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Measurement of Chromosomal Arms and FISH Reveal Complex Genome Architecture and Standardized Karyotype of Model Fish, Genus Carassius

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Abstract: The widely distributed ray-finned fish genus Carassius is very well known due to its unique biological characteristics such as polyploidy, clonality, and/or interspecies hybridization. These biological characteristics have enabled Carassius species to be successfully widespread over relatively short period of evolutionary time. Therefore, this fish model deserves to be the center of attention in the research field. Some studies have already described the Carassius karyotype, but results are inconsistent in the number of morphological categories for individual chromosomes. We investigated three focal species: Carassius auratus, C. carassius and C. gibelio with the aim to describe their standardized diploid karyotypes, and to study their evolutionary relationships using cytogenetic tools. We measured length \( q + p \) length of each chromosome and calculated centromeric index \( i \) value. We found: (i) The relationship between \( q + p \) length and \( i \) value showed higher similarity of C. auratus and C. carassius. (ii) The variability of \( i \) value within each chromosome expressed by means of the first quartile \( Q_1 \) up to the third quartile \( Q_3 \) showed higher similarity of C. carassius and C. gibelio. (iii) The fluorescent in situ hybridization (FISH) analysis revealed higher similarity of C. auratus and C. gibelio. (iv) Standardized karyotype formula described using median value \( Q_2 \) showed differentiation among all investigated species: C. auratus had 24 metacentric \( M \), 40 submetacentric \( sm \), 2 subtelocentric \( st \), 2 acrocentric \( a \) and 32 telocentric \( T \) chromosomes \( 24m + 40sm + 2st + 2a + 32T \); C. carassius: 16m + 34sm + 8st + 42T; and C. gibelio: 16m + 22sm + 10st + 2a + 50T. (v) We developed R scripts applicable for the description of standardized karyotype for any other species. The diverse results indicated unprecedented complex genomic and chromosomal architecture in the genus Carassius probably influenced by its unique biological characteristics which make the study of evolutionary relationships more difficult than it has been originally postulated.

Keywords: chromosome; karyogram; in situ hybridization; \( i \) value; \( q/p \) arm ratio; Carassius auratus; Carassius carassius; Carassius gibelio

1. Introduction

Karyotype analysis is a fundamental approach by which chromosomes are arranged into homologous pairs with a respect to certain morphological categories. In the broader sense, the term karyotype is also referred to as a complete number of chromosomes describing typical taxon, species, biotype or individual [1]. Homologous chromosome pairs and morphological categories are determined based on the ratio of the long \( q \) and short \( p \) arm and the position of the centromere (centromeric index, \( i \) value). If the centromere is situated in median, submedian, subterminal or terminal region of the chromosome, the morphological category might be designated as metacentric \( M, m \), submetacentric \( sm \), subtelocentric \( st \) or acrocentric \( a \)/telocentric \( t, T \), respectively [2]. Two edge points that are located on median and terminal position sensu stricto have centromeric index 50 (assigned as \( M \)) and 0 (assigned as \( T \) chromosomes), respectively [2]. In order to maximize the diagnostic information obtainable from a chromosome preparation, the
precise arrangement of individual chromosomes within a single image might be used as a standardized format. The term karyogram can be also used for a graphical depiction of chromosome complements \cite{3,4}.

Knowledge of the karyotype is necessary in (cyto)genetics, or related fields, in order to study chromosomal rearrangements and abnormalities \cite{5,6}, the identification of sex chromosomes \cite{7,8}, and/or chromosome-specific genes \cite{9}. Although chromosomal changes, (i.e., chromosomal losses, duplications, rearrangements), are strictly linked to a certain locus, without the knowledge of detailed karyotype, it is not possible to precisely identify chromosomal aberration, mutation or syndrome \cite{10,11}. Heteromorphic sex chromosomes (morphologically distinct in male or female individuals) can be distinguished by classic cytogenetic procedures based on Giemsa-staining karyogram of both sexes and/or by chromosome painting and fluorescent in situ hybridization (FISH) \cite{12,13}. Cytogenetic mapping and localization of single-copy gene regions in the genome generally reveal both intra- and interchromosomal rearrangements (i.e., inversions, insertions, deletions or duplications) determined using karyograms \cite{9}. Moreover, gene loci, which have not yet been mapped within a genome, can be assigned to a specific chromosome within a standardized karyogram \cite{14}.

In some model vertebrate species, a low/moderate number of chromosomes, meaning up to approximately 50 chromosomes, of relatively large-size allows for easy identification of each chromosome according to $q/p$ arm ratio and centromeric index \cite{15}. Each chromosome is usually assigned a numeric code, necessary in evolutionary studies pertaining to patterns of orthologous chromosomes between species. Orthologous chromosomes are inferred to be descended from the same ancestral chromosome separated by a speciation event. This concept is also referred to as shared synteny. In a non-model organism with a high number of relatively small chromosomes, identification of chromosomes into categories are inconsistent (e.g., Knytl et al. \cite{16} vs. Kobayasi et al. \cite{17} vs. Ojima and Takai \cite{18}), as is that case with cyprinid fish from the genus \textit{Carassius}. Although a large number of studies describing \textit{Carassius} karyotype, there is no study proposing a standardized karyogram based on exact measurements of the difference between $q$ and $p$ chromosome arm, arm ratio and centromeric index in any \textit{Carassius} species.

