Review

A Fish of Multiple Faces, which Show us Enigmatic and Incredible Phenomena in Nature: Biology and Cytogenetics of the Genus *Carassius*

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Abstract: Sexual vs asexual reproduction—unisexual vs bisexual populations—diploid vs polyploid biotypes—genetic vs environmental sex determination: all these natural phenomena are associated with the genus of teleost fish, *Carassius*. Two *Carassius* entities with completely different biological characteristics exist: one globally widespread and invasive *Carassius gibelio*, and the other *C. carassius* with decreasing trend of natural occurrence. Comprehensive biological and cytogenetic knowledge of both entities, including the physical interactions between them, can help to balance advantages of highly invasive and disadvantages of threatened species. For example, benefits of a wide-ranged colonization can lead toward extinction of native species or be compensated by parasitic enemies and lead to equilibrium. This review emphasizes the comprehensive biology and cytogenetic knowledge and importance of the *Carassius* genus as one of the most useful experimental vertebrate models for evolutionary biology and genetics. Secondly, the review points out that effective molecular cytogenetics should be used for identification of various species, ploidy levels, and hybrids. The proposed investigation of these hallmark characteristics in *Carassius* may be applied in conservation efforts to sustain threatened populations in their native ranges. Also, the review focuses on consequences of co-occurrence of native and non-native species and outlines future perspectives of *Carassius* research.

Keywords: hybridization; sexuality; asexuality; biotype; species; sex determination; ploidy level; *Carassius auratus* complex

1. Introduction

The genus *Carassius* Nilsson, 1832 belongs to a monophyletic group of paleotetraploids from the Cyprinini tribe, [sensu 1] within the family Cyprinidae (Actinopterygii, Teleostei). Two valid, morphologically distinct and phylogenetically diverged species therein, of *Carassius* have been clearly described, including crucian carp (*Carassius carassius* Linnaeus, 1758) and white crucian carp “gengorobuna” (*C. cuvieri* Temminck and Schlegel, 1846). However, despite this distinction between several species of *Carassius* in the literature, taxonomic classification is often inconsistent and are often incorrectly referred to by authors [2,3, e.g.] as various different taxa, species, or holotypes.

Glossary

BIOTYPE A summary term for *Carassius* individuals with a certain ploidy level or reproduction mode (e.g., diploid, triploid, sexual, gynogenetic biotype). Based on sequencing data, it is possible to classify each biotype into separate mitochondrial taxa. 5, 8–10, 12

COMPLEX An evolutionary group of related taxa involving all possible biotypes, hybrids, non-specific genotypes assigned to them due to morphological and genetic similarities between each other. 2, 3, 7, 11, 12

FORM A low taxonomic unit within a species or biotype with unified morphological traits. Often varying in body colour, shape, fin length etc. (e.g., *Carassius* domesticated form, *Carassius* xantic form). 3

HOLOTYPE A single physical example (or illustration) of an organism, known to have been used when the species (or lower-ranked taxon) was formally described. It is either the single such physical example or one of several such, but explicitly designated as the holotype. 3

HYBRID SPECIES Hybridization gives rise to novel allelic combinations that contribute to the spread and maintenance of stabilized hybrid lineages generally recognized as species. The process of hybrid species formation is a hybrid speciation [4]. 9, 11

HYBRID In the strict sense, a hybrid is the first offspring resulting from the reproduction of two or more distinct species. When hybrids mate with parental species the resulting offspring is called backcross. 3, 8–12

NEOTYPE A specimen later selected to serve as the single type specimen when an original holotype has been lost or destroyed or where the original author never cited a specimen. 3

SPECIES A basic (basal) taxon of hierarchical systematic classification defined as a related group of organisms of the same history able to cross between each other (but usually they are not able to cross between another species). 2, 3, 5–12

TAXON (in plural TAXA) A group of one or more populations of an organism or organisms which form a taxonomic unit. 3, 9, 12

2. Taxonomy of the genus *Carassius*

Distinct morphological characteristics have emerged in *C. carassius* and *C. cuvieri*. *Carassius carassius* can be distinguished from all other *Carassius* by the convex angle of the upper edge of the dorsal fin, black dot at the base of caudal peduncle, and whitish peritoneum [5,6]. Despite the relatively clear differences in morphology, the use of the name *C. carassius* has been disputed. Without the use of modern molecular techniques, some authors have previously classified *C. carassius* as Japanese Funa or Black-Funa [sensu 7] , and an ancestor of goldfish [7–9]. However, these classifications are incorrect, based on previous phylogeographical studies which show *C. carassius* (sensu Linnaeus, 1758) as being native to many European freshwaters, with no records of occurrence in Japan [3,10–12]. *Carassius cuvieri* is phylogenetically distinct from *C. carassius* and all other *Carassius* species, representing a basal diverged clade [13,14] with morphological differences which do not correspond with levels of genetic differentiation [15]. Distinct *C. cuvieri* characteristics are large number of gill rakers and endemic occurrence in Lake Biwa in Japan [2]. Until recently *C. cuvieri* was treated as subspecies of *C. auratus* COMPLEX.

