classification is based mainly on morphological and anatomical characteristics, but the taxonomy remains incomplete; in particular, recent molecular phylogenetic and cytogenetic data do not support clear separation between the *Muraena* and *Gymnothorax* genera.<sup>14-18</sup>

Cytogenetically, only approximately 7% of the Muraenidae species has been studied, and for most of them, only karyotype morphology and heterochromatin distribution are known. They show a conserved chromosome number, genome size and AT-content, with the highest values among Anguilliformes.<sup>19-21</sup> Chromosome numerical similarity, with a 42 diploid number reported for all species, is accompanied by great karyotype variation [fundamental numbers (FN) = 42 to 84]; in this group, pericentric inversions may have played an important role in karyotype evolution.<sup>22,23</sup> The pattern of heterochromatin distribution is known for eight Muraenidae species; these few available

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data indicate the presence of centromeric heterochromatic blocks in almost all species; furthermore, large bands in pericentromeric and/or interstitial position are present in some species.<sup>20,22,24</sup> The nucleolus organizer regions (NOR) were localized by silver staining on one chromosome pair in all species studied; furthermore, fluorescence *in situ* hybridization (FISH) mapping of major ribosomal genes and telomeric sequences was carried out in *M. helena* and *G. tile*.<sup>18,25,26</sup> In addition, two repetitive DNA fractions from the *M. helena* genome, were isolated, physically mapped and compared to *G. unicolor*.<sup>25,27</sup> Previous cytogenetic studies characterized the karyotype of the Mediterranean moray eel *Gymnothorax unicolor* (Delaroche, 1809) by heterochromatic (C-, restriction enzyme- and CMA<sub>3</sub>-) and replication- banding and Ag-staining.<sup>20,23,28</sup> Cytogenetic comparison with the other Mediterranean species *Muraena helena* and with the congeneric species *G. tile* pointed out many chromosomal banding similarities, as well as the occurrence of pericentric inversions and changes in heterochromatin amount.<sup>18</sup>

In the present study, we carried out the isolation, molecular characterization and chromosomal mapping of two repeated DNA fractions in the *G. unicolor* genome and localized the

major ribosomal genes and telomeric sequences using FISH. Furthermore, we investigated the presence of these DNA fractions in the *M. helena* genome.

## Materials and Methods

### Chromosomal and genomic DNA preparation

Six individuals of both sexes (four males and two females) of *G. unicolor* were captured along the southwestern Sardinian coast and raised in an aquarium. Blood was collected from the dorsal artery of individuals using a heparinized syringe, and blood cells were used for all experiments. Sex was determined by histological analysis of the gonads of each individual. Metaphase chromosomes were obtained by lymphocyte cultures and chromosome morphology was classified according to arm ratios.<sup>28,29</sup> Genomic DNA was obtained using standard phenol-chloroform procedure.<sup>30</sup> For comparative purposes, *M. helena* metaphase chromosomes and genomic DNA were also used in the same experiments.

### Characterization of genomic DNA: Southern hybridization, cloning, sequencing and sequence analysis

A search for repetitive DNAs was conducted using restriction enzyme digestion of the genomic DNA of *G. unicolor* with different restriction endonucleases (*MboI*, *DdeI*, *HaeIII* and *AluI*). The endonucleases *MboI* and *DdeI* revealed conspicuous bands of about 5 kb and 350 bp, respectively. Briefly, approximately 7 µg of genomic DNA was digested with 14 U of *MboI* or *DdeI* at 37°C for 16 h and subsequently analyzed by gel-electrophoresis using 1% agarose gels containing ethidium bromide (0.5 µg/mL) and TAE buffer (80.04 M Tris-acetate, 0.002 M EDTA) at 25 V for 16 h. The genomic organization of the isolated repetitive fragment was determined using Southern blot hybridization. Genomic DNA was digested with different restriction enzymes (*MboI* and *DdeI*), separated by electrophoresis in 1.5% agarose gel, and the DNA fragments were transferred onto a Hybond N+ nylon membrane (Amersham Biosciences, Uppsala, Sweden) by capillarity. The *MboI* purified band (called *GuMboI* family) was used as a probe and hybridized under conditions of high stringency, using the non-radioactive ECL method (Enhanced Chemiluminescent Kit, Amersham Biosciences) while the *DdeI* 350-bp isolated band (called *GuDdeI* family) was cloned in the One Shot Top 10 competent *E. coli* (Invitrogen Life Technologies,