The genus \textit{Carassius} belongs to the monophyletic paleotetraploid tribe, Cyprinini sensu stricto \cite{19}, within the family Cyprinidae (ray-finned fishes, Teleostei). Several species have been described within the genus \textit{Carassius} and three of them are widely used in cytogenetic research: (i) \textit{Carassius carassius} (Crucian carp), native and threaten in many European countries \cite{20,21}, is diploid with chromosome number $2n = 4x = 100$ \cite{17}, where $n$ refers to the number of chromosomes in each gamete of extant species, and $x$ refers to the number of chromosomes in a gamete of the most recent diploid ancestor of the extant species. Most members of the family Cyprinidae contain 25 chromosomes in each gamete, considered as the most recent diploid ancestral state of extant \textit{Carassius} \cite{22}. (ii) \textit{Carassius auratus} (goldfish), well known for its colourful varieties, bizarre shapes of body and formation of domesticated and feral populations \cite{23}. (iii) \textit{Carassius gibelio} (Silver Prussian carp) has recently spread throughout most continental waters, as a result of relocation from native habitats most likely by humans \cite{24–27}. In addition, \textit{Carassius auratus} and \textit{C. gibelio} form diploid ($2n = 4x = 100$), triploid ($3n = 6x \approx 150$), and tetraploid biotypes ($4n = 8x \approx 200$) \cite{28–30}.

Here we described standardized karyotype of diploid \textit{Carassius} species with 100 chromosomes (\textit{C. auratus}, \textit{C. carassius} and \textit{C. gibelio}) based on measuring of chromosomal $q + p$ arm length, $q/p$ arm ratio, and $i$ value. We performed FISH experiments with ribosomal probes, and analysed values of $q + p$ length and $i$ in order to characterize the inter-species differences between three \textit{Carassius} species, which commonly co-occur in European waters (\textit{C. carassius} and \textit{C. gibelio}), and bred/distributed worldwide as part of the pet trade (\textit{C. auratus}).
2. Materials and Methods

2.1. Fish Sampling and Origin

*Carassius auratus* were obtained via the aquarium trade (transported to Czech Republic from Israel). *Carassius carassius* was captured in the Elbe River basin close to the city Lysá nad Labem, Czech Republic [16,31] and in small pond in Helsinki, Finland [32]. *Carassius gibelio* originated from the Dyje Rive basin, South Moravia, Czech Republic [28].

2.2. Chromosome Analysis

Both males and females of each species (*C. auratus*, *C. carassius*, *C. gibelio*) were used for karyotype analysis. In *C. auratus*, mitotic activity was stimulated by intraperitoneal injection of 0.1% CoCl$_2$ [32]. Somatic chromosomal complements were synchronized in metaphase of cell mitotic division using 0.1% colchicine (Sigma, St. Louis, MO, USA). Chromosome suspension was generated from the cephalic kidney [33] and stored in fixative solution (methanol: acetic acid, 3:1) at −20 °C, before being observed on a glass slide and stained with 5% Giemsa solution in 1x PBS. The chromosome suspensions used in this study have been used in prior analyses of *C. carassius* and *C. gibelio* [16,28,31,32] meaning that chromosomal suspensions were stored 3–7 years in −20 °C. Chromosomal suspensions were spun in a centrifuge, and fresh methanol and acetic acid were added every three months to maintain the constant ratio of methanol and acetic acid in fixative solution.

2.3. Measurements of $q + p$ Chromosomal Arm Length

Photos of mitotic metaphase were taken using a Leica DFC 7000T camera and Leica DM6 microscope equipped with a EL6000 (metal halide) fluorescence illumination. A total of ten high-quality photos were taken for each species, five male and five female metaphases, from which measurements of the length of $q$ and $p$ chromosomal arms were taken. The centromere of each chromosome was identified as the narrowest part of a chromosome (Figure S2). Both arms of each chromatid (i.e., long arm 1 ($q_1$), long arm 2 ($q_2$), short arm 1 ($p_1$), short arm 2 ($p_2$)) were measured using *ImageJ* (V 1.53i) [34]. Chromosomal length ($\text{length}$), difference between $q$ and $p$ arm ($d$), $q/p$ arm ratio ($r$) and centromeric index ($i$) were calculated according following formulas adopted from Levan et al. [2]:

$$
\begin{align*}
p &= \frac{p_1 + p_2}{2} \\
q &= \frac{q_1 + q_2}{2} \\
\text{length} &= p + q \\
d &= q - p \\
r &= \frac{q}{p} \\
i &= \frac{100}{r + 1}
\end{align*}
$$

Measurements of $p$, $q$, $\text{length}$, $d$, $r$, and $i$ were calculated as values in pixels from each image/metaphase and were further analyzed using R software for statistical computing (V 4.1.0) [35] and *RStudio* environment (V 1.4.1717) [36]. The $i$ value was selected as a crucial characteristics for karyotypic analysis because it generally ranges from 0 to 50 [2]. The $r$ value generally ranges from 1 to $\infty$ and thus this value is not suitable for graphical expression of results. The $\text{length}$ and $i$ value were used for calculation of an arithmetic mean of $\text{length}$ ($\text{mean}_\text{length}$) and an arithmetic mean of $i$ ($\text{mean}_i$) separately for each *Carassius* species. The $\text{mean}_\text{length}$ and $\text{mean}_i$ of each species were plotted (plot function in R).

