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Distribution and Heterogeneity of Heterochromatin in the European Huchen (*Hucho hucho* Linnaeus, 1758) (Salmonidae)*

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The chromosomal characteristics, locations and variations of the heterochromatin were studied in the European huchen (*Hucho hucho*, Linnaeus, 1758) karyotype using conventional C- banding, endonuclease digestion banding, silver nitrate ($AgNO_3$), chromomycin A_3 (CMA_3) and DAPI staining techniques. The karyotype consists of 82 chromosomes: 13 pairs of metacentric chromosomes, 2 pairs of submetacentric chromosomes and 26 pairs of subtelo-acrocentric chromosomes (NF=112). Original data on the chromosomal distribution of segments resistant to *Alu* I, *Dde* I and *Mbo* I restriction endonucleases and identification of the C-banded heterochromatin presented here have been used to characterize the huchen karyotype. On the basis of the banding patterns provided in the course of restriction enzyme digestion, $AgNO_3$/CMA_3 staining and C-banding we distinguished twelve types of heterochromatin grouped in four areas of the European huchen chromosomes. One pair of NOR-bearing chromosomes was found to be polymorphic in size and displayed two distinct forms. A combination of various staining techniques provided original data concerning heterochromatin distribution and heterogeneity in the European huchen which enabled better karyotypic definition of this fish species.

Key words: Banding techniques, fish cytogenetics, NORs, Salmonidae.

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Salmonid fishes are considered to be of autotetraploid origin (OHNO 1970; ALLENDORF & THORGAARD 1984; HARTLEY 1987). The tetraploid ancestor of salmonids possessed a karyotype with 96 one-armed chromosomes (NF= 96) (OHNO 1970; PHILLIPS & RAB 2001). Salmonid fish karyotypes have gone through intensive evolutionary changes including chromosomal rearrangements leading to the rediploidization of the genome that has not yet been completed (MAY 1980; ALLENDORF & THORGAARD 1984).

Although, salmonids are one of the best cytogenetically analyzed fish groups, karyotypic data for some species including huchonids (*Hucho* sp.) are still somewhat limited (ARAI 2011). The huchonid fish are represented by five species: *Hucho bleekeri*, *H. hucho*, *H. taimen*, *H. ishikawae* and *Parahucho perryi*, which are distributed in Europe and Asia (FISHBASE 2013). Only three of them have been studied cytogenetically to date (ARAI 2011). The diploid chromosome number of the European huchen (*H. hucho)* specimens from Bosnia and Herzegovina, Slovakia, Yugoslavia and Poland has been stated as 2n=82 (SOFRADZIJA 1979; RAB & LIEHMAN 1982; OCALEWICZ *et al*. 2008). Moreover, RAB & LIEHMAN (1982) reported karyotype polymorphism between Slovakian (26m+4sm+12st+40a) NF=112 and Yugoslavian populations (26m+6sm+12st+38a) NF=114 of the European huchen. Karyotypes of taimen (*H. taimen)* show a variable chromosome number (2n= 82-84, NF=112-116) (DOROFEEVA 1977; VIKTOROVSKIJ *et al.* 1985; FROLOV &

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FROLOVA 2000). Karyotypes of the European huchen and taimen might have evolved by both pericentric inversions and Robertsonian fusions resulting in higher chromosome arm numbers and retained chromosome number close to the karyotype of the hypothetical tetraploid ancestor (DOROFEEVA 1977; RAB & LIEHMAN 1982; VIKTOROVSKIJ *et al.* 1985; FROLOV *et al*. 1999; FROLOV & FROLOVA 2000; OCALEWICZ *et al.* 2008). Karyotypes of these two species show four pairs of characteristic small metacentric chromosomes, called huchonine markers, which probably have been formed in the course of pericentric inversions or other centric shifts. A considerably reduced chromosome number (2n= 62, NF=100) is observed in the exclusively diadromous Japanese huchen (*Parahucho perryi*) which represents a separate monotypic genus more closely related to the *Salmo*+ *Oncorhynchus*+ *Salvelinus* clade (RAB *et al.* 1994; FUJIWARA *et al*. 1998; CRESPI & FULTON 2004). The karyotype of this fish species might have experienced several centric fusions that reduced the chromosome number and retained a chromosome arm number close to that found in the hypothetical salmonid tetraploid ancestor (ANBINDER *et al.* 1982; VASILEV 1983; FUJIWARA *et al*. 1998). Contrary to the European huchen and taimen, the Japanese huchen exhibits only two pairs of small metacenric chromosomes.