Carlsbad, CA, USA) using the Ready To Go pUC18 BAP+ligase kit (Amersham Biotech, Inc., Uppsala, Sweden) and following the manufacturer's recommendations. Five positive clones of the *GuDdeI* family were sequenced using the automated Alpha express DNA sequencer (Pharmacia, Uppsala, Sweden) and the sequences aligned in CLUSTAL W using the default parameters.<sup>31</sup> MEGA version 4 was used to determine nucleotide composition and the genetic distance between sequences calculated using the Kimura 2-parameter (K2P) method.<sup>32</sup> Moreover, a GenBank search was performed in order to compare *DdeI* satDNA with other sat DNAs in the database. One of the clones (called *GuDdeI* 313) was employed as a probe in filter experiments, following the same procedure described for *GuMboI* DNA hybridization.

### Fluorescence *in situ* hybridization procedure and karyotype analysis

FISH procedure was effected using the same probes employed in the filter hybridization (*GuMboI* family and *GuDdeI* 313 clone), the *GuDdeI* family and *Xenopus laevis* 45S rDNA. The probes were labeled with biotin-16-dUTP (Roche Applied Science, Indianapolis, IN, USA) by nick-translation, using the Nick Translation kit (Roche Applied Science) and following the manufacturer's instructions. Hybridization was performed overnight at 37°C in 50% formamide/2xSSC. Post-hybridization washes were carried out at 37°C in 50% formamide/2xSSC for 15 min followed by 15 min washes in 2xSSC at 37°C. Detection was carried out using fluorescein isothiocyanate-conjugated avidin (Vector Laboratories, Inc., Burlingame, CA, USA), with two amplification steps using anti-avidin biotin conjugated (Vector Laboratories, Inc). Chromosomes were counterstained using propidium iodide (Sigma Aldrich, St. Louis, MO, USA) (1 µg/mL) and DAPI (4',6-diamidino-2-phenylindole) (Sigma Aldrich) (0.5 µg/mL). The same procedure was effected for all probes. Telomeric sequences (TTAGGG) were mapped by two-hour-FISH using a telomere PNA (peptide nucleic acid) probe conjugated with FITC (Telomere PNA FISH kit/FITC, DakoCytomation, Glostrup, Denmark), following the manufacturer's instructions.

More than twenty metaphase plates were analyzed for each probe using a Zeiss Imager MI fluorescence microscope; images were captured with a Hamamatsu digital camera C8484 and processed using a karyotyping- and FISH-dedicated image analysis system (Cromowin Plus, TESI Imaging, Pianiga (VE), Italy).

## Results

### Characterization of *MboI*- and *DdeI*- restricted DNA fractions, Southern hybridization and sequencing

Genomic DNA of *G. unicolor* digested with either *MboI* or *DdeI* showed a high mw band of about 21 Kb (Figure 1). Moreover, *MboI* enzyme produced a band of about 5 Kb (*GuMboI*) and a smear composed of DNA fragments of about 4 kb to a few hundred base pairs (Figure 1). Cleavage of genomic DNA of

