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Cytogenetic evidences of genome rearrangement and differential epigenetic chromatin modification in the sea lamprey (*Petromyzon marinus*)

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Abstract :

This work explores both the chromatin loss and the differential genome methylation in the sea lamprey (Petromyzon marinus) from a molecular cytogenetic point of view. Fluorescent in situ hybridization experiments on meiotic bivalents and mitotic chromosomes corroborate the chromatin loss previously observed during the development of the sea lamprey and demonstrate that the elimination affects not only to Germ1 sequences but also to the rpt200 satellite DNA and most part of the major ribosomal DNA present on the germinal line. 5-Methylcytosine immunolocation revealed that the GC-rich heterochromatin is highly methylated in the germ line but significantly less in somatic chromosomes. These findings not only support previous observations about genome rearrangements but also give new information about epigenetic changes in P. marinus. The key position of lampreys in the vertebrate phylogenetic tree makes them an interesting taxon to provide relevant information about genome evolution in vertebrates.

Keywords : Sea lamprey, Chromatin loss, Genome rearrangements, DNA methylation

Introduction

Lampreys (Agnatha, Petromyzontiformes) are one of the major extant lineages of jawless vertebrates (Takezaki et al. 2003). These organisms are crucial in the understanding of early vertebrate evolution because they constitute, together with hagfishes, the most primitive group of living vertebrates and represent the vertebrate ancestral lineage.

Despite its key position at the vertebrate phylogenetic tree, knowledge on the genome organization of lampreys is limited. Chromosome numbers and karyotypes of approximately half of the Petromyzontidae species were described before the 80's (reviewed by Potter and Robinson 1981). With the exception of cytogenetic reports performed in the genus *Lethenteron* (Suzuki et al. 1999), in *Lampetra zanandreai* (Caputo et al. 2011) and in *Petromyzon marinus* (Smith et al. 2010), no new cytogenetic data was published since then. The karyotypes of lamprey species are usually characterized by high numbers of small sized chromosomes (2n = 60 to 180). These features, together with the difficulties in obtaining an appropriate number of well spread metaphase plates, have hindered cytogenetic studies.

This work focuses on the sea lamprey, *Petromyzon marinus* (Linnaeus, 1758), an anadromous species of the North Atlantic and the Mediterranean. Sea lampreys spend most of their life as filter-feeding larvae that undergo metamorphosis to become parasitic adults depending on marine hosts. A diploid chromosome number of 2n = 168 and a karyotype mainly composed of acrocentric and a few metacentric chromosomes were described (Potter and Rothwell 1970). Moreover, a satellite DNA family (*Eco*RI satDNA) has been located at centromeric and paracentromeric positions of several chromosome pairs (Boán et al. 1996). The accomplishment of the first sea lamprey whole-genome sequence assembly (Smith et al. 2013) gave new information about the structure and composition of the genome of *P. marinus* showing a high content of repetitive elements and a richness in GC higher than in other vertebrates (Smith et al. 2013).

On the other hand, the programmed loss of about 500 Mb of DNA was described in several somatic lines during embryonic development of this species (Smith et al. 2009). One of the sequences partially eliminated, *Germ1*, consists of a 5' end somatically rare region, a fragment of the 28S rDNA, the repetitive element *rpt200,* and the whole 18S rDNA (Smith et al. 2009). The elimination processes involve complex, tightly regulated gene networks (Chen 2007) that are, therefore, susceptible of epigenetic control through different modification mechanisms among which DNA methylation is the best known (Reik et al. 2001; Feng et al. 2010). Although changes in DNA methylation levels have been extensively studied in mammals and plants (Feng et al. 2010)

and methylation has been recently identified as a marker of DNA elimination in the ciliate *Oxytricha trifallax* (Bracht et al. 2012; Bracht 2014), the characterization of the DNA methylation patterns in other organisms, including fishes is an area about which few reports have been published.

In order to determine if the programmed chromatin loss in *P. marinus* is related with changes in DNA methylation, we compared the chromosomal methylation levels on germinal and somatic cell lines of the sea lamprey through the immunolocation of 5-methylcytosine on gonadic meiotic bivalents and lymphocyte chromosomes. For a better understanding of the genome organization in sea lampreys and to study the chromosomal rearrangements involved in the DNA loss, we also analyzed mitotic and meiotic chromosomes of the sea lamprey by fluorochrome staining and C-banding and compared the fluorescent *in situ* hybridization (FISH) signal patterns obtained after using *Germ1*, *rpt200, Eco*RI satDNA and 28S rDNA sequences as probes.

Materials and Methods

Biological material

Eight specimens of lampreys were fished in the Miño River (NW Spain). Once in the lab, blood was extracted from their caudal arteries and collected in tubes containing lithium heparin (Sigma). After euthanizing the lampreys with MS-222 (Sigma), samples of muscular and gonadal tissues were excised from each individual and conserved in absolute ethanol. Part of the gonadal tissue was also dissected in order to obtain meiotic chromosome preparations. The experimental procedure was performed with the approval of the Ethics Committee of the University of Vigo, complying with the current laws of Spain. All institutional and national guidelines for the care and use of laboratory animals were followed.

DNA extraction, Polymerase chain reaction (PCR) amplification and probe labeling

Total DNA was extracted from ethanol preserved muscular and gonadal tissues using an extraction kit (Nucleospin®Tissue, Macherey-Nagel) following the supplier instructions.