Other *Carassius* SPECIES and subspecies belong to the *C. auratus* COMPLEX, which is a biologically diverse group [16] with a wide geographical distribution [11,13], different levels of ploidy [17], various modes of reproduction [18], and distinct modes of sex determination [19]. Most notably, Silver carp (gibel carp, *C. auratus gibelio*, Bloch, 1782) and *C. auratus auratus* Linnaeus, 1758) which are pan-globally widespread with invasive character and most abundantly inhabit mainland Eurasia [6]. Additional subspecies occur mainly in the Japanese archipelago and Taiwan (some of them as endemic subspecies): ginbuna (*C. auratus langsdorfii* Temminck and Schlegel, 1846), nigorobuna (*C. auratus grandoculis* Temminck and Schlegel, 1846), okinbuna (*C. auratus buergeri* Temminck and Schlegel, 1846), nagabuna (*C. auratus* subsp. 1) and kinbuna (*C. auratus* subsp. 2) [2]. Some ichthyologists have considered *C. auratus*, *C. gibelio*, and *C. langsdorfii* as separate independent SPECIES [3,6].

The most well known *Carassius*, the goldfish (*C. auratus*), commonly persists in an array of colorful varieties and body morphologies. However, much contention surrounding the taxonomic classification of this species. Identification of *C. auratus* has been inconsistent for a number of reasons, with some proposing several independent origins [13], gene flow from domesticated and feral populations [20], as well as anthropogenic translocation [13] and inter-taxa hybridization [21] contributing to difficulties in classification. Typically, the name *C. auratus* involves either a domesticated FORM of the goldfish or the entire *C. auratus* COMPLEX including *C.* (*auratus*) *gibelio*, *C.* (*auratus*) *auratus*, *C.* (*auratus*) *langsdorfii*, *C.* (*auratus*) *cuvieri*, *C.* (*auratus*) *grandoculis*, *C.* (*auratus*) *buergeri*, *C.* (*auratus*) subsp. 1, and *C.* (*auratus*) subsp. 2. It is difficult to assign distinct "SPECIES" or "subspecies" based on morphological characteristics alone [15], as many exceptions between genetically uniform taxa exist in the *Carassius auratus* COMPLEX (e.g. frequent natural hybridizations) [22]. Even molecular genetic analysis and reconstruction of phylogenetic trees cannot reliably distinguish SPECIES from subspecies [11,13]. Inconsistent identification of *Carassius* SPECIES has persisted since the first identification of *Cyprinus carassius* and *Cyprinus gibelio* by Bloch, 1782. A part of the natural history Museum für Naturkunde der Humboldt Universität zu Berlin (Germany), these two specimens were clearly morphologically indistinguishable from *C. carassius*. In this case, the *Cyprinus gibelio* type specimen was likely lost and replaced by a specimen of *C. carassius* [3,23], making it difficult to judge whether the original identification of *Cyprinus gibelio* by Bloch represents a wholly different SPECIES or a HYBRID between *Cyprinus* and *Carassius*. Because a HOLOTYPE is missing in Bloch's specimen collection, Kalous et al [3] described a NEOTYPE for *C. gibelio* and concluded based on mitochondrial cytochrome *b* sequences that at least one more TAXON has been grouped under the single species *C. gibelio*. This finding indicates the polyphyletic origin of *C. gibelio* and sparks doubt if the sister TAXON still belongs to *C. gibelio*. Other alternative classifications have been proposed, such as *C. gibelio gibelio* or *C. carassius gibelio* [24], but neither of these classifications are widely used.

In this review, we will be keeping the concept of *C. auratus* COMPLEX for all *Carassius* TAXA but *C. carassius* and *C. cuvieri* will be treated as independent SPECIES. Other members of the *C. auratus* COMPLEX will be used under the scientific names *C. auratus*, *C. gibelio*, and *C. langsdorfii*. *Carassius auratus* will be applied in a limited sense to the domesticated goldfish.