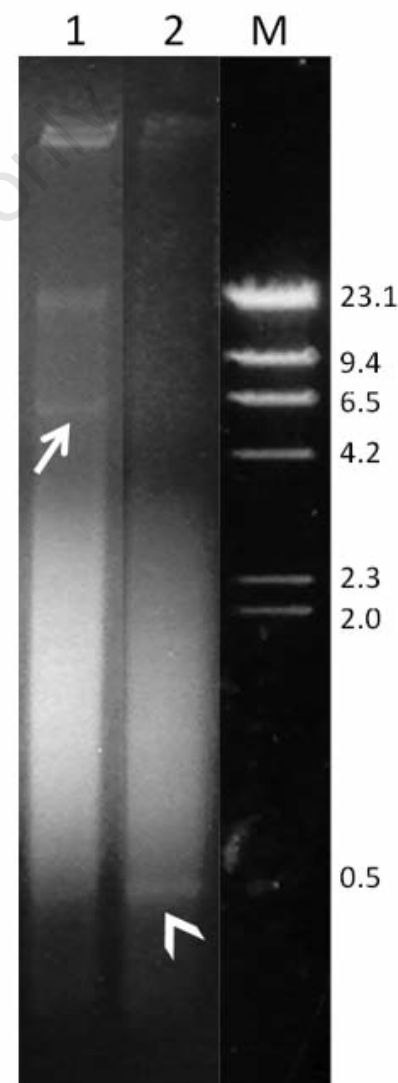


Figure 1. Agarose gel electrophoresis of *Gymnothorax unicolor* genomic DNA digested with restriction endonucleases *MboI* (lane 1) and *DdeI* (lane 2). The arrow indicates the 5kb band detected by *MboI*, and the arrowhead indicates the 350-bp band detected by *DdeI*. M, phage lambda DNA cleaved with *HindIII*, used as a molecular size marker.

the same species with *DdeI* produced an electrophoretic smear, where a band of about 350 bp (*GuDdeI*) was present (Figure 1). When *GuMboI* DNA was used as a probe in *G. unicolor* *MboI*- digested DNA, a band of about 5 Kb and other fainter bands of lower mw, ranging from 5 Kb to about 200 bp, were observed (Figure 2a). The Southern hybridization of *GuMboI* probe on *M. helena* genomic DNA digested with the same enzyme produced a band of about 10 Kb and other bands of lower mw (Figure 2a).

The cloning and sequencing of the *GuDdeI* band revealed DNA fragments of 248-340 bp. Analysis of the sequenced segment of the clones showed that the sequences were very different, and the *GuDdeI* 313 clone was selected for studies. Southern hybridization using *GuDdeI* 313 probe on *G. unicolor* genomic DNA cleaved with *DdeI* showed a ladder of bands, whose mw ranged from about 6.5 Kb to about 400 bp; furthermore, a large band of 313 bp (i.e. the *GuDdeI* 313 band) was observed (Figure 2b). These results indicate a tandem arrangement of repeating units typical of satellite DNA. When this probe was used on *M. helena* genomic DNA cleaved with *DdeI*, a series of bands ranging from about 6.5 Kb to about 600 bp and a band of about 310 bp was observed, the latter being thinner than that observed in *G. unicolor* (Figure 2b). The complete nucleotide sequence of the *GuDdeI* 313 clone is shown in Figure 2c; it was 313 bp long and composed of 58,1% AT bases. No similarity between the *GuDdeI* 313 and any sequence deposited in the nucleotide sequence databases was detected.

### Cytogenetic mapping of repetitive DNA sequences

FISH using the *GuMboI* probe produced positive signals in the centromeric region of most chromosomes of *G. unicolor*, including a large acrocentric pair (Figure 3a), the latter region being DAPI negative (Figure 3c). 45S rDNA sites were located in the pericentromeric region of a single acrocentric pair (Figure 3b) and this region was also dull after DAPI staining (Figure 3d). The hybridization of *GuDdeI* 313 probe produced bright positive signals in the centromeric region of some *G. unicolor* chromosomes, mostly acrocentric ones (Figure 3e), while *GuDdeI* probe showed positive signals in all centromeres (Figure 3f). None of the probes (*GuMboI*, *GuDdeI* and *GuDdeI* 313) produced any positive signal on *M. helena* chromosomes (*data not shown*). FISH with the telomeric probe (TTAGGG)<sub>n</sub> revealed hybridization signals on each telomere of all chromosomes, and interstitial telomeric sites (ITS) were not detected (Figure 3g).