FISH probes were obtained by PCR. Amplifications were performed in 20 µl of a solution containing 50 ng DNA, 1x PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 1 µM each primer and 1 U BIOTAQ DNA polymerase (Bioline). The combinations of primers used in the amplification experiments appear in Table 1. Universal primers (Vilgalys 2005) were used to amplify a fragment

of the 28S rRNA gene of the major rDNA repeat. The amplifications of the *rpt200* element and the *Eco*RI satellite were performed using primers described in Smith et al. (2009) and Boán et al. (1996), respectively. Amplifications were realized in a GeneAmp PCR system 9700 (Applied Biosystem) using the conditions shown in Table 2. All PCR products were examined by electrophoresis on 2 % agarose gels.

In order to detect exclusively the 5' end of the "somatic rare region of *Germ1*" (Smith et al. 2009), specific primers were designed using Primer3 v.0.4.0 designing tool (Rozen and Skaletsky 2000). The primers (Table 1) allowed amplifying the 5' end of *Germ1*, the part of the sequence devoid of *rpt200*, 28S rDNA and 18S rDNA.

Germ1 and *rpt200* amplifications were confirmed by cloning in a pGEM-T vector System (Promega) and sequencing using a dRhodamine terminator cycle sequencing kit (Applied Biosystems) and an automated sequencer ABI PRISM 310.

28S rDNA was labeled with biotin 16-UTP by nick translation. *rpt200*, 5' *Germ1* and *Eco*RI satDNA were directly labeled by PCR either with biotin-16-UTP or digoxigenin-11-UTP. The labeled PCR products were precipitated before FISH.

Chromosome preparation

Meiotic prophase I bivalents were obtained from male individuals showing well developed gonads. Fragments of tissue excised from the gonads were immersed in a hypotonic solution containing 0.05 % colchicine in 0.4 % KCl for 1 h. The tissue was then fixed with a solution of ethanol and acetic acid (3:1) for 1 h. Chromosome spreads were prepared by dissociating small pieces of tissue in 60 % acetic acid and dropping the cellular suspension onto clean slides heated to 58 ºC.

Mitotic chromosome preparation from lymphocyte cultures followed the protocol described by Fujiwara et al. (2001) with slight modifications. Two ml of heparinized blood were mixed with 5 ml of culture medium (Iscove, Sigma) containing 12 % fetal bovine serum (Sigma) and 1 % of an L-glutamine, penicillin and streptomycin solution (Sigma). The mixture was centrifuged at 1200 rpm for 10 min. The upper layer, corresponding to lymphocytes, was collected and added to 5 ml of the supplemented culture medium. After lymphocyte stimulation with a mixture of phytohemagglutinin (167 µg/ml, Sigma) and lipopolysaccharide (1.67 µg/ml, Sigma), cells were cultured for six days at room temperature. 5-Bromo-2'-deoxyuridine (20 µg/ml) and colchicine (0.2 µg/ml) were added to the cultures 8 and 3 h, respectively, before harvesting. After centrifugation at 1200 rpm for 10 min, cells were treated with 0.075 M KCl for 20 min at room

temperature and fixed with a mixture of ethanol and acetic acid (3:1). Chromosome slides were obtained by dropping the cellular suspension on clean slides and air drying.

Fluorochrome staining and C-banding

Some preparations were stained for 2 h with CMA (0.25 mg/ml) and counterstained with DAPI (0.14 µg/ml) for 8 min. Once washed with distilled water, slides were air-dried and mounted with antifade (Vectashield, Vector). After visualization and photography, preparations were washed and re-stained with a combination of DAPI (0.14 µg/ml) and PI (0.07 µg/ml). The slides were then washed in distilled water, air-dried, mounted in antifade and photographed again.
C-banding was performed following the technique of Sumner (1972). The chromosomes were stained with acridine orange (0.05 mg/ml) instead of Giemsa as proposed by Bella et al. (1986).

Immunolocation of 5-methylcytosine (5-mC)

Methylated cytosines were detected using a mouse antibody against 5-mC (Eurogentec) following the protocol indicated by the supplier with minor modifications. Mitotic and meiotic chromosome preparations were treated with a blocking solution (1 % bovine serum albumin (BSA) in phosphate saline solution Tween-20 (1xPBST)) for 30 min and then incubated with the 5-mC antibody for 90 min at 37 ºC. After washing in blocking solution, the 5-mC antibody was detected with a FITC (fluorescein isothiocyanate)-conjugated goat anti-mouse secondary antibody (Sigma).

Fluorescent in situ hybridization (FISH)

Single and double FISH experiments followed the methods published by Pérez-García et al. (2010) with minor modifications. Preparations were denatured at 69 ºC for 2 min and hybridized overnight at 37 ºC. Stringency washes were performed with 50 % formamide in 2xSSC (saline-sodium citrate) at 45 ºC for 15 min, 1xSSC at 60 ºC for 15 min and 2xSSC at room temperature for 5 min.
Signal detection was carried out with fluorescein avidin and biotinylated anti-avidin (Atom) for the biotinylated probes and with mouse anti-digoxigenin, goat anti-mouse TRITC (Tetramethylrhodamine isothiocyanate) and rabbit anti-goat TRITC (Sigma) for the digoxigenin-labeled probes. Slides were counterstained with DAPI or DAPI/PI and mounted in antifade.

Slide visualization and photography were performed with a Nikon Eclipse-800 microscope equipped with an epifluorescence system. Separated images for each fluorochrome were obtained with a DS-Qi1Mc CCD camera (Nikon) controlled by the Nis-elements software (Nikon). Merging of the images was performed with Adobe Photoshop. Chromosome counting was performed using the Nis-elements software (Nikon).