The European huchen chromosomes have been studied to date by traditional and molecular cytogenetic techniques and have led to the identification of the nucleolus organizer regions (NORs) and localization of the minor and major rRNA genes as well as telomeric DNA sequences (RAB *et al*. 1994; FUJIWARA *et al*. 1998; OCALEWICZ *et al.* 2008). Surprisingly, no data concerning chromosomal location and arrangement of the heterochromatin is available for this species even though various methods capable of characterizing such chromatin have been adjusted to study fish chromosomes (OCALEWICZ *et al.* 2003). As information related to the distribution and structure of the heterochromatin is important to understand the genomic organization of the studied species, the main purpose of this paper was to provide detailed characteristics of the heterochromatin in the European huchen using conventional cytogenetic techniques and restriction enzyme digestion.

Material and Methods

Fish

Fourty-six one-year-old specimens of the European huchen, comprising 20 females and 26 males, were studied cytogenetically. Fish were obtained from the fish hatchery Lopuszna (Polish Anglers Association – PZW) (southern Poland), which is the only available source for the European huchen stocking material in Poland.

Chromosome preparation

Metaphase plates were prepared from pooled cephalic kidney cells by the conventional air-drying technique, as described by JANKUN *et al.* (1998). Briefly, fishes were injected with 0.1% colchicine solution (1 ml/100 g body weight). After 60 min they were killed by an overdose of the anesthetic 2-Phenoxyethanol (Sigma). The kidney was removed, dissected in 0.075 M KCl and the cell suspension free of tissue fragments was hypotonized for 60 min in 0.075 M KCl, fixed in 3:1 methanol: acetic acid fixative, washed twice in fixative, and finally spread onto slides.

Banding techniques

The constitutive heterochromatin blocks were identified using the C-banding technique as described by SUMNER (1972) with some modifications. In short, prepared slides were hydrolyzed in 0.1 N hydrochloric acid for 3 min, dipped into saturated $Ba(OH)_2$ at 50°C for 8 to 10 min and incubated in 2xSSC at 65°C for 2 h. After washing in distilled water they were mounted with antifade with DAPI (Vectashield). For identification of AT-rich chromatin regions, chromosomes were stained with 4',6-diamidino-2-phenylindole DAPI. Three drops of antifade solution Vectashield (Vector, Burlingame, USA) containing DAPI (1.5 μg /ml) were dropped onto a slide and covered with a coverslip (OCALEWICZ *et al.* 2003). Restriction endonuclease (RE) banding was performed by digestion of fresh chromosome spreads with *Alu*I (AG↓CT), *Dde* I (C↓TNAG) and *Mbo* I (↓GATC), employing a conventional technique described by JANKUN *et al.* (2004). Briefly, restriction enzymes suspended in the appropriate buffers were put on the chromosome slides and covered with 24 x 32 mm coverslip. The concentration of each enzyme (*Alu* I, *Dde* I, *Mbo* I) varied from 0.3 to 0.5 U/μl, depending on its activity. Then, slides were incubated in a moist chamber at 37°C, washed with distilled water, and stained with 5% Giemsa for 15 min. The optimal incubation times for *Alu* I, *Dde* I, and *Mbo* I endonucleases were 2 h, 3.5 h and 6.5 h, respectively. Silver nitrate staining of the nucleolus organizer regions was performed by the technique of HOWELL & BLACK (1980). The chromomycin (CMA_3) method fluorescence banding following the procedure of SOLA *et al.* (1992), was applied, with simultaneous counterstaining by DAPI (Vectashield).