2.4. Standardized Carassius Karyotype

Two highest $i$ values (one chromosomal pair) were dissected from each of the metaphases (ten metaphases in total) and put into additional data frame as a numeric vector. This group of $i$ values were identified as chromosome 1 (hereafter chr1). The third and fourth highest $i$ values were dissected from each metaphase and identified as chr2 and so on up to two chromosomes with the lowest $i$ values identified as chr50. This function(){ is named Select_chrome and shown in the supplemental results. All $i$ values of each identified chromosome were plotted (boxplot function in R) separately in
each species. The default interquartile range 1.5 was used for elimination of extreme values (errors). Output value ($\text{stats}$) was called in R. The $i$ values within the the minimum ($Q_0$), first quartile ($Q_1$), median ($Q_2$), third quartile ($Q_3$) and maximum ($Q_4$) were investigated in detail, especially if some $i$ values of each individual chromosome were shared among focal Carassius species. Chromosomal categories ($M, m, sm, st, a$ and $T$) were determined according $Q_2$ of $i$ values in each Carassius species. A morphological category of each chromosome was determined according chromosomal nomenclature Levan et al [2]. The following Table 1 shows boundaries between each morphological category:

| Centromeric Position | Arm Ratio | Centromeric Index | Chromosome Category |
|----------------------|-----------|-------------------|---------------------|
| median sensu stricto | 1.00      | 50                | M (metacentric sensu stricto) |
| median               | 1.01–1.70 | 49.9–37.51        | m (metacentric)      |
| submedian            | 1.71–3.00 | 37.50–25.01       | sm (submetacentric)  |
| subterminal          | 3.01–7.00 | 25.00–12.51       | st (subtelocentric)  |
| terminal             | >7.01     | 12.50–0.01        | a/t (acro-/telocentric) |
| terminal sensu stricto | $\infty$ | 0                  | T (telocentric sensu stricto) |

2.5. Preparation of 5S and 28S Ribosomal Probes

New 5S PCR primers 5S_F (5′-GAGGCTTTATGGCCGTAGG-3′) and 5S_R (5′-AGCCGGCATCTGCTGTGAT-3′) were designed according to the 5S gene of the western clawed frog, Xenopus tropicalis. The X. tropicalis genomic sequence is available on Xenbase, accessed on 11 June 2020 (http://www.xenbase.org). The 28S primers 28S_A (5′-AAACTCTGTGAGGCTCCGT-3′) and 28S_B (5′-CTTACAAAAGTGGCCCACTA-3′) used in this study were designed by Naito et al. [37]. Total genomic DNA (gDNA) was extracted from pectoral fin tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Carassius gibelio and X. tropicalis gDNA were used as a template for 5S and 28S PCR amplification, respectively. Primers were made by Integrated DNA Technologies (Coralville, IA, USA). The PPP Master Mix (Top-Bio, Prague, Czech Republic) was used for efficient amplification of ribosomal gene fragments. The temperature profile for the non-labelling amplification of the 5S and 28S loci followed Top-Bio instructions: initial denaturation step for 1 min at 94 °C, followed by 35 cycles (94 °C for 15 s, 53 °C for 15 s and 72 °C for 40 s) and a final extension step at 72 °C for 7 min. The obtained PCR products were separated on a 1.25% agarose gel with TA buffer and extracted from the gel using MicroElute Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) according to manufacturer’s instructions. The PCR amplicons were sequenced and mapped using blastn algorithm for finding out of the locus- and species-specificity of amplification. Subsequently, the 5S and 28S ribosomal DNA (rDNA) loci were indirectly labelled by Digoxigenin-11-dUTP (Roche, Mannheim, Germany) and Biotin-16-dUTP (Jena Bioscience, Jena, Germany), respectively, by PCR reaction again. Taq DNA polymerase (Top-Bio) was used for labelling instead of The PPP Master Mix which was used for non-labelling amplification. Conditions for labelling PCR of the 5S and 28S loci were adopted from Sember et al. [38] and slightly modified as follows: initial denaturation step for 3 min at 94 °C, followed by 30 cycles (94 °C for 30 s, 53 °C for 30 s and 72 °C for 40 s) with final extension step at 72 °C for 10 min. The PCR product was separated on an 1% agarose gel with TBE buffer and purified using E.Z.N.A. Cycle Pure Kit (Omega Bio-tek) according to manufacturer’s instructions.

2.6. Fluorescent In Situ Hybridization

Cell suspensions prepared from each Carassius species were spread onto clean microscopic slides, which were subsequently used for FISH on the same day. The 5S probe from C. gibelio was used for chromosomal spreads of C. auratus, C. carassius and C. gibelio.
The 28S probe from *X. tropicalis* was used for *C. auratus*, *C. carassius* and *C. gibelio* chromosomal spreads. In total 44 µl of the hybridization mixture containing 100 ng of either 5S or 28S rDNA probe, 50% deionized formamide, 2x SSC, 10% dextran sulphate and water was placed on a slide and covered with a 24 x 50 mm coverslip. Both probe and chromosomal DNA were denatured in a PCR machine with special block for slides at 70 °C for 5 min [9]. Hybridization, post-hybridization washing, blocking reaction and visualization of 5S and 28S rDNA signals were carried out as described in Knytl et al. [39]. The Digoxigenin-11-dUTP/Biotin-16-dUTP labelled probe was detected by Anti-Digoxigenin-Fluorescein (Roche)/ CY™3-Streptavidin (Invitrogen, Camarillo, CA, USA), respectively, diluted according to manufacturer’s instructions. Chromosomes were counterstained with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). At least 20 metaphase spreads in total were analysed per individual.

3. Results

3.1. Karyotype Analysis

Number of chromosomes in all studied species (*C. auratus*, *C. carassius* and *C. gibelio*) was uniform (2n = 4x = 100). We did not find any differences in chromosomal morphology between male and female individuals. This finding confirmed homomorphic sex chromosomes at least in diploid biotypes of the genus *Carassius*.