Statistical evaluation

In order to compare the degree of methylation of the GC-rich bands in meiotic and mitotic cells, we determined the number of 5-mC antibody in 32 mitotic metaphase and 32 meiotic prophase I plates (Table 1 in Supplementary Files). The numbers of *Eco*RI satDNA, *rpt*200 and 28S rDNA FISH signals were also determined in the same cells. In order to assess the possible reduction of these genome marks, the numbers of the mitotic signals were compared with the number of expected signals, calculated as the double of the meiotic signals, applying a Wilcoxon signed-rank test using an IBM software (IBM corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0).

Results

Fluorochrome staining and C-banding

Mitotic metaphase chromosome analysis of all specimens of *P. marinus* shows a diploid complement of 2n = 168 chromosomes whereas meiotic prophase I plates present a haploid complement of n= 99 chromosomes (Fig. 1). Combined DAPI/CMA staining of mitotic chromosomes and meiotic bivalents reveals CMA bright bands at one of the terminal regions of every meiotic bivalent and mitotic chromosome (Fig. 1a, b, g, h). These CMA positive regions are also DAPI negative (Fig. 1c, i), coincident with C-positive bright bands (Fig. 1d, j) and showing also bright fluorescence after PI staining (Fig. 1e, k). DAPI staining also reveals a banding pattern on the chromosomes; while some of the mitotic chromosomes are poorly or intensely stained with DAPI, others show alternate dull and bright regions. These differences are clearer on meiotic plates in which most bivalents show a pattern of alternate dull and bright DAPI bands (Fig. 2a, c). Some of these bright DAPI bands are coincident with faint C-positive bands (Fig. 1d, j).

Immunolocation of 5-methylcytosine

The methylation patterns of the chromosomes of the sea lamprey *P. marinus* were analyzed on 200 mitotic metaphase and meiotic prophase I plates from eight individuals. Strong 5-mC signals appear on terminal regions of all bivalents on meiotic plates. These signals are coincident with the CMA+/PI+/DAPI- regions (Fig. 2b, some signaled by arrows). Intercalary signals were also present on some regions showing dim DAPI fluorescence (Fig. 2a, b, arrowheads). 5-mC signals are also located on the terminal CMA+/PI+/DAPI- regions of the mitotic chromosomes (Fig. 2c, d, arrows) but they are fainter and not present in every single chromosome of the mitotic plate. No signals were detected in at least 20 mitotic chromosome pairs (Fig. 2c, d, arrowheads). After carefully counting the number of positive signals in 32 meiotic and 32 mitotic plates (Table 3), the application of the Wilcoxon signed-rank test to the data showed that the number of mitotic 5-mC signals shows a significant reduction ($Z = -4.938$, $p < 0.001$) with respect to the number of signals expected, that is the double of the number detected in meiotic plates counts.

Fluorescent in situ hybridization

FISH experiments using a biotin labeled *Eco*RI satDNA probe were performed in both meiotic bivalents and mitotic chromosomes from 8 specimens of *P. marinus*. Analysis of 200 chromosome plates showed signals on 17 bivalents and 31-34 mitotic chromosomes (Fig. 1e, k). The detection of all 34 signals in a single mitotic metaphase plate was difficult, mostly due to the small size of some chromosomes and its high number. *Eco*RI satDNA signals appear on bright DAPI bands, close to the CMA/PI positive regions (Fig. 1e, k). In five of the DAPI-bright bivalents and mitotic chromosome pairs, the signals cover most part of the chromosome (Fig. 1c, e, i, k, arrowheads) while in others the size of the signal is smaller (Fig. 1e, f, arrows). In mitotic chromosomes some of these small signals seem to overlap the CMA positive regions (Fig. 1k, l, arrows). The numbers of FISH signals detected in 32 meiotic prophase I and 32 mitotic metaphase plates appear resumed in Table 3. In contrast with the reduction of the 5-mC signals, the Wilcoxon signed-rank test revealed that the number of *Eco*RI satDNA FISH signals is not significantly different ($Z=-1.738$, $p=0.082$) from the double of those detected in meiotic plates.

The repetitive element *rpt200* was mapped by FISH with a digoxigenin labeled probe. Meiotic prophase I plates show signals on DAPI-dull regions of 20 bivalents. Most signals are close to the bright CMA/PI regions (Fig. 1f, asterisks). In contrast, only 20 to 30 mitotic chromosomes, and not the expected 40, present *rpt200* signals. Most of these signals seem to overlap the CMA/PI positive regions on mitotic chromosomes (Fig. 1l, asterisks). Table 3 shows the number of *rpt200* FISH signals in 32 meiotic prophase I and 32 mitotic metaphase plates. The number of *rpt200* FISH

signals is significantly lower (Z= -4.963, $p < 0.001$) than expected taking into account the number of signals detected in meiotic plates.

Double FISH experiments demonstrated that signals for *Eco*RI satDNA and *rpt200* map to different meiotic bivalents and chromosome pairs (Fig. 1f, l). In the case of *Eco*RI satDNA, posterior C-banding of the same plates confirmed that some signals are located on C positive bands on meiotic bivalents and on mitotic chromosomes (Fig. 1d, j).

