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Research Article

First cytogenetic characterization of the sub-arctic marine fish *Mallotus villosus* (Müller, 1776), Osmeriformes, Osmeridae

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Abstract

The capelin, *Mallotus villosus* (Osmeriformes, Osmeridae), is an ecological and commercial key component of the sub-arctic ichthyofauna. Here, we provide the first cytogenetic information on the species based on both conventional karyotyping and chromosomal mapping of 45S and 5S ribosomal genes through fluorescence *in situ* hybridization (FISH). The capelin genome displayed a diploid number of 54 with the karyotypic formula 26m/sm+28st/a and a fundamental number (FN) = 80. Both classes of ribosomal genes appeared to be spread out to multiple chromosomal locations, *i.e.* the 45S and 5S rDNA clusters were detected on six and seven chromosome pairs, respectively. A linked chromosomal organization of the major and minor ribosomal genes classes has been visualized in most of the rDNAs chromosomal locations. A comparative analysis of the available cytogenetic data for the family Osmeridae reveals diploid numbers higher than 48 and high fundamental numbers. This suggests that a rearranged karyotype is a shared feature within this family.

Key words: capelin, chromosomes, Osmeridae, ribosomal genes.

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Introduction

The capelin, *Mallotus villosus* (Müller, 1776), is a smeltlike fish species widely distributed in sub-arctic waters (Gjøsæter, 1998). The monospecific genus *Mallotus* belongs to the family Osmeridae (*sensu* Johnson and Patterson, 1996). The precise position of *Mallotus* within the Osmeridae has been challenged, however. Johnson and Patterson (1996) included the genus within the tribe Salangini, whereas it appears to be closer related to the Osmerini/Hypomesinae clade according to a recent analysis of mitochondrial DNA sequences (Fu *et al.*, 2005).

In the Barents Sea, capelin is an abundant component of the ichthyofauna. It is the main plankton-feeder and prey for Atlantic cod, *Gadus morhua*, and harp seal, *Phoca groenlandica* (Gjøsæter, 1998; Nilssen *et al.*, 2000). The life cycle of Barents Sea capelin is characterized by extensive seasonal feeding and spawning migrations: juveniles and adults spend most of their life offshore and migrate towards the spawning grounds along the Norwegian and Russian coasts during sexual maturation in spring (Magnússon *et al.*, 2005). The species appears to be semelparous (Gjøsæter and Loeng, 1987), although females from fjord populations may survive to spawn a second time (Christiansen and Siikavuopio, 1998). As the focal target of a major industrial fishery, the Barents Sea capelin has been extensively studied in terms of population dynamics, stock assessments, and management (*e.g.* Gjøsæter, 1998; Hjermann *et al.*, 2004). Biological investigations have mainly focused on trophical ecology (Gjøsæter and Loeng, 1987; Gjøsæter, 1998), bioenergetics of reproduction (Christiansen and Siikavuopio, 1998; Karamushko and Christiansen, 2002), and on the swimming costs associated with spawning migrations (Behrens *et al.*, 2006).

Despite the relevance of capelin in the ichthyofauna of the sub-arctic regions, several basic biological aspects, including cytogenetics, remain to be investigated. Actually, chromosomal studies on Arctic and sub-arctic fishes are surprisingly scanty (Klinkhardt *et al.*, 1995; Ghigliotti *et al.*, 2005), although cytogenetic data can provide valuable information on species diversification and diversity in fish communities (*e.g.* Galetti *et al.*, 2006; Pisano *et al.*, 2007b). Molecular cytogenetics allows resolution of the finer details of chromosome structure in terms of spatial arrangements of genes and DNA sequences. Some genomic

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regions such as the telomeric DNA, the centromeric satellites, and the ribosomal genes are largely recognized as powerful chromosomal markers for both inter-specific (*e.g.* Almeida-Toledo *et al.*, 2002; Mazzei *et al.*, 2004) and intra-specific (*e.g.* Gromicho and Collares-Pereira, 2004) analyses. In addition, recent advances in the field have largely increased the potential of fish cytogenetics in the study of genome organization and comparative genomics (Martins *et al.*, 2004; Fischer *et al.*, 2004; Phillips, 2007).