3.2. Interspecies Variability Based on Chromosomal Length

Detailed description of R analysis including R scripts are given in Supplementary Results. All steps outlining how the measured values were calculated and processed into tables and plots are shown on the *C. auratus* dataset. The mean_i and mean_length of each individual chromosome were plotted onto x axis and y axis, respectively, (Figure 1). Morphological chromosomal categories m, sm, st, a and T are present in each species. Extreme category, such as T, is represented by 19, 26, and 25 chromosomes of *C. auratus*, *C. carassius* and *C. gibelio*, respectively (i.e., 19, 26, and 25 points lie on gray dashed vertical lines of interval 0). The T chromosomes have small mean_length, approaching 0. Other chromosomal categories, such as M, are not represented in any species—no i value reached 50, with the highest i of 48.778 found in chromosome 1 of *C. gibelio*. Chromosomal length of *C. auratus*, *C. carassius*, and *C. gibelio* range from 23.340–40.614, 26.799–44.092, and 15.205–29.496, respectively. It is evident that chromosomes of *C. gibelio* are generally smaller (mean of q + p length was 21.36) than those of both *C. auratus* (31.53) and *C. carassius* (35.05). For the minimum of errors we calculated mean_length and mean_i from a group of ten measured q + p values, i.e., each dot on Figure 1 is arithmetic mean resulted from ten measured q + p values. Potential influences on chromosomal length are discussed (Section 4).

3.3. Interspecies Variability Based on Centromeric Index Linked to Individual Chromosomes of the Whole Chromosomal Complement, and Standardized Carassius Karyotype

The i values were assigned to each individual chromosome by dissection of chr1–50 from each metaphase (also Section 2.4, R protocol is described in the section of R analysis of Supplementary Results) and plotted onto x and y axis (Figure 2). Each box depicts a group of chromosomes dependent on i values ordered from most metacentric chromosomes on the left side of plots (chr1) to most telocentric chromosomes on the right side of plots (chr50). In general, each species has six to seven chromosomes that were highly variable in i, i.e., chr31–37 in *C. auratus*, chr27–33 in *C. carassius* and chr23–29 in *C. gibelio*. The Q1 and Q3 cover i values from 0 to 29.342 in *C. auratus*, from 0 to 23.9 in *C. carassius* and from 0 to 25.747 in *C. gibelio*. *Carassius auratus* has the highest variability of i value.
Figure 1. Relationship between centromeric index ($i$) and chromosomal length ($\text{length}$). Top plot shows chromosomes of *Carassius auratus* in yellow, middle plot shows chromosomes of *C. carassius* in red and bottom plot shows chromosomes of *C. gibelio* in blue. Chromosomal categories are bounded by gray dashed vertical lines which define intervals 0–12.5, 12.5–25, 25–37.5 and 37.5–50 corresponding to acrocentric ($a$), subtelocentric ($st$), submetacentric ($sm$) and metacentric ($m$) chromosomes, respectively. Both plotted $i$ value and $\text{length}$ are presented as an arithmetic mean of each chromosome.

Figure 2. Intrachromosomal variability of $i$ displayed on a whole chromosomal complement. Top plot shows haploid chromosomal complement (50 chromosomes) of *C. auratus* in yellow, middle plot shows 50 chromosomes of *C. carassius* in red and bottom plot shows 50 chromosomes of *C. gibelio* in blue. Each chromosome is linked to $i$ value (y axis). Upper and lower whiskers show extreme values, the minimum ($Q_0$) and maximum ($Q_4$), respectively, boxes involve the first quartile ($Q_1$) and the third quartile ($Q_3$) group of values. Black line within box indicates median value of the dataset ($Q_2$). Outliers (errors) are drawn as black points.
If some i values are shared among C. auratus, C. carassius and C. gibelio, the range of i values of each individual chromosome (chr1–50) from ten metaphases were compared with the range of i values of other two orthologs of each of other two species (ten metaphases from each species). The Q1–Q3 range of i value within chr1 of C. auratus was compared with the Q1–Q3 range of i value within chr1 of C. carassius and C. gibelio, similarly the Q1–Q3 range of i value within chr2 of C. auratus was compared with the Q1–Q3 range of i value of C. carassius and C. gibelio etc. up to the Q1–Q3 range of i value within chr50 of C. auratus, C. carassius and C. gibelio. As an additional analysis, multi-plot with 50 separate box plots (chr1–50) was generated, each box plot was composed of orthologous chromosomes of all three species (Figure 3).

Based on i value (Figure 1 and 3), we concluded that:

1. Each orthologous chromosome of C. carassius and C. gibelio shared i values within Q1–Q3 range and therefore we consider that karyotypes of C. carassius and C. gibelio to be more similar based on i value.
2. Chromosomes 12–30 of C. auratus were represented by different i values within Q1–Q3 range those of C. carassius and C. gibelio. Therefore, we consider the karyotypes of C. auratus to be most variable for this parameter.
3. Chromosomes 8, 11 and 31 of C. auratus were represented by different i values within Q1–Q3 range those of C. gibelio but C. carassius shared i values in these chromosomes with both C. auratus and C. gibelio.