Microscopy processing

Slides were analyzed using the Zeiss Axio Imager.A1 microscope equipped with epifluorescence and a digital camera. Chromosomes were scored under bright field and fluorescent light using appropriate filters for DAPI and CMA_3. The metaphase plates were karyotyped by size and position of the centromere in accordance with the system of LEVAN *et al.* (1964) using Band-View software (Applied Spectral Imaging). Metacentrics and submetacentrics were classified as bi-armed, whereas subtelocentrics and acrocentrics as mono-armed chromosomes. The fundamental arm number described in literature was recalculated in the same manner.

At least 10 metaphases per individual were studied. Hardy-Weinberg equilibrium was checked by chi-square test based on the frequencies of NOR-bearing chromosome isoforms.

Results

The diploid chromosome number of the European huchen under study was determined as 2n= 82. The karyotype complement of the examined fish comprised 26 metacentric, 4 submetacentric, 12 subtelocentric and 40 acrocentric chromosomes (NF= 112). The presence of 4 pairs of small-sized metacentric chromosomes called hucho markers were also observed (chromosome pairs nos. 10, 11, 12 and 13) (Fig. 1). Differences between karyotypes of males and females were not found.

C-banding revealed the distribution of the constitutive heterochromatin which was located in the telomeric and centromeric regions in most of the chromosomes. Additionally, entire q arms of small metacentric chromosome pairs (no. 10-13) were C-positive. Centromeric regions of two metacentric chromosomes (nos. 7 and 13) and two acrocentric chromosomes (nos. 27 and 37) did not show

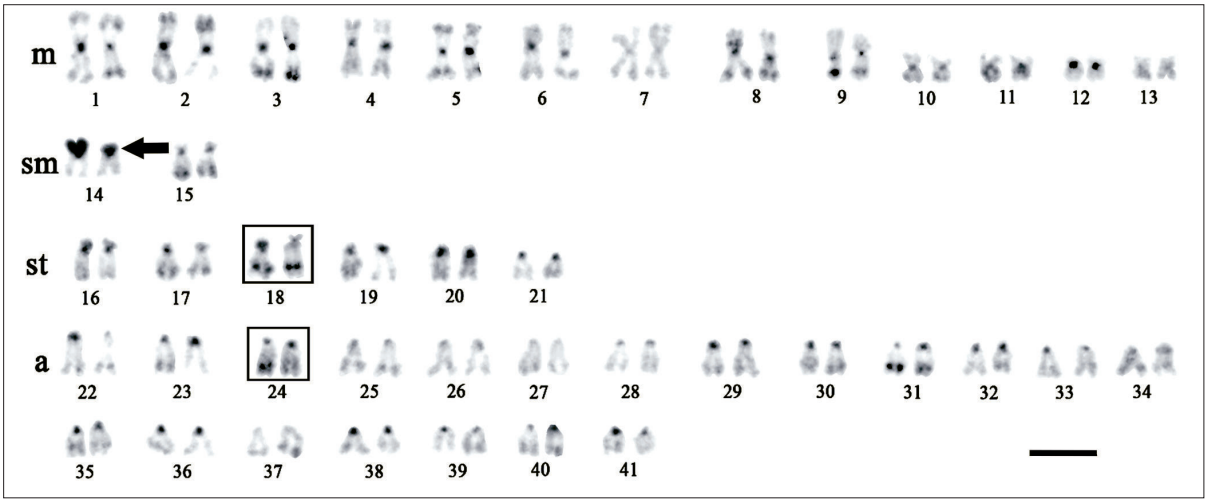

Fig. 1. C-banded karyotype of the European huchen (*Hucho hucho*). Chromosome pairs no. 18 and 24 with interstitial positive bands on q arms are framed. An arrow indicates NOR-bearing chromosomes. Scale bar = 10μm.