As a contribution to the biological and taxonomical knowledge of capelin, we have undertaken a cytogenetic study by using both conventional karyotyping and molecular cytogenetics. Here we provide the karyotype and the localization of 45S (major or nucleolar) and 5S (minor or extranucleolar) ribosomal genes through cytogenetic mapping. Apart from preliminary data presented as conference abstracts, no cytogenetic information is available for capelin.

Materials and Methods

Animal sampling and chromosome preparation

A total of 28 sexually mature capelin *Mallotus villosus* (14 males and 14 females) from the Barents Sea population were studied. Live specimens were sampled by bottom trawl from the R/V "Jan Mayen" along the Finnmark coast (Ingøy 71 02 °N, 24 13° E; Loppa 70 29 °N, 21 52 °E), northern Norway and transported to the research facilities at the University of Tromsø. The fish were kept in aquaria with running, aerated sea water. After treatment of specimens with colchicine, suspensions of mitotic cells were obtained from the head kidney and spleen following the method of Doussau de Bazignan and Ozouf-Costaz (1985) with slight modifications. Fixed chromosome preparations were stored at -20 °C for later analysis.

Karyotyping

Chromosome spreads were treated for conventional karyotyping after DAPI staining. Characterization of chromosomal morphology followed the standard nomenclature according to the centromeric position and arm ratio (Levan *et al.*, 1964; Klinkhardt *et al.*, 1995). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) or acrocentric (a) and arranged in the karyotypes according to morphology and size.

Ribosomal probes

The 28S probe for detecting the major ribosomal genes consisted of a 400 bp sequence containing the C1 and C2 conserved domains and a more divergent domain D1. The DNA fragment used as a probe for detecting the 5S ribosomal genes is a 120 bp sequence corresponding to the 5S coding region (highly conserved among fishes and other vertebrates). The probes were prepared as previously described in Mazzei *et al.* (2004).

The 28S and 5S rDNA probes were labelled with biotin-16 dUTP and digoxigenin-11 dUTP respectively by use of a nick translation kit (Roche Diagnostics) following the manufacturer instruction. The labelled probes were purified by ethanol precipitation and dissolved individually or together in the hybridization buffer (50% formamide/ 2×SSC, 40 mM KH_2PO_4, 10% dextran sulfate) to yield final concentrations of 10 ng/µL (28S rDNA) and 20 ng/µL (5S rDNA).

Fluorescence *in situ* hybridization (FISH)

One-color FISH and two-color FISH were performed as described previously (Mazzei *et al.*, 2004). Briefly, the chromosomes were denatured by heating at 70 °C for 30 s in 70% (v/v) formamide/2×SSC (pH 7), dehydrated in a cold ethanol series, and air-dried. The probes were applied to chromosomal spreads (20 µL per slide) and the slides were incubated overnight in a moist chamber at 37 °C. High-stringency post-hybridization washing was performed in 2×SSC at 72 °C (5 min) followed by 2 min in 1×PBD (MP Biomedicals) at room temperature. Bound single probe was detected by incubation of chromosomal spreads with streptavidin-FITC (MP Biomedicals); fluorescence signals of two-color FISH were visualized by co-application of the rhodamine-anti-digoxigenin antibody (Roche Diagnostics) and streptavidin-FITC (MP Biomedicals). The chromosomes were counterstained in 0.3 µg/mL DAPI/2×SSC and mounted in a standard antifade solution (Vector).

Microscopic analyses

The metaphases were analyzed by an Olympus BX61 or a Zeiss Axiophot equipped with CCD cameras for digital imaging. Micrographs were processed either by Genus Software (Applied Imaging) or by Adobe Photoshop and Corel Photopaint image analysis softwares.