The Q2 range (median) of i value was used for determination of a standardized karyotype formula. Chromosome1 of C. auratus, C. carassius and C. gibelio with median of i value 47.87 (CAU_median_i column of Table 2), 48.28 (CCA_median_i) and 48.55 (CG1_median_i), respectively, were identified as m chromosomes in all three Carassius species (CAU_category, CCA_category, CG1_category columns of Table 2). Karyotypes all three Carassius species were different in the number of chromosomal categories sensu Levan et al. [2]. The number of chromosomes in categories found out by arithmetic mean (Figure 1) slightly differed from the number of chromosomes in categories determined by Q2 range (Figure 2). The Q2 range eliminated errors and thus we inferred standardized karyotype formula according to the Q2 range.

\[
\text{Carassius auratus} : 24m + 40sm + 2st + 2a + 32T \\
\text{Carassius carassius} : 16m + 34sm + 8st + 42T \\
\text{Carassius gibelio} : 16m + 22sm + 10st + 2a + 50T
\]

3.4. Fluorescent In Situ Hybridization with 5S and 28S Ribosomal Probes

The PCR amplification of 28S and 5S rDNA locus resulted consistently in approximately 300 bp long fragments. Searches, using the blastn algorithm, confirmed the locus- and species-specificity of each amplicon: 100% identity with 28S rRNA of X. tropicalis (accession numbers XR_004223792–XR_004223798), 95% identity with sequence of 5S rRNA of C. gibelio (accession number DQ659261). The amplified 5S rDNA sequence was deposited to the NCBI/GenBank database (accession number BankIt2492026 5s, MZ927820). Mapping of the 5S and 28S loci showed different patterns in the number and position within each investigated Carassius species (Figure 4). No differences between males and females were detected. The q + p arms of the FISH images were measured on chromosomes that bear positive rDNA loci because the FISH protocol involves denaturation step after which chromosomal structure is disrupted due to high temperature. The rDNA positive loci are mostly accumulated at the pericentromeric chromosomal region in Carassius and another cyprinid fishes [32,40,41] and thus the identification of the centromere and measurement of the q + p arms are precise on rDNA positive chromosomes. The i value of rDNA positive chromosomes (arrows on Figure 4) were assigned to the closest i value of the standardized karyotype from Table 2.
Figure 3. Box plots composed of orthologous chromosomes of each species. Chromosomes are ordered decreasingly according to the $i$ value. Chromosomes on the top left of the figure are the most metacentric, chromosome 1 (chr1), chromosomes on the bottom right of the figure are the most telocentric (chr50). Yellow, red and blue boxes represents *C. auratus*, *C. carassius* and *C. gibelio*, respectively. Each chromosome is linked to $i$ value (y axis). Upper (Q3) and lower (Q1) whiskers show extreme values, boxes involve Q1 and Q3 group of values. The black line within boxes indicates median value of the dataset (Q2). Outliers (errors) are drawn as black points. The grid in some box plots represents significantly different $i$ values within the Q1–Q3 range. Box plots without the grid share some $i$ values within the Q1–Q3 range.
Table 2. Table of values used for determination of standardized karyotype. Each median ($Q_2$) of $i$ values corresponds to certain chromosomal category of chr1–50. The medians of $i$ value are sorted decreasingly. CAU = C. auratus, CCA = C. carassius, CGI = C. gibelio, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, T = telocentric sensu stricto.

| chromosome  | CAU_median_i | CCA_median_i | CGI_median_i | CAU_category | CCA_category | CGI_category |
|-------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 1           | 47.87        | 48.28        | 48.55        | m            | m            | m            |
| 2           | 47.07        | 45.68        | 46.12        | m            | m            | m            |
| 3           | 45.74        | 44.74        | 44.99        | m            | m            | m            |
| 4           | 44.81        | 43.52        | 43.47        | m            | m            | m            |
| 5           | 43.46        | 42.11        | 42.53        | m            | m            | m            |
| 6           | 42.43        | 41.08        | 40.89        | m            | m            | m            |
| 7           | 41.63        | 39.58        | 39.89        | m            | m            | m            |
| 8           | 40.84        | 37.90        | 38.42        | m            | m            | m            |
| 9           | 40.13        | 36.81        | 36.91        | m            | sm           | sm           |
| 10          | 39.67        | 36.17        | 35.84        | m            | sm           | sm           |
| 11          | 38.75        | 35.79        | 35.41        | m            | sm           | sm           |
| 12          | 37.65        | 35.19        | 34.73        | m            | sm           | sm           |
| 13          | 36.77        | 34.86        | 34.25        | sm           | sm           | sm           |
| 14          | 36.19        | 34.36        | 33.36        | sm           | sm           | sm           |
| 15          | 35.88        | 33.66        | 32.61        | sm           | sm           | sm           |
| 16          | 35.71        | 32.70        | 31.90        | sm           | sm           | sm           |
| 17          | 35.48        | 32.22        | 28.62        | sm           | sm           | sm           |
| 18          | 35.21        | 31.23        | 26.86        | sm           | sm           | sm           |
| 19          | 34.87        | 30.54        | 26.07        | sm           | sm           | sm           |
| 20          | 34.33        | 29.11        | 24.42        | sm           | sm           | sm           |
| 21          | 33.45        | 28.54        | 23.45        | sm           | sm           | sm           |
| 22          | 33.03        | 27.41        | 22.83        | sm           | sm           | sm           |
| 23          | 32.78        | 26.86        | 21.37        | sm           | sm           | sm           |
| 24          | 31.95        | 26.25        | 16.52        | sm           | sm           | sm           |
| 25          | 31.41        | 25.22        | 9.19         | sm           | sm           | a            |
| 26          | 31.08        | 24.00        | 0.00         | sm           | st           | T            |
| 27          | 30.70        | 22.57        | 0.00         | sm           | st           | T            |
| 28          | 30.11        | 20.62        | 0.00         | sm           | st           | T            |
| 29          | 29.75        | 18.13        | 0.00         | sm           | st           | T            |
| 30          | 28.96        | 0.00         | 0.00         | sm           | T            | T            |
| 31          | 28.17        | 0.00         | 0.00         | sm           | T            | T            |
| 32          | 26.87        | 0.00         | 0.00         | sm           | T            | T            |
| 33          | 24.41        | 0.00         | 0.00         | st           | T            | T            |
| 34          | 11.19        | 0.00         | 0.00         | a            | T            | T            |
| 35          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 36          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 37          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 38          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 39          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 40          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 41          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 42          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 43          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 44          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 45          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 46          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 47          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 48          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 49          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
| 50          | 0.00         | 0.00         | 0.00         | T            | T            | T            |
Figure 4. Double-colour fluorescent in situ hybridization with 5S and 28S ribosomal probes. DAPI-counterstained metaphase spreads show 100 chromosomes (B&W) in (a) *C. auratus* (CAU), (b) *C. carassius* (CCA) and (c) *C. gibelio* (CGI). The 5S (green) probe shows two more intensive (strong) signals and eight, six and eight less intensive (weak) signals in (d) *C. auratus*, (e) *C. carassius* and (f) *C. gibelio*, respectively. The 28S rDNA probe (red) reveals two strong signals and two, four and two weak signals in (d) *C. auratus*, (e) *C. carassius* and (f) *C. gibelio*, respectively. Green and red arrows correspond to 5S and 28S ribosomal loci, respectively.