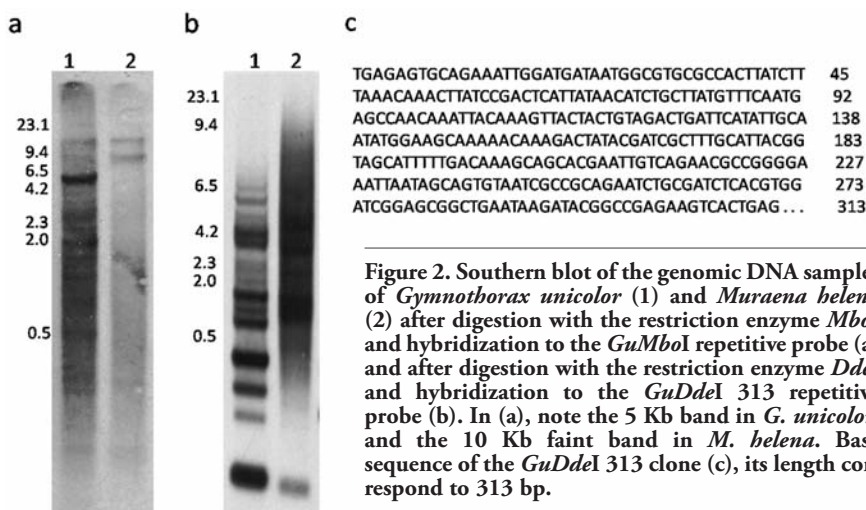


Figure 2. Southern blot of the genomic DNA samples of *Gymnothorax unicolor* (1) and *Muraena helena* (2) after digestion with the restriction enzyme *MboI* and hybridization to the *GuMboI* repetitive probe (a) and after digestion with the restriction enzyme *DdeI* and hybridization to the *GuDdeI* 313 repetitive probe (b). In (a), note the 5 Kb band in *G. unicolor*, and the 10 Kb faint band in *M. helena*. Base sequence of the *GuDdeI* 313 clone (c), its length correspond to 313 bp.

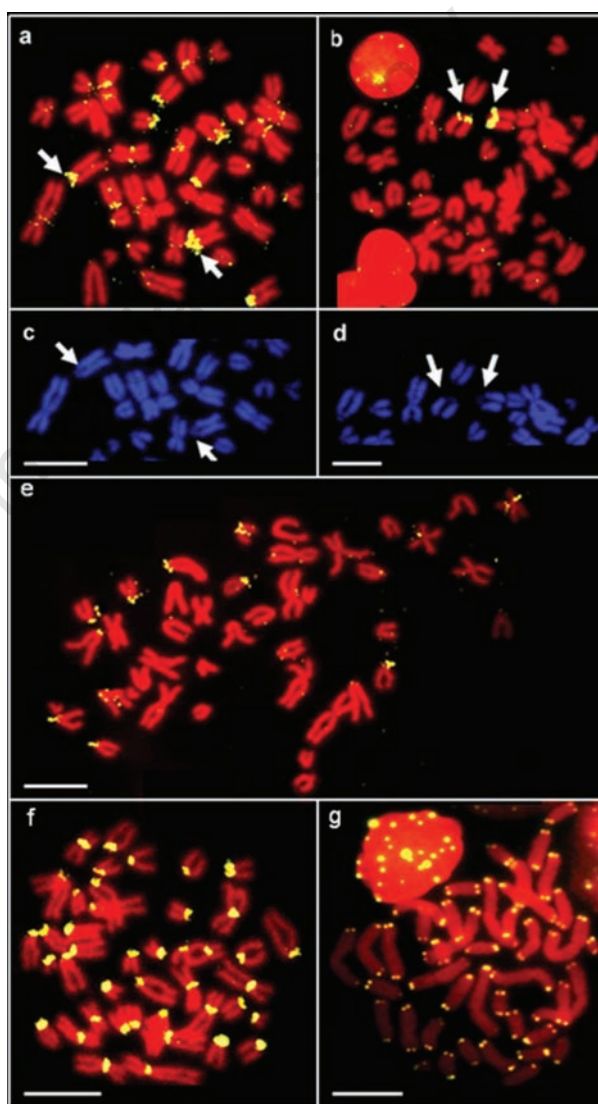


Figure 3. Fluorescence *in situ* hybridization of *Gymnothorax unicolor* metaphase chromosomes hybridized with different repetitive DNA sequences (yellow signals) and counterstained with propidium iodide. They include *GuMboI* (a), 45S rDNA sites (b), the sequential DAPI counterstaining of the same metaphases (c) and (d), *GuDdeI* (e), *GuDdeI* 313 (f) and (TTAGGG)<sub>n</sub> sequences (g). The arrows indicate the DAPI-region in the chromosome no.9 (c,d), which corresponds to the NOR site (a,b). Scale bars: 10  $\mu$ m.