Major ribosomal genes were mapped by FISH using a biotin labeled fragment of the coding region of the 28S rDNA (Fig. 3a, c, d, f). This 28S rDNA probe covers part of the 28S rDNA segment included in the *Germ1* sequence. FISH signals were detected in 9 bivalents (Fig. 3a). In contrast, a single location in one chromosome pair was present in mitotic metaphase plates (Fig. 3d). Double FISH experiments using 28S rDNA and *rpt200* probes labeled differently revealed that all 28S rDNA signals coincide with *rpt200* signals, but there are some *rpt200* signals devoid of 28S rDNA signals, too (Fig. 3c, f). The 5' end somatically rare region of *Germ1* probe was used to determine the true localization of the *Germ1* sequence with respect to the 28S rDNA position (Fig. 3g, h, j, k). Double FISH experiments revealed that 5' end somatically rare region of *Germ1* and 28S rDNA signals co-locate in both meiotic and mitotic chromosomes (Fig. 3i, l). A schematic representation of the main types of meiotic and mitotic chromosomes, attending to their cytogenetic markers, is shown in the Figure 4.

Discussion

Programmed loss of chromatin, far from an unusual event, seems to be a common process of cell differentiation and development. Chromatin elimination has been reported in somatic cell lines during embryonic development of nematodes, copepods, insects, marsupials, hagfish (reviewed by Kloc and Zagrodzinska (2001)) and in the sea lamprey *P. marinus* (Smith et al. 2009; Smith et al. 2010). As previously described by Smith et al. (2010), discrepancies in haploid complement were detected between lymphocytes (n = 84) and germ-line cells (n = 99), indicating that chromosomal fusion and/or chromosome loss occurred during development. The lymphocyte chromosome counts obtained in this work (2n = 168) coincide with the numbers previously described by Potter and Rothwell (1970), but differs with respect to the diploid chromosome number (2n = 164) reported by Smith et al. (2010). Given the small size of the sample and the difficulties in accurately counting large numbers of small chromosomes, the differences might be due to variation among individuals but also to a loss of chromosomes during the spreading caused by strong hypotonic treatment.

The FISH experiments presented here corroborate the *Germ1,* 28S rDNA and *rpt200* loss observed by Smith et al. (2009) in the somatic line but also show that *rpt200* appears in locations devoid of *Germ1*, confirming that *rpt200* is not exclusively a part of the *Germ1* sequence. Taking into account that *Germ1* and major rDNA signals co-localize with *rpt200* signals in meiotic bivalents (Fig. 4), most of the *rpt200* signal loss squares with major rDNA and *Germ1* elimination (Smith et al. 2009). The *rpt200* signals still present in the somatic line mostly correspond to *rpt200* loci not associated to *Germ1* with the exception of the single remaining nucleolar organizer region of mitotic chromosomes.

Chromatin loss has been associated to elimination of heterochromatic regions in hagfishes, copepods and nematodes (Nakai et al. 1991; Müller and Tobler 2000; Grishanin 2014). Nonetheless, the terminal heterochromatic regions detected in all meiotic bivalents of *P. marinus* are still present in somatic mitotic chromosomes. The presence of these GC rich heterochromatic regions is consistent with the high GC content of the genome of *P. marinus* (Smith et al. 2013). Moreover, we also detected significant differences in the degree of methylation of germ and somatic cell lines. DNA methylation has been associated with the structural maintenance of the genome (Matzke et al. 2000; Jones 2012) and the control of repetitive elements (Suzuki and Bird 2008; Feng et al. 2010). Some of the DNA methylation signals detected could be involved in those processes, but the significant reduction in their number probably indicates that most of the signals are related with the control of the chromatin loss. In this sense, methylation has been recently identified as a marker of DNA elimination in the ciliate *Oxytricha trifallax* (Bracht et al. 2012; Bracht 2014). Although the reduction in the 5-mC signals could also be partly attributed to technical problems associated to the differences in chromosome compaction of meiotic bivalents and mitotic chromosomes, the absence of differences in the number of *Eco*RI satDNA FISH signals in the same cells indicates that it is not the case. Moreover, specific cell-type DNA methylation patterns due to regulatory landscape have been described in some vertebrates (Laurent et al. 2014). However, most of the DNA methylation status remains unchanged in all tissues and only a small portion is considered tissue-specific (Dricu 2012). Nevertheless, further studies are necessary to determine the implication of these processes on the methylation differences detected in lampreys.

Although the presence of bright CMA bands at terminal positions on the chromosomes of *P. marinus* points to the presence of GC-rich DNA satellites in this species, the only satellites thus far characterized in lampreys, *Eco*RI and *rpt200*, are relatively rich in AT. Both satellites map to different chromosome pairs and the signals are contiguous to the terminal CMA+/DAPI-/C- bands in meiotic prophases, but on mitotic chromosomes seem to overlap these regions. These

apparent overlapping detected in mitotic chromosomes can be attributed to limitations in the resolution power of the technique partly due to the high condensation degree of these chromosomes. *Eco*RI satDNA signals always appear on DAPI+ regions, as expected from a 59.19 % AT-richness. On the other hand, *rpt200* signals map to chromosomal regions without differential DAPI staining despite their richness in AT (54.68 %). The hybridization results obtained for *Eco*RI satDNA are in accordance with those provided by Boán et al. (1996) but clearly differ from those found in *Lampetra zanandreai,* in which the *Eco*RI satDNA only appears at the centromeric region of a single chromosome pair (Caputo et al. 2011). In contrast with most other eukaryote species, the heterochromatin detected by C-banding in *P. marinus* appear on both AT-rich (CMA^0/DAPI+), and GC-rich (CMA+/DAPI-) regions. Thus far, GC-rich heterochromatin have only been previously reported in the cyprinid *Gobio gobio* (Kirtiklis et al. 2005), in several other fish species of the genus *Chromaphyosemion* (Völker et al. 2005) and in the lamprey *Lampetra zanandreai* (Caputo et al. 2011). As also happens in the fish *Piaractus mesopotamicus* (Almeida-Toledo et al. 1998), the bright signals obtained after 5-methylcytosine immunolocation are located in the GC-rich heterochromatic regions, demonstrating that on meiotic chromosomes these regions are highly methylated and, therefore, transcriptionally repressed (Jaenisch and Bird 2003).