Summary of 5S rDNA (corresponding to non-nucleolar region). We found:

- Two more intensive (strong) 5S rDNA signals at the *p* arm of *sm* chromosomes (chr28, *i* value = 30.06) and eight weak signals in *C. auratus*.
- Two strong 5S rDNA signals at the *p* arm of *sm* chromosomes (chr19, *i* value = 30.60) and six weak signals in *C. carassius*.
- Two strong 5S rDNA signals at the *p* arm of *sm* chromosomes (chr17, *i* value = 30.00) and eight weak signals in *C. gibelio*.

Summary of 28S rDNA (corresponding to nucleolar organizer region). We found:

- Two strong 28S rDNA signals at the *p* arm of two *sm* chromosomes (chr16, *i* value = 35.69) and two weak signals in *C. auratus*.
- Two strong 28S rDNA signals at the *p* arm of two *sm* chromosomes (chr13, *i* value = 34.72) and four weak signals in *C. carassius*.
- Two strong 28S rDNA signals at the pericentromeric region of two *T* chromosomes (in the range of chr26–50, *i* value = 0) and two weak 28S rDNA signals in *C. gibelio*.

4. Discussion

Diploid vs. polyploid biotypes—sexual vs. asexual reproduction—genetic vs. environmental sex determination: All these natural phenomena make *Carassius* a valuable experimental model for evolutionary studies. The study of these aforementioned unique characteristics of *Carassius* require basic cytogenetic techniques such as chromosome prepa-
rations and/or karyotype. Diploid biotypes of the genus *Carassius* have a karyotype consisting of 100 chromosomes (e.g., [28], this study), with some exceptions, i.e., 50, 94, 98, 102 or 104 chromosomes [22,42–46]. Karyotype formula of diploid *Carassius* is inconsistently defined by several authors (Table 3) and q + p arms have not been measured. An application of a technique of measurement, and the following determination of standardized karyotype is needed in order to clear up genome architecture and its evolution.

Table 3. Previously published karyotypes of diploid *C. auratus, C. carassius* and *C. gibelio* including information about sex and locality of the investigated individuals. NA = information not available, F = female, M = male.

| Karyotype | Sex | Locality | References |
|-----------|-----|----------|------------|
| *C. auratus* | | | |
| 2n = 94 | NA | Japan | [42,47] |
| 2n = 96–104 | F, M | NA | [48] |
| 2n = 100(12m + 36sm + 52st–a) | F, M | Japan | [49,50] |
| 2n = 104(46m + 16sm + 42a) | F, M | NA | [22] |
| 2n = 104(20m + 72sm–st + 12a) | NA | NA | [44] |
| 2n = 100(20m + 40sm + 40a) | F, M | NA | [17] |
| 2n = 100(16m + 84sm–a) | NA | NA | [51] |
| 2n = 100(12m + 36sm + 52st–a) | F, M | China | [18,52,53] |
| 2n = 100(22m + 30sm + 48st–a) | F, M | China | [54–56] |
| *C. carassius* | | | |
| 2n = 104(20m + 72sm–st + 12a) | NA | NA | [44] |
| 2n = 100(20m + 40sm + 40a) | F, M | Netherlands | [17,57–59] |
| 2n = 100(20m + 44sm + 36a) | NA | France | [60] |
| 2n = 100 | F, M | Bosnia | [61] |
| 2n = 50(20m + 12sm + 18st–a) | NA | Romania | [45] |
| 2n = 100 | F, M | Czech Republic | [62] |
| 2n = 100(20m + 36sm + 44st–a) | F, M | Czech Republic | [16,31] |
| 2n = 100(20m + 36sm + 44st–a) | F, M | Poland | [40] |
| 2n = 100(20m + 36sm + 44st–a) | M | Finland | [32] |
| *C. gibelio* | | | |
| 2n = 94 | F, M | Belarus | [43] |
| 2n = 100(20m + 40sm + 40a) | NA | River Amur | [63] |
| 2n = 98(48m–st + 50a) | NA | Romania | [45] |
| 2n = 102(24m + 36sm–st + 42a) | F | Yugoslavia | [46] |
| 2n = 104(24m + 36sm–st + 44a) | M | Yugoslavia | [46] |
| 2n = 100(14m + 24sm + 62st–a) | F, M | Poland | [64] |
| 2n = 100(26m + 38sm + 36st–a) | F, M | Poland | [65] |