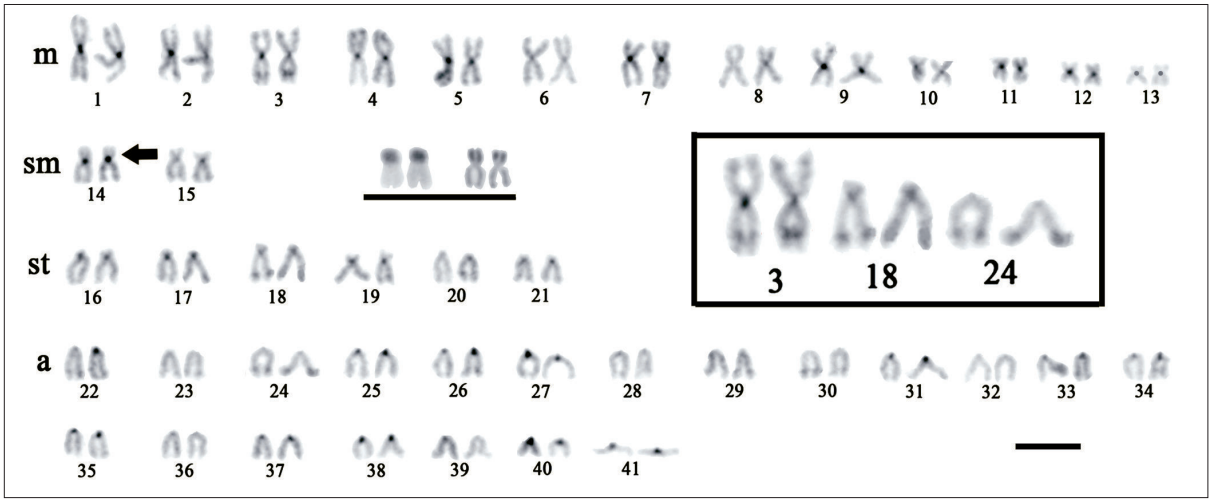

Fig. 2. Karyotype of the European huchen (*Hucho hucho*) stained with DAPI (13m+2sm+6st+20a). An arrow indicates NOR-bearing chromosomes. The same chromosome pair was sequentially stained by CMA_3-left and Ag-right (underlined). Chromosome pairs with distal DAPI positive bands on the short arms were zoomed (frame). Scale bar = 10μm.