Results

The main results of karyotyping and molecular cytogenetic characterization are summarized in Figure 1. Chromosomal counts of multiple metaphase plates from all the specimens gave a consistent number of 54 elements. The corresponding karyotype was established by arranging the 27 pairs of homologous DAPI-stained chromosomes into two series: the meta/submetacentric series composed by 13 pairs of chromosomes and the subtelo/acrocentric series made up of 14 pairs of elements (Figure 1a). Chromosome pairs in each series were arranged according to their size. A certain morphological ambiguity, mostly due to the variability in the condensation of chromosomes in different plates, made it difficult to classify some elements as submetacentrics or acrocentrics. This morphological variability was observed for chromosome pairs 4, 5, and 6, finally assigned as submetacentrics, and in the homologs of pair 17,

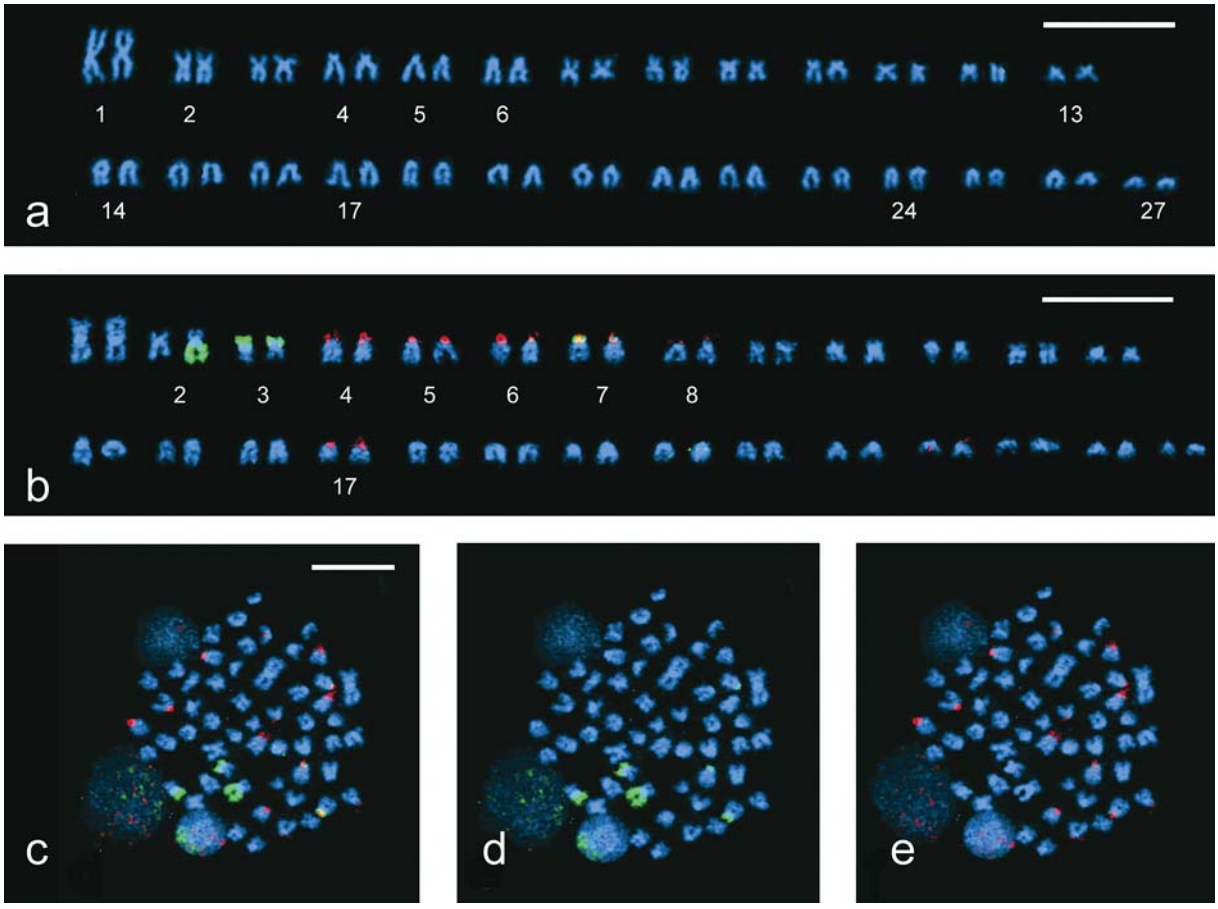

Figure 1 - Cytogenetics of the Barents Sea capelin *Mallotus villosus*. DAPI stained chromosomes arranged in the karyotype (a); pattern of 45S (green) and 5S (red) ribosomal genes onto the karyotype after two-color FISH (b); simultaneous visualization of 45S and 5S rDNA loci onto metaphase chromosomes (c); separate visualization of major (d) and 5S (e) ribosomal sequences on the same hybridized metaphase. Bars = 10 μm.

classified as acrocentrics. As a result, the karyotypic formula for capelin is 26m/sm+28st/a and the fundamental number (FN) is 80. The chromosomes composing pair 1 were easily recognizable in all metaphases in being the largest elements of the set. The size of the chromosomes for this pair is at least twice the size of pair 2 and four times the size of the smallest elements of the karyotype (pair 27). Recognizable polymorphisms were not observed except for a size heteromorphism between the homologs of pairs 2 and 24 in some specimens.