We generated a revised karyotype as a result of novel measurement and using statistical programming we described standardized karyotype of three species (*C. auratus, C. carassius* and *C. gibelio*). We measured the length of each individual chromosome of each species and identified each chromosome using i value. Groups of i values were divided into quartiles Q0–Q4. Our results based on median (Q2) of i value for each chromosome revealed three different karyotypes; not one of these three karyotypes corresponded to any of the previously published karyotypes from Table 3. The karyotype of *C. auratus* had 24m, 40sm, 2st, 2a and 32T chromosomes (shortened formula 24m + 40sm + 36st–T). The karyotype of *C. carassius* had 16m, 34sm, 8st and 42T chromosomes (shortened formula 16m + 34sm + 50st–T). The karyotype of *C. gibelio* possessed 16m, 22sm, 10st, 2a and 50T chromosomes (shortened formula 16m + 22sm + 62st–T). These standardized karyotypes are likely highly reproducible and could be applied to the reconstruction of cytogenetic maps, comparative cytogenetics and genomics, or cytotaxonomy and karyosystematics. The designed R scripts (Supplementary Results) could be applied to the description of
standardized karyotype for another species with relatively high number of chromosomes similar to that of Carassius.

In the wider range of $i$ values ($Q_1$–$Q_3$) for each chromosome, we found karyotypes of C. carassius and C. gibelio more similar, as both C. carassius and C. gibelio shared some $i$ values within $Q_1$–$Q_3$ range of corresponding orthologous counterparts. The $i$ values in $Q_1$–$Q_3$ of chr8 and chr11–31 in C. auratus were significantly different those of $i$ values in $Q_1$–$Q_3$ of C. carassius and C. gibelio (Figures 2 and 3). This finding indicates higher divergence of C. auratus karyotype based on $i$ value. The $i$ value divergence might be supported by distinct origin of the samples used in this investigation—C. auratus used in this study was imported from Israel and was domesticated in ancient China [23]. Both C. carassius and C. gibelio used in this study originated from Europe and mostly from the Czech Republic (only four individuals of C. carassius were caught in Finland). A phylogenetic study of Asian–European Carassius suggests a closer evolutionary relationship exists between diploid C. auratus and diploid C. gibelio compared to C. carassius and diploid C. auratus/gibelio [66]. Thus, phylogenetic distance does not reflect $i$ value divergence in C. carassius, as demonstrated in this study. The higher similarity between C. carassius and C. gibelio $i$ values could be caused by hybridization, as previously confirmed by molecular genetic and cytogenetic tools in several European Carassius populations [16,66,68]. Although, we can not exclude a hybrid origin for diploid Carassius individuals.

In addition, female meiotic drive might also affect $i$ values [69]. Chromosomes of particular morphology might be preferentially transmitted to the egg during meiosis and the chromosomal complement of new offspring can be rapidly changed. Interestingly, polyploid Carassius biotypes have variable numbers of microchromosomes [16] which results in odd or variable chromosome numbers within polyploid Carassius [28]. The uniform chromosome numbers, we have described in diploid Carassius, are in accordance with expectations that female meiotic drive has never been shown in diploid Carassius.

We also showed a relationship between the mean of $i$ value and the mean of $q + p$ chromosomal length. Chromosomes of C. gibelio had smaller size (mean of chromosomal length was 21.36) those of C. auratus (31.53) and C. carassius (35.05, Figure 1). This difference in chromosomal length in Carassius might be promoted by altered chromatin spiralisation and condensation during cell cycle, especially during interphase and mitosis [70–72]. In this study, chromosomes were synchronized in metaphase by colchicine but some chromosomes can be fixed in prometaphase, early metaphase or late metaphase and we assume that slight differences in chromosomal length can be present in different Carassius species. The length of chromosomes might theoretically be influenced by storage time if the ratio or concentration of acetic acid and methanol is slightly changed [72]. To avoid these inaccuracies and preserving the chromosome suspension, we used a fixative solution with an acetic acid : methanol ratio of 1:3. This ratio was restored through the addition of fresh acetic acid and methanol. In addition, we statistically processed metaphases with different storage times, for which we found no difference in chromosome length. For the case of standardized karyotype, all previous negative effects that influence $q + p$ arm length can be discounted because even if the length of chromosome had changed, the position of the centromere remains identical, thus $i$ values never change [2]. In addition, the $i$ value errors were eliminated through analysis of the median $Q_2$ range.

The correct order of chromosomes in Carassius karyograms remains difficult to determine. For our purposes, we arranged chromosomes according to decreasing $i$ values. As such, chr1 has the highest $i$ value and chr50 has the lowest $i$ value. Using this approach describes a shared synteny of $i$ value among C. auratus, C. carassius and C. gibelio (Figure 3). This method of numbering of chromosomes according $i$ value in Carassius can be modified in future studies such as the numbering in human decreasingly according to $q + p$ chromosomal length [73].