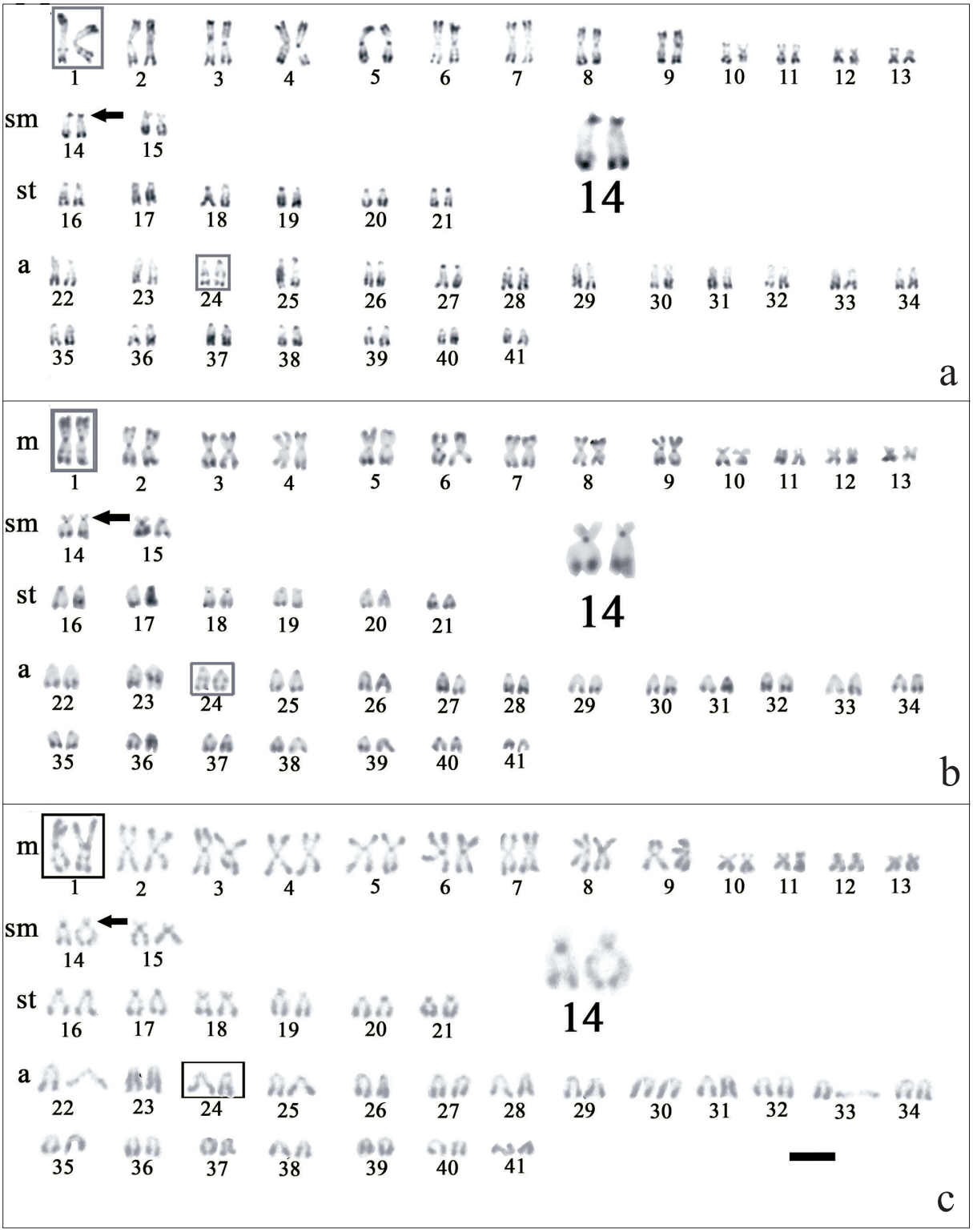

Fig. 3. Karyotype of the European huchen (*Hucho hucho*) chromosomes digested with *Mbo* I (A), *Alu* I (B) and *Dde* I (C) restriction enzymes. Chromosome pairs no. 1 and 24 with interstitial positive bands on q arms are framed. Arrows indicate NOR-bearing chromosomes which were presented in enlargement on the right. Scale bar = 10μm.

any C-bands (Fig. 1). Interstitial bands at the distal part of the long (q) arms were observed on two chromosome pairs (nos. 18 and 24) (Fig. 1). The DAPI counterstaining approach showed that all of the European huchen chromosomes had centromeric bright DAPI signals. Moreover, distal DAPI positive bands were observed on the long arms of chromosomes nos. 3, 18 and 24 (Fig. 2).

The banding patterns obtained for each restriction enzyme applied were very similar. Chromatin resistant to the restriction endonucleases was confined to the telomeric and pericentromeric regions of most of the European huchen chromosomes, entire q arms of chromosomes no. 10-13 and interstitial regions observed on the q arms of chromosome no.1 and 24 (Fig. 3). Pericentromeric regions from five metacentric (no. 7, 10-13) and eight acrocentric chromosomes (no. 22, 33, 29-33 and 41) were completely digested after *Mbo* I, *Alu* I and *Dde* I treatment (Fig. 3). In turn, pericentromeric areas of chromosome 1 and five acrocentric chromosomes (no. 35, 36 and 38-40) were fully digested after *Mbo* I and *Dde* I treatment but remained untouched after *Alu*I action (Fig. 3a, c).