The results of the *in situ* hybridization indicated that ribosomal genes are spread at several chromosomal locations (Figure 1b, c). The hybridization signals of the 28S rDNA probe (Figure 1d) revealed that large amounts of major ribosomal gene clusters are located on the submetacentric chromosomes of pairs 2 and 3. On the homologs of pair 2, the ribosomal gene signals extend along the longest arms and, interestingly, they have consistently been detected in only one of the two chromosomes of the pair. On pair 3, the clusters of major ribosomal genes occupy the entire short arms of both the homologs. Minor amounts of major ribosomal genes were always detected on the short arms of the chromosomes composing pair 7. Small hybridization signals were also observed on the short arms of chromosomes number 4, 5, and 6, in most of the metaphases. Hybridization signals of 5S rDNA (Figure 1e) were detected on several chromosomes at the sub-centromeric position and extended along the small arms in the submetacentric elements. A differential intensity of the signals on the various chromosomes indicated that the 5S ribosomal gene loci differ in number of repeated gene clusters. According to the intensity of the FISH signals, the bulk of 5S rDNA sequences is located on pairs 4, 5, 6, and 17. Other loci, representing a minor number of 5S genes, are located on chromosomes 7 and 8. For most of the locations, the 5S rDNA colocalized with the major rDNA, except for pairs 8 and 17. A small amount of 5S rDNA genes colocalized with the major ribosomal genes on the short arms of the chromosomes of pair 3 but, interestingly, 5S rDNA sequences were not detected on the chromosomes of pair 2, which are the elements bearing the largest amount of 45S ribosomal genes in the complement.

No recognizable differences were noticed either in karyotypes or in the patterns of ribosomal genes between the studied males and females.

Discussion

The karyotype of capelin

Among the 23,637 fish species of the division Teleostei (Nelson, 1994) only approximately 12% have been cytotaxonomically studied (Klinkhardt *et al.*, 1995). Thus, data are still insufficient to draw a general picture on the chromosome evolution in fishes. Nevertheless, by re-examining the available karyological data, Brum and Galetti (1997) proposed that the putative ancestral teleost karyotype of 48 acrocentrics (Ohno, 1974) should be restricted to the Euteleostei and Clupeomorpha. However, the hypothesis of Ohno (1974) was supported by Jaillon *et al.* (2004). These authors demonstrated that the entire genome duplication, which leads to a diploid number of 48 chromosomes, occurred in the ray-finned fish (actinopterygians) lineage after the separation from tetrapods.

The karyotype of capelin is composed of 54 chromosomes and appears slightly derived with respect to the possible ancestral set of 48 chromosomes. On the other hand, the high fundamental number (80), due to the occurrence of many biarmed elements, suggests that the present chromosomal morphology is the result of a complex history of rearrangements. The reconstruction of the steps that might lead to the present karyotype would require detailed comparative analyses among phylogenetically related species. Unfortunately, few cytogenetic data are available for the family Osmeridae, and they comprise mainly the basic characterization in four species from three genera (*Hypomesus, Osmerus,* and *Sprinchus*) (Table 1). The limited number of studied osmerid taxa doesn't provide sufficient data either to support hypotheses on chromosomal rearrangements that could have led to the present condition in capelin, or to reconstruct any karyotypic trend for the family. However, the occurrence of diploid numbers above 48 and high fundamental numbers in the four taxa studied (Table 1) suggest that species of the family Osmeridae are characterized by highly rearranged karyotypes.