3. Overview of the early cytogenetic studies focused on *Carassius* karyotype

A landmark cytogenetic study was published by Makino [25] in 1934 focused on chromosome number and karyotype of the genus *Carassius*. This work led to the first description of diploidy in goldfish (*C. auratus*) $2n = 4x = 94$ from Japan, where "n" refers to a 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. Descriptions of the karyotypic diversity in *Carassius* was further extended by Makino [7], who reported the karyotypes of 13 colorful gold-fish as well as *C. carassius*. From this work, it can be concluded that *C. carassius* belongs to the TAXON *C. auratus* due to the Japanese origin of the original samples. The karyotype of *C. gibelio* was first recorded by Cherfas [26], revealing that ploidy level of this SPECIES is represented by diploid and triploid individuals with chromosome numbers $2n = 4x = 94$ and $3n = 6x = 141$, respectively. Karyotypes of diverse across *Carassius* (Table 1), with descriptions of polyploidy in *C. auratus*, *C. gibelio*, *C. langsdorfii*, *C. cuvieri*, *C. buergeri*, and *C. grandoculis*. Numbers of

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chromosomes and karyotype formulas in diploid *C. auratus*, *C. carassius*, and *C. gibelio* are reviewed in Table 3 in Knytl and Fornaini [27]. Knytl and Fornaini [27] also published standardized karyotypes for diploid *C. auratus*, *C. carassius*, and *C. gibelio*.

Table 1. Previously published karyotype formulas within *C. auratus* complex including information about sex and locality of the investigated individuals. Karyotype data are ordered chronologically. *Carassius carassius* and diploid *C. auratus* and *C. gibelio* are not involved. m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, B = B chromosome/microchromosome, NA = information not available, F = female, M = male.

Species and karyotype	**Sex**	**Locality**	**Reference**
Carassius auratus (goldfish)			
$3n = 6x = 162\ (33m + 53sm + 76st\text{-}a)$	F	China	[28,29]
$3n = 6x = 156\ (30m + 46sm + 80st\text{-}a)$	NA	China	[30]
$3n = 6x = 162\ (36m + 56sm + 70st\text{-}a)$	NA	China	[31]
Carassius gibelio			
$3n = 6x = 141$	F	Belarus	[26]
$3n = 6x = 156\ (34m + 62sm + 60a)$	NA	River Amur	[32]
$3n = 6x = 150$	F	Bosnia	[33]
$3n = 6x = 166\ (46m + 82sm\text{-}st + 32a + 6B)$	F	Czechoslovakia	[34]
$3n = 6x = 160\ (15m + 28sm + 126st\text{-}a + 1B)$	F	Yugoslavia	[35]
$3n = 6x = 160$	F	Czechia	[36]
$3n = 6x = 162\ (32m + 52sm + 78st\text{-}a)$	NA	NA	[30]
$3n = 6x = 158\ (36m + 54sm\text{-}st + 68a)$	F	Yugoslavia	[37]
$3n = 6x = 156\ (42m + 74sm + 40st)$	F, M	China	[38]
$3n = 6x = 150\ (26m + 50sm + 74st\text{-}a)$	F	Poland	[39]
$3n = 6x = 156\ (36m + 54sm + 60st\text{-}a + 6B)$	M	China	[40]
$3n = 6x = 162\ (42m + 54sm + 60st\text{-}a + 6B)$	F		
$3n = 6x = 154\ (24m + 54sm + 72st\text{-}a + 4B)$	F	Poland	[41]
$3n = 6x = 160\ (33m + 48sm + 75st\text{-}a + 4B)$	F		
$3n = 6x = 160\ (34m + 58sm + 62st\text{-}a + 6B)$	M		
$3n = 6x = 156\ (30m + 54sm + 66st\text{-}a + 6B)$	F	Czechia	[22]
Carassius langsdorfii			
$2n = 4x = 100\ (20m + 40sm + 40a)$	F, M	Japan	[42]
$3n = 6x = 156\ (34m + 62sm + 60a)$	F		
$4n = 8x = 206\ (44m + 82sm + 80a)$	F		
$3n = 6x = 156\ (34m + 62sm + 60a)$	F	Japan	[43]
$2n = 4x = 100\ (20m + 40sm + 40a)$	F, M	Japan	[44]
$3n = 6x = 157\ (32m + 59sm + 62a + 4B)$	F		
$3n = 6x = 165\ (44m + 82sm + 80a + 9B)$	M		
$3n = 6x = 156\ (34m + 62sm + 60a)$	F	Japan	[45]
$4n = 8x = 206\ (44m + 82sm + 80a)$	F		
$3n = 6x = 156$	F	Czechia	[46]
Carassius cuvieri			
$2n = 4x = 100\ (12m + 36sm + 52a)$	F, M	Japan	[47]
$2n = 4x = 100\ (20m + 40sm + 40a)$	F, M	Japan	[44]
$2n = 4x = 100\ (12m + 36sm + 52a)$	F, M	Japan	[48]
$2n = 4x = 100\ (12m + 36sm + 52st\text{-}a)$	F, M	Japan	[49]
Carassius buergeri			
$2n = 4x = 100\ (20m + 40sm + 40a)$	F, M	Japan	[43]
$3n = 6x = 156\ (34m + 62sm + 60a)$	F		
$2n = 4x = 100\ (12m + 36sm + 52st\text{-}a)$	F, M	Japan	[49]
Carassius grandoculis			
$2n = 4x = 100\ (20m + 40sm + 40a)$	F, M	Japan	[43]
$2n = 4x = 100\ (12m + 36sm + 52st\text{-}a)$	F, M	Japan	[49]