Nucleolus organizer regions (NORs) were located on the short (p) arm of the submetacentric chromosome pair (no. 14). CMA_3 staining displayed GC rich heterochromatic blocks exactly overlapping with the Ag-NOR (Fig. 4). In all metaphases studied two signals of CMA_3 and two $AgNO_3$ deposits were found. The entire p arm of NOR-bearing chromosomes was strongly stained by C-banding (Fig. 1). Moreover, a NOR-site was visible as a pale area after *Alu* I digestion, whereas it was completely digested after *Dde* I treatment. Furthermore, *Mbo* I did not find any specific recognition sites in the NOR location (Fig. 3). The studied group of fish showed interindividual size variation of the short arm of the NOR-bearing chromosome pair. Two forms of this chromosome were found: L – chromosome with the p arm twice shorter than the q arm, and S – chromosome with p arm three times shorter than the q arm (Fig. 4). Only four LL homozygotes were found among the studied fish, 23 heterozygotes LS and 19 homozygotes SS. The frequencies of the NOR-bearing chromosome types in the studied fish group were as follows: $f(L) = 0.337$ and $f(S) = 0.663$. The Chi-square test showed that differences between observed and expected chromosome type frequencies were not statistically significant ($P<0.05$, $df=1$) so the studied stock of the European huchen was in Hardy-Weinberg equilibrium.

Discussion

Heterochromatin is a complex composition of various types of repetitive sequences, currently recognized as an important part of the eukaryotic genome, the functions of which include chromosome segregation, nucleus organization and the regulation of gene expression, among others (GREWAL & JIA 2007; VARRIALE 2008; BUHLER 2009). In comparison with other salmonid fish species, the chromosome pattern obtained after C-banding revealed a relatively large amount of heterochromatin in the European huchen genome. Cytogenetic analysis of closely related vertebrate species including fishes showed that heterochromatin may be lost during evolution (KORNFIELD *et al.* 1979). Thus, a higher amount and diversity of constitutive heterochromatin are considered ancestral (ELDER & TURNER 1995). This might also be true for the European huchen, the karyotype of which is rather archaic when compared to other species from the subfamily Salmoninae (PHILLIPS & RAB 2001).

Application of restriction enzymes digestion, $AgNO_3/CMA_3$ staining and C-banding distinguished at least twelve types of heterochromatin grouped in four areas of the European huchen genome including telomeric sites, pericentromeric regions, interstitial locations and NORs (Fig. 5). Constitutive heterochromatin confined to the telomeric sites of the European huchen chromosomes was rather homogenous and usually remained untouched after the restriction endonucleases applied here. Contrastingly, pericentromeric constitutive heterochromatin studied here exhibited huge heterogeneity, in agreement with previously published data concerning the diversity of such heterochromatin in vertebrates (MAISTRO *et al.* 2000; MANTOVANI *et al.* 2000; MARTINS *et al.* 2013; SILVA *et al*. 2013). In comparison to telomeres, centromeric DNA sequences do not have defined DNA sequences and are not conserved among different species (MEHTA *et al.* 2010). Centromeric heterochromatin is composed of a large amount of various repetitive DNA sequences interspersed with AT-rich DNA fractions and mobile elements (PIDOUX & ALLSHIRE 2005). Pericentromeric regions in the European huchen were usually DAPI positive however their resistance to particular endonucleases differed between chromosomes (Figs 2 & 5). Some types of heterochromatin were unique for the centromeric locations of single chromosomes while other fractions were common for groups of chromosomes. Such interchromosomal variation might be explained by accumulation of different repetitive DNA sequences in the heterochromatin from the pericentromeric regions of different chromosomes or groups of chromosomes.