Chromosomal organization of ribosomal genes

Ribosomal genes sequences (rRNA genes) have begun to play an important and innovating role as markers for karyotypic and chromosomal characterization for both freshwaters fishes (*e.g.* Vera *et al.*, 2003; Hatanaka and Galetti, 2004; Kavalco *et al.*, 2004; Mantovani *et al.*, 2005) and, to a lesser degree, for marine species (Jankun *et al.*, 2001; Pardo *et al.*, 2001; Sola *et al.*, 2003; Vitturi *et al.*, 2005; Rossi *et al.*, 2005; Galetti *et al.*, 2006), including polar fishes (Mazzei *et al.*, 2004). The chromosomal patterns of rRNA genes have been investigated in model and domestic fishes such as *Tetraodon nigroviridis* (Fischer *et al.*, 2000), *Oreochromis niloticus* (Martins *et al.*, 2000), *Danio rerio* (Phillips and Reed, 2000; Sola and Gornung, 2001), *Xiphophorus maculatus* (Ocalewicz, 2004), *Oncorhynchus mykiss*, and *O. kisutch* (Iturra *et al.*, 2001). The repeated nature of both classes of ribosomal genes makes them easier to detected on the chromosomes by use of molecular cytogenetic techniques such as fluorescence *in situ* hybridization (FISH). According to the available data, the chromosomal patterns of ribosomal genes in fishes are much diversified. The rRNA gene clusters have been detected either in single or in multiple chromosomal locations (*e.g.*, Sola *et al.*, 2000; Almeida-Toledo *et al.*, 2002), and the two classes of genes themselves have been found in linked or separate configurations (reviewed in Martins and Galetti, 2001; Martins, 2007). Inter-specific and intra-specific variability in the number of chromosomal loci has been described for both the major and minor ribosomal genes (Zhuo *et al.*, 1995; Almeida-Toledo *et al.*, 2002; Boron *et al.*, 2006 among others). In some species the integration of cytogenetic mapping and sequence analyses provided important insights into the structure and organization of both 45S rDNA (*e.g.* Reed and Phillips, 2000) and 5S rDNA

Table 1 - Available cytogenetic information on species within the family Osmeridae.

Species	2n	FN	Formula	45S rDNA*	5S rDNA*
Mallotus villosus[1]	54	80	26m/sm+28a	6	7
Hypomesus olidus[2]	56	78	22m/sm+34st/a		
Hypomesus pretiosus[3, 4]	50	60			
	52				
Hypomesus transpacificus[5]	56	82	26sm+30a		
Osmerus eperlanus[6, 7, 8, 9]	56	68	12m/sm+44st/a		
	54	70	16m+38a		
	58	68	10m+48a		
	56	70	10m+18sm+14a		
Sprinchus starksi[10, 3]	50				
	50	60			

*Number of chromosome pairs bearing rDNA signals.
[1]Present study; [2]Vasiliev, 1985; [3]Ohno, 1974; [4]Ohno *et al.*, 1969; [5]Kitada *et al.*, 1980; [6]Lajus, 1992; [7]Nygren *et al.*, 1971; [8]Svärdson, 1945; [9]Ocalewicz *et al.*, 2007; [10]Ohno, 1970.

(Martins and Galetti, 2001; Wasko *et al.*, 2001), and has revealed the occurrence of sequence variants at different chromosomal loci. The cytogenetic and molecular data as a whole indicated that, despite overall evolutionary conservation, the organization of ribosomal genes in fishes is highly variable. This phenomenon has also been observed in a variety of organisms including other animals and plants (*e.g.* De Lucchini *et al.*, 1997; Reed and Phillips, 2000; Pedrosa-Harand *et al.*, 2006).

The present ribosomal gene mapping for the osmerid capelin indicated that both the major and the 5S ribosomal genes are organized in multiple chromosomal loci, thus confirming the heterogeneous chromosomal organization of such sequences in fish species. A number of mechanisms has been proposed to explain the spread of rDNA sequences at various chromosomal locations as well as the origin of new rDNA loci (*e.g.* Reed and Phillips 2000; Pedrosa-Harand *et al.*, 2006). Mechanisms mainly include structural chromosomal rearrangements such as translocations (*e.g.* Hayashi *et al.*, 2001). However, the dispersion of rDNA repeats, either through intra-chromosomal recombination and insertion of circular intermediate DNA into a new locus (*e.g.* Dubcovski and Dvorak 1995; Martins, 2007), or by transposon mediated rDNA transfer (Schubert 1984; Raskina *et al.*, 2004), has also been suggested. Changes in number and size of rDNA sites can also occur through ectopic recombination (interlocus unequal crossing over), especially when the rDNA sites are located in the chromosome terminal regions or close to other classes of repetitive DNA sequences. In capelin, rDNA signals indicate that most of the rDNA sequences are close to the centromeric regions: the contiguity of rDNA to the repetitive centromeric sequences may have enabled homologous recombination to take place between non-allelic sites, thus facilitating the distribution of ribosomal gene clusters to a large number of chromosomes.