Carassius carassius, *C. cuvieri*, and *C. grandoculis* are exclusively diploid with 100 chromosomes ($2n = 4x = 100$) [49–51]. *Carassius auratus*, *C. gibelio*, and *C. langsdorfii* form diploid ($2n = 4x = 100$), triploid ($3n = 6x \approx 150$) and tetraploid BIOTYPES ($4n = 8x \approx 200$) [42,46,52,53]. *Carassius* tetraploids are not listed in Table 1. Tetraploid *Carassius* have been usually generated by interspecific crossing triploid *Carassius* female with diploid heterospecific male [54,55], or naturally occurring pure *Carassius* tetraploids have not been cytogenetically described. Biologically diploid *Carassius* BIOTYPES are interestingly considered as evolutionary paleo-tetraploids [50,56] because the most members of the family Cyprinidae contains 25 chromosomes in each gamete (50 chromosomes in a somatic cell).

4. Synergy of conventional chromosome banding and ribosomal fluorescent in situ hybridization

The G-, C-, R-, Q-, Ag-,CMA_3-banding techniques have served as useful tool for efficient and precise analyses of karyotypes in various animals beyond the routine Giemsa method level and have promoted the development of cytogenetics of *Carassius* SPECIES to a great extent especially in the end of the last century [31,36,49,57]. Nowadays conventional banding techniques together with more advanced molecular cytogenetics (e.g., in situ hybridization) are widely utilized in many SPECIES and may determine a tempo of karyotype evolution focused on repetitive ribosomal DNA (rDNA) loci, microsatellite motifs, ratio of parental genomes within a hybrid genome etc. [58–60]. Banding specific chromosomal patterns are helpful in identification of homologous chromosome pairs in karyotypes in which morphological chromosomal characteristics (length, arm ratio), distinguishing individual chromosomes between each other, are not evident [61]. Therefore, conventional banding approach will continue to be an integral and additional part of future cytogenetic research.

Fluorescent in situ hybridization (FISH) method has a diverse utilization in a study of evolutionary processes due to many modifications focused on various types of targeted nucleotide sequences. The FISH method, in which rDNA probes are hybridized on chromosomal DNA, reveals exact chromosomal positions where the rDNAs are situated. Ribosomal DNA regions can be easily amplified using PCR and labelled with specific nucleotides using labelling PCR [62] or nick translation [63]. These regions encoding rRNA are arranged into tandemly repeated units: major 45S unit composed of *18S*, *5.8S*, and *28S* genes, and minor 5S unit which forms independently. The 45S unit creates nucleolar secondary constriction which is called nucleolar organizer region (NOR) present during the M phase of the cell cycle, thus detectable using rDNA FISH technique (as well as using Ag-NOR and CMA_3 staining). The ribosomal genes is highly conserved in the term of sequence similarity across SPECIES [64]. Therefore one identical probe can be used for rDNA FISH for multiple SPECIES analysis, e.g. Spoz et al. [51] used the human and loach (genus *Cobitis* Linnaeus, 1758) *5S* and *28S* rDNA probes for hybridization with *C. carassius* chromosomal DNA; Knytl et al. [65] used pike (*Esox lucius* Linnaeus, 1758) *5S* rDNA probes for hybridization on *C. carassius* chromosomes. The number of positive *5S* rDNA loci within the *Carassius* karyotype are not consistent because of the high number of these loci. Spoz et al. [51], Knytl et al. [65] and Knytl and Fornaini 2021 [27] found from eight to 18 positive *5S* rDNA loci in *C. carassius*. Knytl and Fornaini [27] revealed ten *5S* rDNA signals in diploid *C. auratus* and *C. gibelio*. Zhu et al. [66] revealed 9-21 positive *5S* rDNA loci in triploid *C. gibelio*, Knytl et al. [65] detected 27 positive *5S* rDNA signals in triploid *Carassius*. The number intensively stained *5S* rDNA loci agreed on the ploidy level in *Carassius*, i.e., two intensively fluorescent loci in diploids, three in triploids [27,65,66] and four in tetraploids [67]. The number of NORs is usually four within diploid *Carassius* BIOTYPES [50,51,65,68] but polyploid *Carassius* BIOTYPES show different numbers of NORs. Triploid *C. gibelio* has four [65] or five NORs [69,70], tetraploid *Carassius* bears six [69,70] or eight NORs [68]. The number, size and localization of rDNA patterns in *Carassius* are not consistently specific for each SPECIES, but the intensity of these rDNA positive signals could serve as a specific