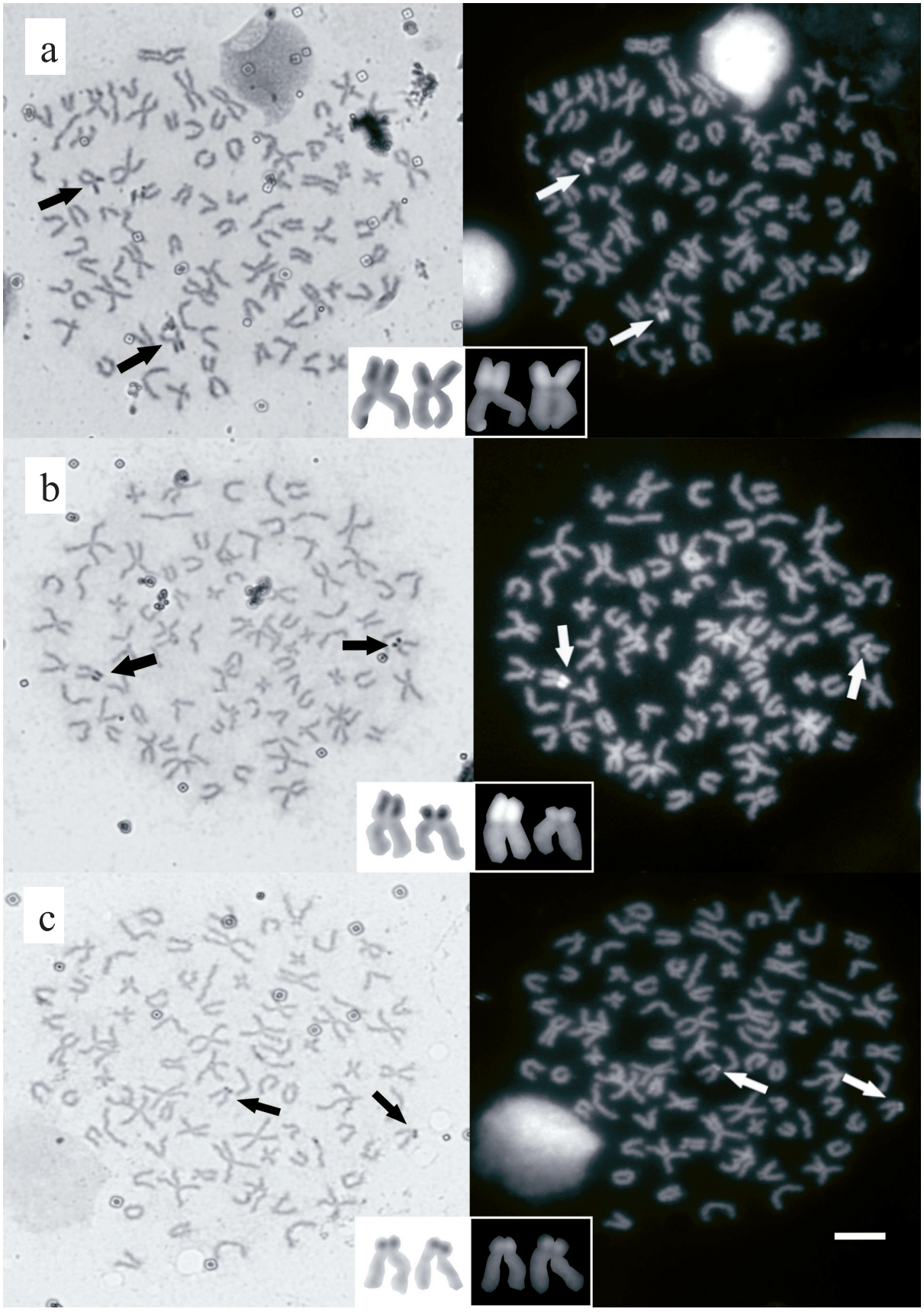

Fig. 4. Metaphase chromosomes of the European huchen (*Hucho hucho*) sequentially stained by $AgNO_3$ (left) and CMA_3 (right), displaying individuals with isoforms LL (A), LS (B) and SS (C). Arrows indicate NOR-bearing chromosomes that are enlarged in insets. Scale bar = 10μm.