The phylogenetic relationships between lampreys and the rest of the vertebrate groups and the deepness of their lineage ancestry, makes genome characterization of lampreys an interesting subject that might provide relevant information about genome evolution in vertebrates. Here we confirmed the chromatin loss in the somatic line and demonstrated that the elimination affects not only to *Germ1* sequences but also to the *rpt*200 satellite DNA and most part of the major ribosomal DNA present on the germinal line. We also found that the GC-rich heterochromatin regions of germ and somatic lines show different methylation patterns. The change in the methylation pattern, its GC-rich heterochromatin and the elimination of chromatin during its development show that lampreys are an intriguing taxonomic group in the tree life meriting further research.

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339
340 **Conflict of interest**
341 The authors declare that they have no conflict of interest.
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Figure Legends

Fig. 1 Sequential DAPI/CMA/PI staining, C-banding and FISH using *Eco*RI sat and *rpt200* probes on the chromosomes of *Petromyzon marinus*. Combined DAPI/CMA staining shows CMA bright bands at one of the ends of every meiotic bivalent (**a**, **b**) and mitotic chromosome (**g**, **h**). These CMA+ bands are also DAPI- (**c**, **i**), coincident with C-positive bands (**d, j**) and showing bright fluorescence after PI staining (**e**, **k**). Fainter C-bands are also located in some CMA^0 regions (arrowheads **d, j**). DAPI staining also shows variations in fluorescence intensity along the chromosomes, some remarked with arrowheads (**c**, **i**). *Eco*RI satellite signals (SAT) appear on DAPI+ regions (arrowheads in **e**, **k**) close to the CMA+/PI+ bands. The number of *rpt200* signals decreases from meiotic prophase to mitotic metaphase (asterisks in **f**, **l**). Double-color FISH using *Eco*RI satDNA (SAT) and *rpt200* probes show that the signals for each probe appear on different chromosomes (**f**, **l**). Scale bars, 10 µm

Fig. 2 Immunolocation of 5-methylcytosine (5-mC) on meiotic bivalents and mitotic chromosomes of *Petromyzon marinus* counterstained with DAPI and PI. Strong 5-mC signals appear on every single CMA+/PI+ region on meiotic bivalents (arrows in **b**) and are also present at some intercalary DAPI faint bands (arrowheads in **a**, **b**). 5-mC signals are also coincident with CMA+/PI+ regions on mitotic chromosomes but the signals are both less intense and not present in all chromosomes (arrowheads in **c**, **d**). Scale bars, 10 µm

Fig. 3 Chromosomal location of major rDNA, *rpt200* and 5' *Germ*1 in *Petromyzon marinus.* 28s rDNA probe yielded signals on nine different bivalents (arrows in **a**) but in only a single mitotic chromosome pair (arrows in **d**). *rpt200* signals appear on DAPI-dull regions of some bivalents, most of them close to the bright CMA/PI regions (**b**), and in a lower number on mitotic chromosomes (**e**). Double FISH experiments using 28S rDNA and *rpt200* probes labeled differently revealed that all 28S rDNA signals coincide with *rpt200* signals, but there are *rpt200* signals devoid of 28S rDNA signals, too (arrowheads **c**, **f**). Double-color FISH experiments using 5' *Germ* 1 and 28S rDNA probes always show overlapping signals in both meiotic and mitotic chromosomes (**i**, **l**). Single FISH images for 5' *Germ*1 (**g, j**) and 28s rDNA (**h, k**). Scale bars, 10 µm

Fig. 4 Schematic representation of the chromosomal markers on meiotic and mitotic chromosomes of *Petromyzon marinus*. *Germ1* is eliminated in 8 chromosome pairs (a) and kept in one (b).