FISH analysis revealed two large non-nucleolar 5S loci and two large nucleolar 28S ribosomal loci in each of the Carassius species that were investigated; this indicates functional diploidy in evolutionary tetraploid biotype. Two strong rDNA signals were found
in other studies which described *Carassius* karyotype [32,40,74]. The number of 5S rDNA loci (strong and weak signals in total) ranged from eight to ten, and 28S rDNA loci ranged from four to six, with a single chromosome-bearing rDNA locus differing in each *Carassius* species. We found higher similarity in a number of rDNA loci in *C. auratus* and *C. gibelio*, i.e., ten 5S and four 28S rDNA signals in these two species. *Carassius carassius* possessed eight 5S and six 28S rDNA signals. Knytl et al. [32] identified 18 5S and four 28S rDNA loci in diploid *C. carassius* originating from Finland waters. Spoz et al. [40] pointed out ten 5S and four 28S rDNA loci in *C. carassius* from Poland. Chinese *C. auratus* has 2–8 5S rDNA signals [74]. This study brought the first report of the FISH analysis used on diploid European *C. gibelio*, which belongs to phylogenetically very diverse taxonomic group [66]. Our results showed variability in the number and position of ribosomal tandem repeats in *Carassius* which is in accordance with other rDNA studies [32,40,74]. This variability in rDNA loci has been shown between species of the same genus [38] and even between individuals of the same species for 18S and 28S loci [75,76]. The present variability of rDNA loci can be attributed to degree in heterochromatin condensation and nucleolar activity during mitosis [77].

Overall, we have described a standardized karyotype of three species of *Carassius* genus: *C. auratus*, *C. carassius* and *C. gibelio*. We compared these three species using cytogenetic tools: The arithmetic mean of the length of *q + p* chromosomal arm showed higher similarity of *C. auratus* and *C. carassius*, and a difference of *C. gibelio* (Figure 1). Analysis of median of the *i* value showed higher similarity of *C. carassius* and *C. gibelio*, and higher difference of *C. auratus* (Figures 2 and 3). FISH confirmed a higher similarity of *C. carassius* to *C. gibelio*, compared to *C. auratus* (Figure 4). Thus, we can conclude that the genus *Carassius* has a very complex cytogenetic background, which can be distinguished using cytogenetic tools, with some inconsistencies in measuring propinquity and congeniality. The findings presented here are consistent with the presence of great genome variability and plasticity. Such genome variability could be attributed to the occurrence of rare natural phenomena such as interspecies hybridization, polyploidization, and/or alternation of reproduction modes within the genus *Carassius* [16,43,78,79].

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/cells10092343/s1, Figure S1: Giemsa-stained chromosome metaphases (B&W) used for long (*q*) and short (*p*) chromosome arms measurement. The Figure shows one representative metaphase for each sex of each species. Each chromosome has a working numeric code 1–100. Metaphases show 100 chromosomes in (a) *Carassius auratus* (CAU) male, (b) *C. auratus* female, (c) *C. carassius* (CCA) male, (d) *C. carassius* female, (e) *C. gibelio* (CGI) male and (f) *C. gibelio* female, white circles in (f) indicate centromeric regions assigned as the narrowest part of chromosome. Figure S2: Giemsa-stained chromosome dissected from metaphase. Graphical example of the measurement of metacentric chromosome with four chromosomal arms (two chromatids). Two thin lines which lead through all four arms cross in the centromeric region—the narrowest part of chromosome. Long arm 1 (*q1*), long arm 2 (*q2*), short arm 1 (*p1*), short arm 2 (*p2*) have 14.8, 13.6, 12.6 and 12.4 pixels, respectively. Calculation of the *q + p* arm length is shown at the top right of the figure. Table S1: *Carassius auratus* *q + p* lengths were dissected from each metaphase and added into separate table. The last column (*mean_length*) shows arithmetic mean of length used for dot plot (Figure 1). Table S2: *Carassius auratus* *i* values were dissected from each metaphase and added into separate table. The last column (*mean_i*) shows arithmetic mean of the *i* value used for dot plot (Figure 1).

**Author Contributions:** Conceptualization, M.K.; methodology, M.K. and N.R.F.; software, M.K.; validation, M.K.; formal analysis, M.K.; investigation, M.K. and N.R.F.; data curation, M.K.; writing—original draft preparation, M.K.; writing—review and editing, M.K., N.R.F.; visualization, M.K.; supervision, M.K.; project administration, M.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** All experimental procedures involving fish were approved by the Institutional Animal Care and Use Committee of the Czech Academy of Sciences, Institute of
Animal Physiology and Genetics, according to the directives from the State Veterinary Administration of the Czech Republic, permit number 124/2009, and by the permit number CZ 00221 issued by the Ministry of Agriculture of the Czech Republic. M.K. is a holder of the Certificate of professional competence to design experiments according to §15d(3) of the Czech Republic Act No. 246/1992 coll. on the Protection of Animals against Cruelty (Registration number CZ 03973), provided by the Ministry of Agriculture of the Czech Republic.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Sanger sequencing data are available on-line at the NCBI database, accessed on 26 August 2021 ([https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/)). All data generated by R Stud.io are not presented in this study and they may be obtained on request from the corresponding author. All steps generating data frames and plots in R were summarized on-line on GitHub, accessed on 26 August 2021 ([https://www.github.com/](https://www.github.com/)) available on request. Basic q + p arm measurements can be provided in the form of RData file as R workspace, also upon request.

**Acknowledgments:** We are very grateful to Evans lab (Department of Biology, McMaster University, Canada) which helped us with the basis of computational programming in R (namely Ben J. Evans, Caroline M.S. Cauret and Xue-Ying Song). We thank very much Adrian Forsythe (Department of Zoology, Evolutionary Biology Center, Uppsala University, Sweden) for English correction. We thank Abhishek Koladiya (Department of Immunomonitoring and Flow Cytometry, Institute of Hematology and Blood Transfusion, Czech Republic) for improvement R scripts. Furthermore, we thank MDPI Production Team (especially Liu and Jing) for figuring out ISIPX issues, and Editorial Board of Cells for quick and positive feedback.

**Conflicts of Interest:** The authors declare no conflict of interest.

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