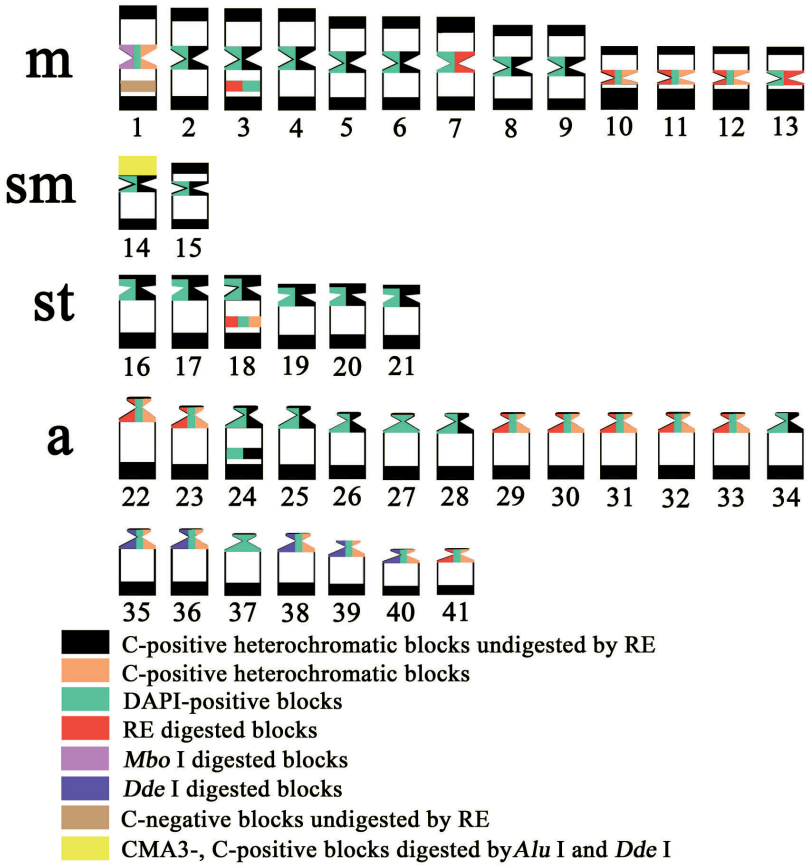

Fig. 5. Ideogram showing the distribution of heterochromatin in the European huchen (*Hucho hucho*) from Poland.

Interstitial heterochromatic bands detected on chromosomes 3 and 18 showed characteristics similar to some of the pericentromeric heterochromatin fractions. Such interstitial heterochromatin might have appeared in the course of (1) chromosome rearrangements that involved centromeres and accompanied karyoevolution of the European huchen and/or (2) dispersion of the centromeric heterochromatin driven by (retro)transposons (SCHWEIZER & LOIDL 1987). In turn, the interstitial band on chromosome 1 that is not heterochromatic but resistant to the endonucleases applied here is unique for this chromosome.

Association of NORs and C-positive heterochromatin are a rather common feature in vertebrates including fishes (FUJIWARA *et al* 1998; SCHNEIDER *et al*. 2013; GOUVEIA *et al.* 2013). In many fish species including the European huchen, rDNA sequences (18S, 5.8S and 28S) clustered in the NOR sites correspond to the GC-rich CMA_3 positive sites (e.g. RAB *et al.* 1999; JANKUN *et al.* 2003; among others). In the European huchen, NOR/CMA_3 carrying chromosomes were incorrectly classified as metacentric chromosome no. 10 (OCALEWICZ *et al.* 2008). After careful inspection we suggest that the chromosome with NORs should rather be classified as submetacentric chromosome no. 14. Current results also show that NORs in the European huchen are built with heterochromatin enriched in the restriction sites recognized by *Dde* I and *Alu* I. Similar observations have been reported in other fish species (SANCHEZ *et al.* 1991; SALVADORI *et al.* 1997; JANKUN *et al.* 1998; DI *et al.* 2006). Additionally, similar interindividual size heteromorphism of the NOR-bearing chromosome short arms observed in the European huchen was also recognized in the Siberian taimen (FROLOV & FROLOVA 2000). Variation in length of NORs is usually considered as a consequence of unequal crossing-over or amplification of the NOR related chromatin as both are listed as common mechanisms acting in the evolution of multiple tandem arrays (WOZNICKI & JANKUN 1994; CASTRO *et al.* 2001; JANKUN *et al.* 2001; MARTINEZ *et al.* 2009).

To conclude, our study has shown that a combination of cytogenetic procedures including C-banding, $AgNO_3$ and CMA_3 staining and chromosomal digestion with restriction endonucleases provided original data on heterochromatin struc-

ture and distribution in the European huchen chromosomes, enabling better karyotypic definition of this fish species.

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