Figure1
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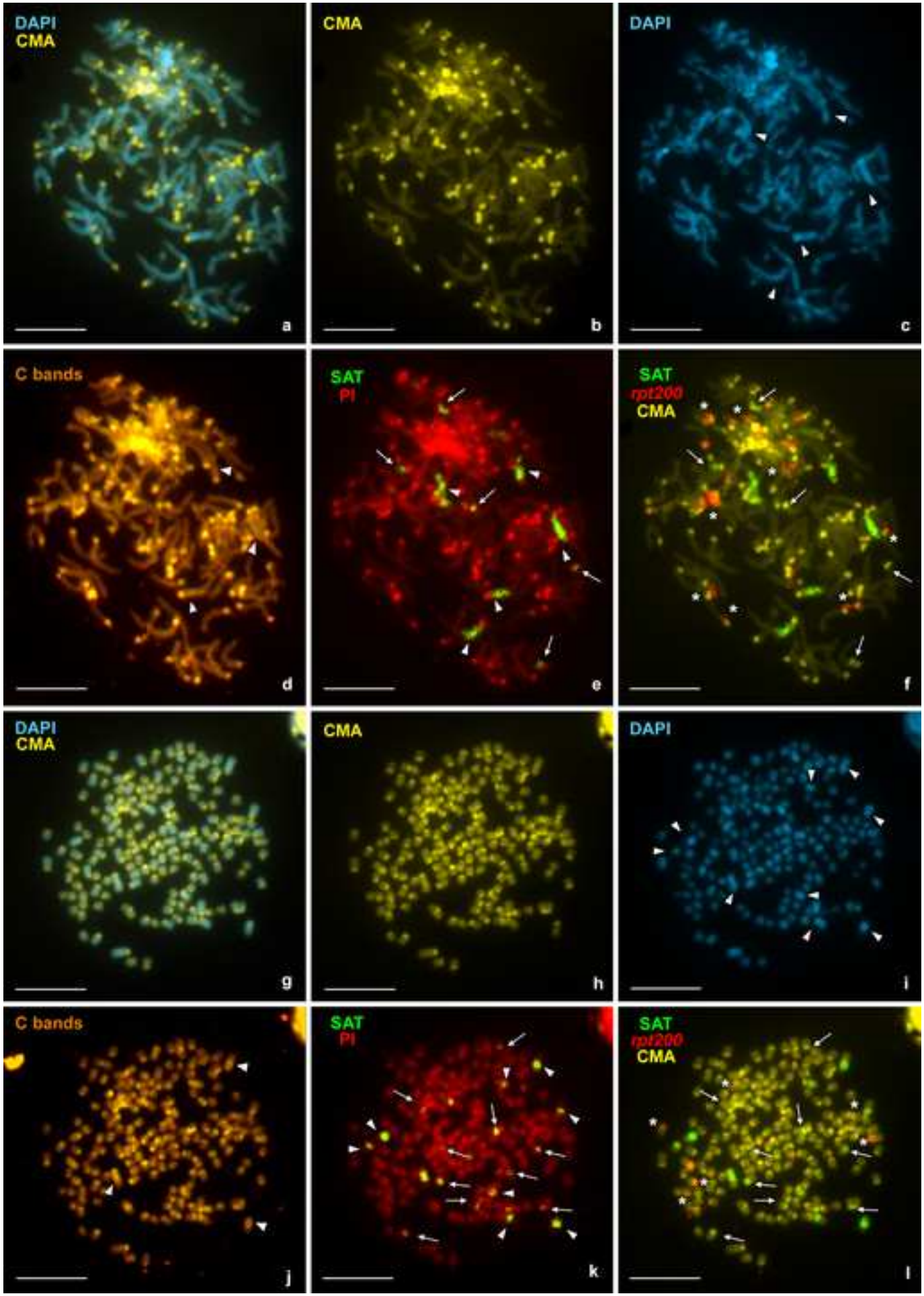

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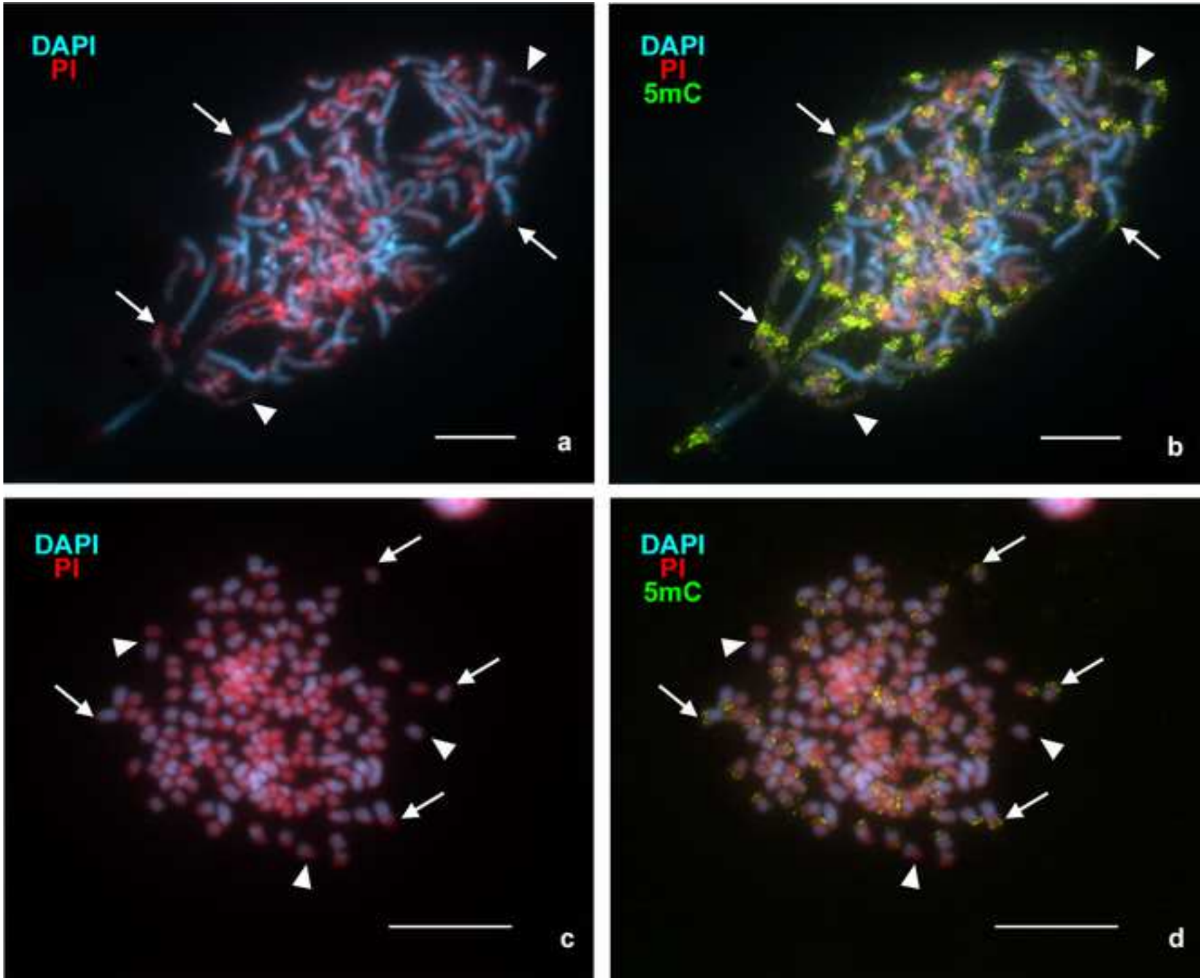