## Discussion

The digestion of *G. unicolor* DNA with the enzymes *MboI* and *DdeI* showed the presence of distinct bands in agarose gels, as observed in other fish species after genomic DNA digestion with restriction endonucleases.<sup>33-36</sup> Analysis of membrane immobilized genomic DNA of *G. unicolor* hybridized to the *GuMboI* family and to the *GuDdeI* 313 clone indicates that they correspond to highly repetitive elements, predominantly clustered in the genome. The comparison of satellite *GuDdeI* DNA sequences with nucleic acid sequences available on databases revealed no similarities to any known DNA sequence. Comparative Southern hybridization revealed that these repeated DNAs are present in both *G. unicolor* and *M. helena*, with species-specific features. Among Muraenidae, repetitive DNA fractions were characterized only in *M. helena*, and in particular two *MboI*-restricted DNA fractions, called B2 and B3; Southern blotting comparative analysis on the *G. unicolor* genome pointed out the presence of these DNA fractions in both species, although showing characteristic features in each species.<sup>25,27</sup>

The two isolated repetitive elements are clustered in the centromeric heterochromatin of many chromosomes of the complement, previously identified by C-banding.<sup>20</sup> The repetitive sequences are not randomly distributed in the genome, suggesting a pattern of compartmentalization on chromosomes. In particular, the *GuMboI* sequence was localized in two different types of C-positive areas: in many centromeres and the pericentromeric region of acrocentric pair no. 9, co-localized with the Ag-NOR localization previously obtained.<sup>28</sup> FISH mapping of the major ribosomal gene family confirms the presence of only one NOR-bearing pair in the *G. unicolor* genome; this situation is also found in all the Muraenidae studied and is the most frequent condition among Anguilliformes.<sup>18,22,37</sup> The *GuMboI* fraction might be a heterogeneous repetitive DNA associated with ribosomal sequences; this hypothesis could account for the lack of signals in cross-species *GuMboI* FISH on *M. helena* chromosomes. A NOR-related DNA fraction was also obtained in *M. helena*, and the association between ribosomal cistrons and repetitive DNA is commonly found in eukaryotes.<sup>25</sup> The *G. unicolor* *GuDdeI* DNA fraction was localized in the centromeric region of all *G. unicolor* chromosomes, while the *GuDdeI* DNA 313 probe hybridized in only some centromeres, indicating that this satellite DNA family is composed of several types of centromeric repetitive DNAs, also confirmed by the great differences in nucleotide sequences

found among clones. Repetitive DNAs are frequently localized in centromeric regions of chromosomes in both animals and plants and represent the main component of functional centromeres.<sup>38-42</sup> Although their real function is still being debated, satellite DNA is required for centromere composition and organization.<sup>43</sup> Centromeric location of repetitive DNAs is also the most frequent situation among fish, and the distribution pattern in all centromeres had already been found in *M. helena* and other fish species, like some species of the genus *Sparus* and some species of Cichlidae.<sup>25,44-48</sup> In the Erythrinid fish, *Hoplias malabaricus*, 5*SHindIII* is a satellite DNA family specific to the *H. malabaricus* genome, and is located only in the centromeric region of some chromosome pairs. This satellite family has spread to the centromeric region of several chromosomes and has been favored during evolution due to a possible role of the repetitive sequences of the centromeric region in centromere structure and function.<sup>10,11,34</sup> In order to establish the degree of conservation of the *GuMboI* and *GuDdeI* DNA fractions, FISH was performed on *M. helena* chromosomes; the lack of positive signals suggests that these sequences are species-specific and might have originated after the divergence of *G. unicolor* from the other Muraenidae species. These results agree with the heterochromatin heterogeneity detected in the two species by *in situ* restriction enzyme banding.<sup>20</sup>

Telomeric (TTAGGG)<sub>n</sub> sequences 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, involved in the evolution of the chromosomes.<sup>49</sup> FISH with the telomeric probe (TTAGGG)<sub>n</sub> revealed hybridization signals on each telomere of all chromosomes of *G. unicolor*, and interstitial telomeric sites (ITS) were not detected, which could indicate that Robertsonian fusions or chromosomal translocations were probably not involved in the karyotypic differentiation of this species. The absence of ITS had already been reported for the other Muraenidae species *M. helena* and *G. tile*.<sup>18,26</sup> On the other hand, two Anguilliform species studied (*Anguilla anguilla* and *A. rostrata*) presented telomeric sequences colocalized with the NOR region.<sup>26</sup>

In conclusion, the results obtained provide information useful in comparative genomics at the chromosomal level and add to the cytogenetic knowledge regarding this fish family, contributing to the molecular and cytogenetic characterization of *G. unicolor* for studying karyotype evolution in Muraenidae. A more extensive cytogenetic survey is needed to allow evaluation of Muraenidae cytotaxonomy.

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