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marker for ploidy level determination and for determination how many times the genome has been duplicated (Figure 1).

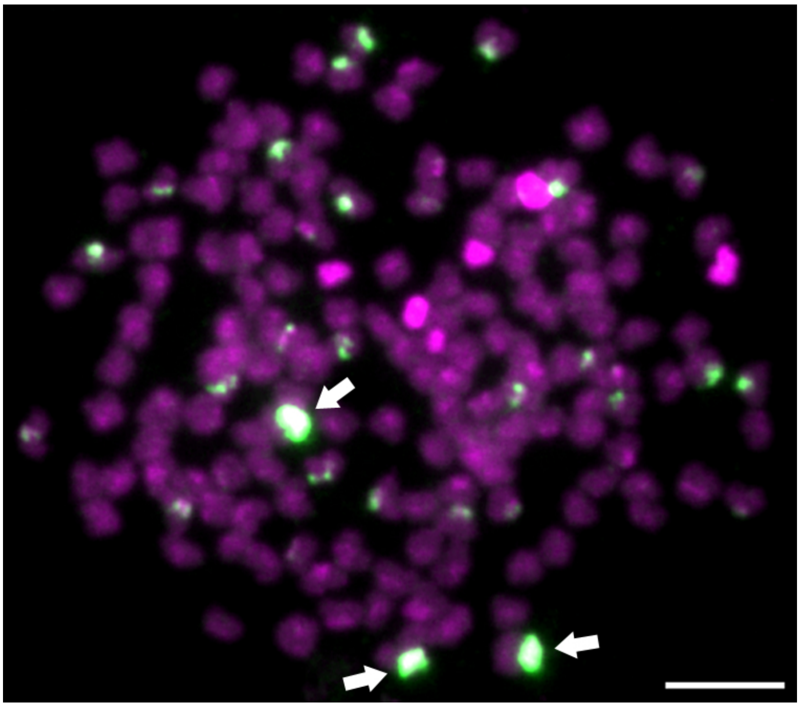

Figure 1. Fluorescent in situ hybridization (FISH) with *5S* ribosomal probes on *Carassius* hybrid female with 156 chromosomes. Three highly intensive *5S* gene loci in green (arrowheads) out of 27 show triploid origin of this female. Chromosomes are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in blue and red. Scale bar represents 10 μ M. Figure was modified according Knytl et al. [65].