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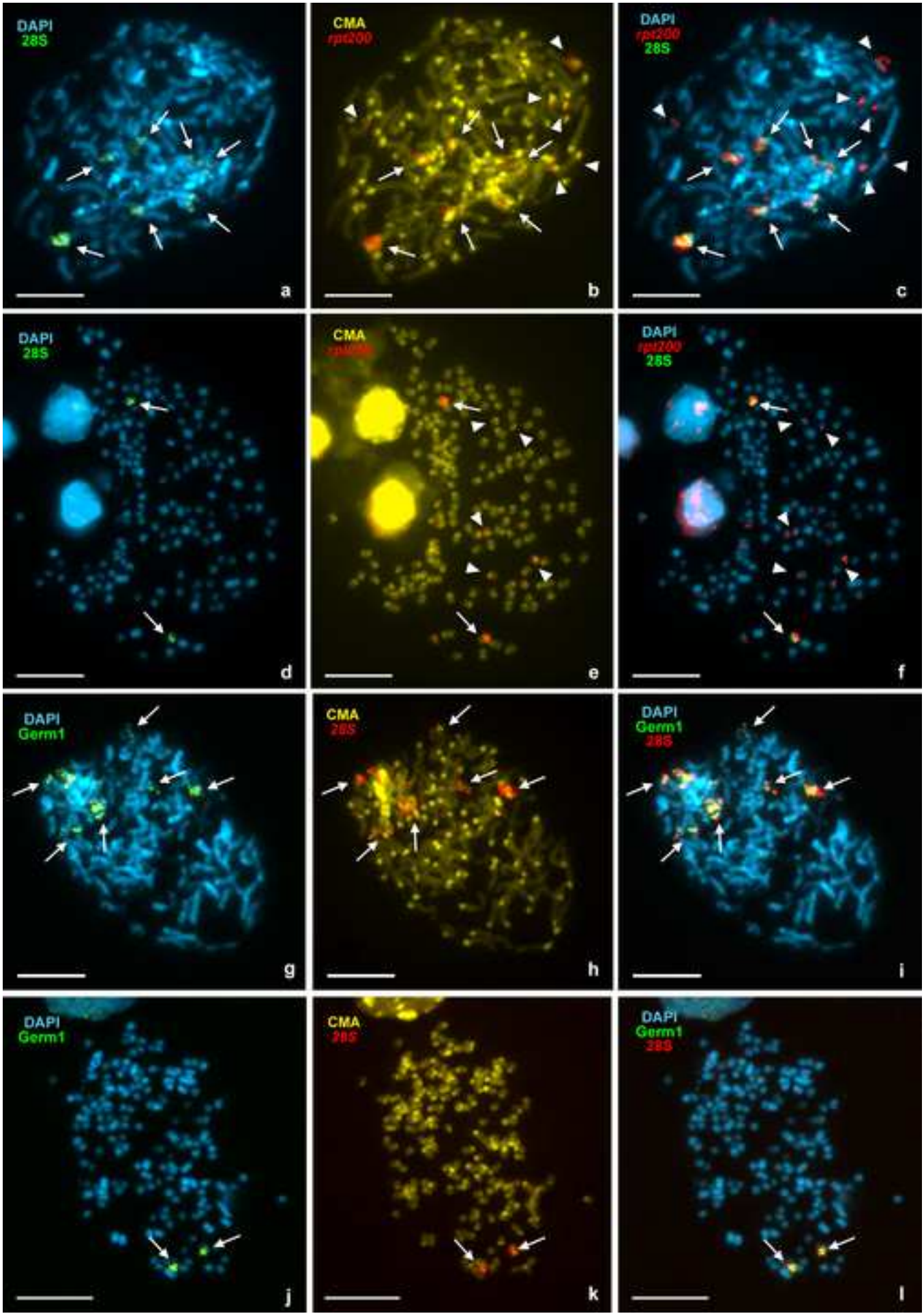