To fully understand the pathways of ribosomal gene distribution on the chromosomes in capelin, comparable mapping data from other species of the family Osmeridae would be necessary. To our knowledge such data are presently lacking except for the species *Osmerus eperlanus*. Recent studies on this species indicated a single locus for the 45S rDNA (indirectly detected through silver staining) and the spread of 5S rDNA on four chromosome pairs (Ocalewicz *et al.*, 2007). Therefore, in the absence of more comprehensive data for phylogenetically related species, we can not assess the direction of the rDNAs spreading in the osmerid genome and explain the diversified rDNA chromosomal loci patterns. It would be interesting to examine, through complementary molecular analyses, if the multiple loci detected in capelin contain rDNA sequence variants.

A striking feature concerning the distribution of ribosomal genes in capelin is the detection of the larger cluster of major ribosomal genes in only one of the two homologs of pair 2, in all the specimens analyzed. It is well known that, owing to differences in the amount of cistrons, the homologous chromosomes bearing 45S rDNA genes can exhibit size polymorphisms (*e.g.* Vinas *et al.*, 1996; Mazzei *et al.*, 2004). In the light of the above evidence, our findings in capelin may be explained in terms of a strong heteromorphism of pair 2, which hampers detection of FISH signals in one of the two homologues when a very low amount of ribosomal gene sequences is present. Alternatively, the odd rDNA signal found in pair 2 could indicate a heterozygous condition of such genes, but this has to be investigated in depth through further analysis in a larger number of specimens.

The chromosomal linkage of the major and minor ribosomal genes classes, detected in most of the rDNAs locations in the capelin, is not in agreement with most observations in vertebrates, including fishes, where divergent locations of nucleolar and 5S rDNA have been reported (*e.g.* Martins and Galetti, 2001). Although the linkage between the two rRNA gene families is not functionally required, the growing literature on the topic suggests that such an association (at least at the chromosomal level) is more frequent than previously assumed: *e.g.* Salmoniformes (Fujiwara *et al.*, 1998; Rossi and Gornung, 2005), Cypriniformes (Inafuku *et al.*, 2000; Boron *et al.*, 2006), Characiformes (Almeida-Toledo *et al.*, 2002; Hatanaka and Galetti, 2004; Mantovani *et al.*, 2005), and Perciformes (Mazzei *at al*., 2004). There is not enough information to answer whether the 5S and major rDNA clusters are more efficient in linked or separated configurations, but we may speculate that possible functional interferences between too closely arranged 45S and 5S rDNA sequences (Martins and Galetti, 1999) could be compensated by increased amounts of clusters and/or by the spreading of sequences at multiple chromosome positions, as already suggested by Mazzei *et al.* (2004).

Conclusions

The present study provides the first basic chromosomal features of the ecologically and commercially important capelin. The karyologically derived chromosome set suggests a complex history of chromosome evolutionary changes in the family Osmeridae that would require comparable studies in related species to be understood. The interesting multiple location ribosomal pattern stresses the need to improve our knowledge of the organization on rDNA in the highly diversified teleostean lineages, including a better understanding of the mechanisms leading to the spread of rDNA sequences and the frequency of possible rDNA sequence variants. Further studies on the capelin and phylogenetically related species may give new insights to the evolution of rDNA in fishes, especially if cytogenetic studies are integrated with molecular sequence analyses. The combination of cytogenomic methods appears to be a particularly promising approach in fish cytogenetics and

comparative genomics (Jaillon *et al.*, 2004; Volff, 2005; Pisano *et al.*, 2007b).

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