5. Asexuality, unisexuality, and sexuality

One of other characteristics of the genus *Carassius* is an ability to reproduce asexually [26]. Gynogenesis (equivalently sperm dependent parthenogenesis or sexual parasitism) is asexual mode of reproduction in which paternal sperm is needed for activation of egg and embryonic development but sperm does not genetically contribute to the offspring and the offspring is distributed clonally [71]. It is generally known that gynogenetic females produce unreduced eggs, e.i. eggs with the same ploidy as ploidy in somatic cells [72]. In *Carassius*, an embryonic development stimulated by sperm of heterospecific (i.e., from another related SPECIES) sexual host gives rise to gynogenetic and clonal progeny consists of females only, thus unisexual populations arise [26]. An exception to the rules of unisexuality and asexuality has appeared by an occurrence of *Carassius* polyploid males in nature [73]. The exception has brought new enigmatic biological questions, such as the origin of polyploid *Carassius* males, or an incorporation or expression of paternal genetic material to a polyploid gynogenetic offspring with variable numbers of chromosomes [17]. Jiang et al. [73] proved a leakage of genetic information from heterospecific sperm into gynogenetic clones. An allogynogenetic offspring showed effect of enhanced growth, skewed sex ratio, different liver isoenzyme patterns and variation in body colour [73]. Allogynogenesis has become a cause of occasional formation of a small proportion of males in gynogenetic offspring [74]. Significant incidence of natural triploid *Carassius* males has been up to 23% compared to incidence of triploid females [53] but older literature introduced up to 10% [75]. Diploid *Carassius* males are common in nature with occurrence 1:1 for males and females within diploid population [53]. Triploid males of *C. gibelio* have served as valuable experimental material for artificial crosses with the aim to find out a mechanism of allogynogenesis/paternal leakage. If conspecific (i.e., from the same

SPECIES) sperm from triploid *C. gibelio* inseminates egg, the responding reproduction mode will be sexual with generation of bisexual recombinant offspring. If heterospecific sperm from *Cyprinus carpio* activates *C. gibelio* egg development, the result is unisexual clonal lineage formed by gynogenesis [75,76]. Similar situation, all female progeny and gynogenesis would occur if sperm from goldfish initiates egg and embryonic development of *C. langsdorfii* [77]. One small difference was evidenced in the development of *C. gibelio* [76] from the development of *C. langsdorfii* [77] eggs. An egg of *C. langsdorfii* retained somatic ploidy by retention of the first polar body [77] and an egg of *C. gibelio* extruded the first polar body and somatic ploidy level was restarted using extra DNA endoreplication without cytokinesis during the first mitosis in the stimulated egg [76]. Therefore *C. auratus* COMPLEX demonstrates coexistence of dual mode of reproduction—sexual (recombinant) and asexual (gynogenetic) with formation of unisexual (females only) and bisexual (both sexes) offspring [75].

6. Tell me your ploidy and I will tell you who you are: variability in ploidy levels and chromosome numbers

In general, it is possible to conclude that diploid *Carassius* females produce eggs with reduced amount of genetic information in nucleus. The spawning partners are conspecific males that fertilize eggs (true fertilization) and resulted offspring is recombinant [17,78]. Triploid *Carassius* females can produce both gynogenetic and recombinant progenies dependent on heterospecific and conspecific sperm interaction, respectively [75]. Tetraploid *Carassius* females reproduce gynogenetically [45]. Exceptionally occurring tetraploid males have low motility spermatozoa, thus poor fertilization capacity has been discovered [79]. Therefore, the knowledge of the ploidy level can tell us the origin of the examined *Carassius* individual (i.e., whether it is a clonal product of gynogenesis or result of recombination) and subsequent reproductive mode of the examined individual (in what mode its progeny will be produced). Therefore, determination of ploidy level in *Carassius* can serve as an additional marker helpful for SPECIES identification and important marker for determination of reproductive mode.

Artificial crossing experiments of *Carassius* individuals with different ploidy levels have been performed in order to find out variability or stability in chromosome numbers. Kalous and Knytl [17] performed crossing experiment of diploid *C. gibelio* male with 100 chromosomes and triploid female with 159 chromosomes. Resulted F1 offspring possessed 150, 151, 156, 158, and 159 chromosomes. The mode of reproduction was not studied but there was evident paternal leakage and European *C. gibelio* showed high genome plasticity and diversity in chromosome numbers. Variability in number of chromosomes in Asian *Carassius* confirmed Zhou and Gui [40]. They cytogenetically investigated Chinese *C. gibelio* F1 generation after crossbreeding experiment between triploid 156 chromosomal male and triploid 162 chromosomal female. The progeny bore 159 chromosomes which is the intermediate number between paternal 156 and maternal 162 chromosomes. In addition, SCAR (sequence character amplified region) markers indicated recombination in offspring and originality of each parent from different hybridization events [40]. Both previous works indicates close relationship between triploid karyotypes of European and East Asian *C. gibelio* populations, i.e., an ability to form diverse number of chromosomes.