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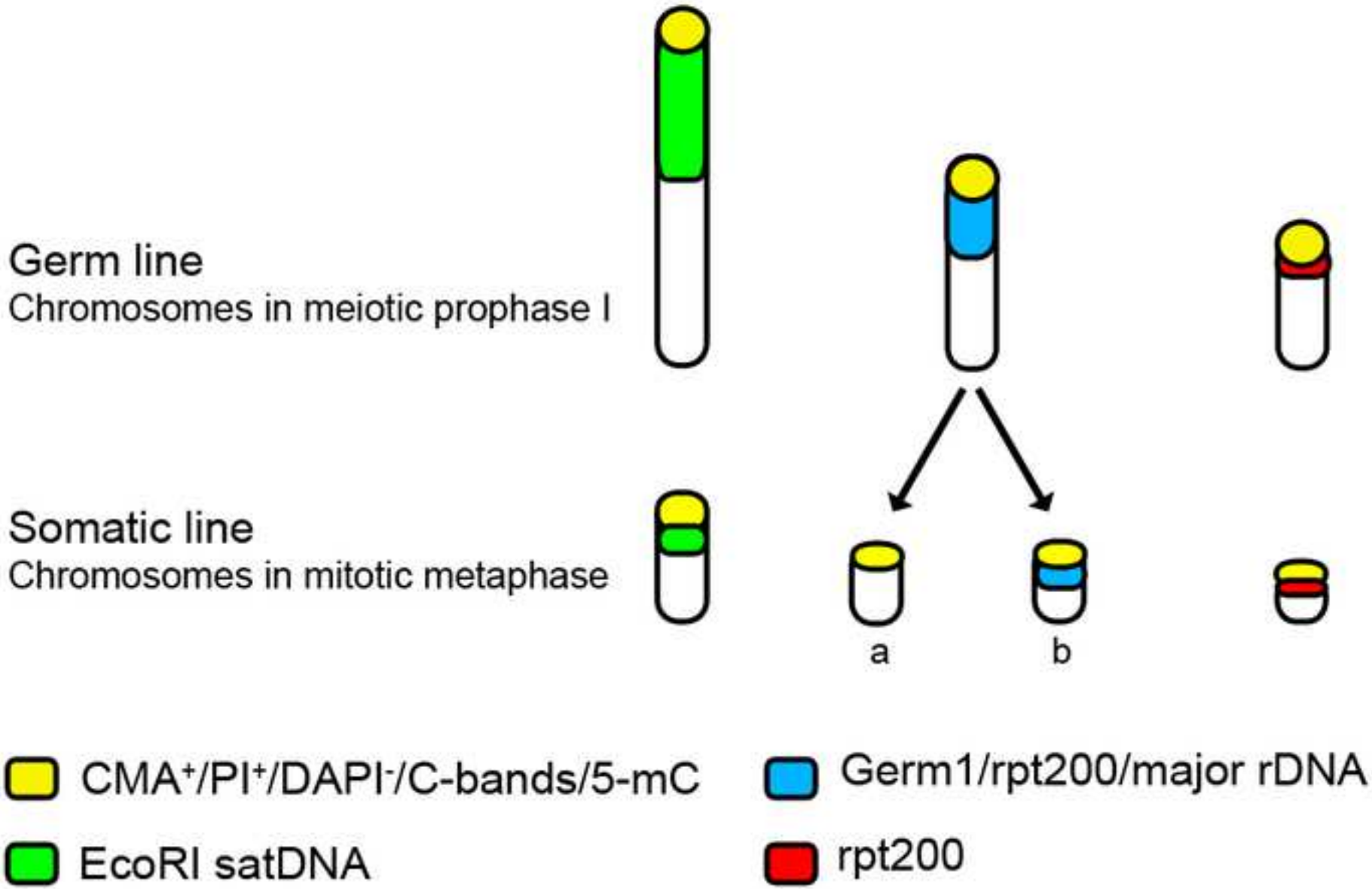

Table 1
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Table 1 Combinations of primers used in the PCR amplification

Region	Primers	Primer sequence (5'→3')
28S rDNA	*LR10R*	GACCCTGTTGAGCTTGA
	LR12	GACTTAGAGGCGTTCAG
rpt200	*rptF*	GAAATGCATGTGCACTCAAAA
	rptR	ATGGGGTTGAATGCTTTTTG
***Eco*RI satDNA**	*satF*	TCTCCCCGTCTGGACGAGAGGAA
	satR	GACCCAAACCGAGAGGATTCTGGG
5' *Germ1*	*5' germF*	ACGTATCGATGAGGGAGCAC
	5' germR	TGTTCCACCGTCAAAAGTGA

Table 2
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Table 2 Parameters of the PCR amplification

Probe	Initial denaturation	30 cycles			Final elongation
		Denaturation	Hybridization	Elongation	
28S rDNA	95 ºC, 5 min	95 ºC, 20 s	48 ºC, 20 s	72 ºC, 20 s	72 ºC, 7 min
rpt200	94 ºC, 5 min	94 ºC, 45 s	59 ºC, 45 s	72 ºC, 30 s	72 ºC, 7 min
***Eco*RI satDNA**	95 ºC, 5 min	95 ºC, 20 s	57 ºC, 20 s	72 ºC, 20 s	72 ºC, 7 min
5' *Germ1*	95 ºC, 5 min	95 ºC, 8 s	60 ºC, 8 s	72 ºC, 8 s	72 ºC, 7 min

Table 3
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Table 3 Number of 5-mC, EcoRI satDNA, rpt200 and 28 S signals in meiotic prophase I and mitotic metaphase plates of sea lamprey. The number of expected mitotic signals are also given

	5-mC			**EcoRI satDNA**			**rpt200**			**28S**		
	Meiotic plates	Mitotic plates		Meiotic plates	Mitotic plates		Meiotic plates	Mitotic plates		Meiotic plates	Mitotic plates	
		Observed	Expected		Observed	Expected		Observed	Expected		Observed	Expected
Mean	76.75 ± 0.87	69.94 ± 1.00	153.50 ± 1.74	16.22 ± 0.20	31.31 ± 0.26	32.44 ± 0.41	19.94 ± 0.12	28.16 ± 0.30	39.88 ± 0.24	10.91 ± 0.16	2.00 ± 0.00	21.81 ± 0.32
Median	77.50	65.00	155.00	16.00	31.00	32.00	20.00	29.00	40.00	11.00	2.00	22.00
Mode	71	64	142	16	32	32	20	27	40	10	2	20
Range	70-85	57-76	140-170	14-19	28-34	28-38	19-21	25-30	38-42	10-12	2	20-24

Supplemental File

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