Many studies claim variability in *Carassius* numbers of chromosomes [e.g. 17,40,78], also see Table 1. Some studies uncovered variability in diploid chromosome numbers [e.g. 80,81], reviewed in Table 3 in Knytl and Fornaini [27]. This variability can be caused by karyotyping errors from a period beginning cytogenetics. Data about chromosome numbers of triploids and tetraploids ranged from 141 [26] to 166 [34] and from 200 [52] to 240 [82], respectively. The variability of chromosome numbers in polyploid *Carassius* is not probably affected by karyotyping errors but by the mechanism of allogynogenesis/paternal leakage [74,83]. Another possible reason why in some studies *Carassius* chromosome numbers differ from each other is a high number of tiny chromosomes in karyotype and a presence of extra chromosomal elements such as microchromosomes, also called

B chromosomes. These B chromosomes are difficulty countable [17]. Despite the fact that B chromosomes can make counting of chromosomes more difficult we can conclude that polyploid *Carassius* BIOTYPES have variable number of chromosomes and *Carassius* diploids have 100 chromosomes.

7. Fluorescent in situ hybridization vs. cryptic interspecies hybridization

Various ploidy levels can be formed by two mechanisms. The first one is autopolyploidization when new polyploid BIOTYPE arises from single parental ancestor. Environmental factors such as temperature shock prevents extrusion of the polar body in meiosis II and promotes polyploidization [84]. Chemical factors e.g., presence of bisphenol S induces aberrant formation of spindle and thus chromatin could be divided unequally during oogenesis [85]. Colchicine is widely used for preparation of chromosomal suspension with the aim to defend chromosomal cleavage and subsequent migration of each chromatid towards centrosomes [e.g. 86,87] and can be used for induction of polyploidy [88]. Factors mentioned above can theoretically lead to natural elevation of ploidy level in *Carassius*.

The second mechanism generating higher ploidy levels is allopolyploidization when two or more distinct SPECIES hybridize. The precise mechanism of a heterospecific sperm genome incorporation into *Carassius* egg is unknown yet but several factors, which influence chromosomal segregation or promote retention of polar body during cell division, have been described [76].

In general, asexual polyploid BIOTYPES, that form unisexual populations, are considered to be of a hybrid origin [89], i.e., to be a product of allopolyploidization. *Carassius* parental SPECIES have not been identified. Interspecies hybridization in *Carassius* is a spontaneously ongoing process and several HYBRIDS were identified in nature [22,90–92] as well as artificially allotetraploid HYBRIDS were generated [69,70]. Exact ratio of maternal and paternal chromosomes within hybrid genome was specified in naturally occurring *Carassius* allotetraploid by genomic in situ hybridization (GISH) [22]. Mitochondrial DNA confirmed *C. gibelio* maternal origin and GISH technique identified 50 *C. carassius* chromosomes out of 206 chromosomes of hybrid *Carassius* female (Figure 2).

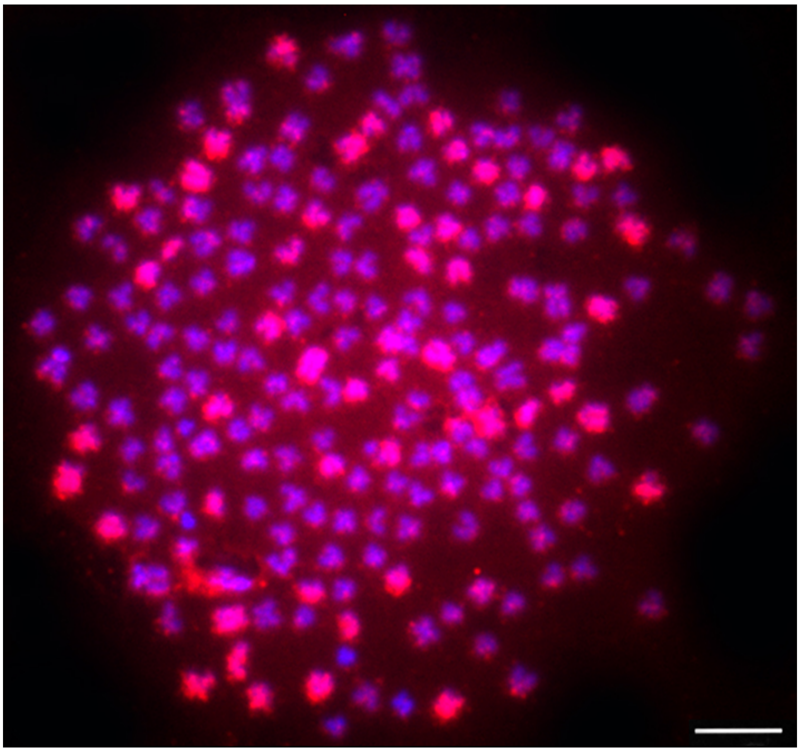

Figure 2. Genomic in situ hybridization (GISH) identificatified naturally occurring *Carassius* hybrid female with 206 chromosomes. Whole genomic painting probe labelled 50 paternal *C. carassius* chromosomes in red. Other 156 maternal chromosomes in blue originated from *C. gibelio*. Figure was modified according Knytl et al. [22]

Other allotetraploid *Carassius* HYBRIDS were artificially generated and cytogenetically investigated. *Carassius* with 206 and 212 chromosomes contained 156 and 162 *C. gibelio* chromosomes, respectively [69,70]. Both allotetraploids contained paternal 50 *Cyprinus carpio* chromosomes ($2n = 4x = 100$) which were incorporated to the maternal genome during fertilization. In all three cases of the *Carassius* hybrids, sperm genome was added into an egg of gynogenetically reproducing triploid *Carassius* female.

Analysis of microsatellite loci proposed by Hanlfing et al. [21] has been used for fast distinction of pure natural *Carassius* SPECIES from HYBRIDS. Same microsatellite markers were used by Papoušek et al. [92] and Wouters et al. [91] for genetic screening of natural *Carassius* populations, and cryptic hybridization between invasive and native *Carassius* TAXA was unraveled. Moreover, cryptic hybridization was proposed as one of the possible reasons for the declining trend of *C. carassius* occurrence in Europe [65,93]. Unfortunately, the ploidy level of naturally occurring cryptic *Carassius* HYBRIDS was not determined in the studies Papoušek et al. [92] and Wouter et al. [91] and we can only speculate if tetraploid *Carassius* BIOTYPES were prevalent or exclusively present within HYBRIDS. Mezhzherin et al. [94] investigated Ukrainian *Carassius* population structure by SPECIES and ploidy level. High occurrence of cryptic hybridization (by biochemical markers) was uncovered within all diploid, triploid, and tetraploid ploidy levels. Tetraploid individuals have been exclusively represented by interspecific HYBRIDS and no pure tetraploid *Carassius* BIOTYPE was identified. These data indicate that tetraploid *Carassius* BIOTYPES are interspecific HYBRIDS generated by sperm genome addition of sexual host SPECIES, usually addition of haploid 50-chromosome set into triploid maternal 150-chromosomal set. There is the question whether asexual *Carassius* tetraploids are evolutionary dead-end or whether we are observing rapid ongoing *Carassius* evolution—creation of allotetraploid HYBRID SPECIES able to reproduce gynogenetically and exploit "sexual parasitism". Owing to their ability to be fertile and produce fertile allotetraploid gynogenetic offspring [70] distribution in nature might be rapid and effective.

Another way to distinguish maternal and paternal genome complements between each other within HYBRIDS offers FISH with whole chromosome painting probes. Individual chromosomes [95] or morphologically similar chromosomal groups [58] can be separately dissected and used as a probe for in situ hybridization. Intensity of fluorescent signal of painting probes can indicate evolutionary distance and distinguish parental subgenomes in BIOTYPES that originate from interspecies hybridization [96]. B chromosomes can be easily distinguished and dissected from *Carassius* karyotype. Yi et al., [74] dissected B chromosomes from metaphase spreads of allotriploid *C. gibelio* and detected eight positive signals on telomeric and pericentromeric regions of *Megalobrama amblycephala* (Yih, 1955) chromosomes. *Megalobrama amblycephala* was a parental SPECIES thus Yi et al. [74] brought evidence of allogynogenetic introgression (paternal leakage) of the paternal *M. amblycephala* genome to the triploid *C. gibelio* maternal genome. No chromosomal microdissections have been carried out on naturally occurring *Carassius* and it would be a prospective challenge for better understanding of paternal leakage and determining parental SPECIES of polyploid *Carassius* BIOTYPES.

8. Equilibrium between quantital gynogenesis and qualital sexuality (Red Queen hypothesis)

Gynogenetic reproduction mode and production of all female populations without a need of coupling with conspecific males caused rapid invasivity and expansion of polyploid *Carassius* BIOTYPES almost all over the world [13,90,97–99]. Excessive *Carassius* expansion entailed negative consequences due to successful competition, interspecies hybridization, and occupation of new areas [100,101]. Negative consequences are declining numbers or even extinction of the native ichthyofauna. After reaching a peak incidence in the population, the most common (clonal gynogenetic) phenotype is becoming more vulnerable to attacks by biological enemies than a far less frequent sexual population [102]. Herpesviral hematopoietic necrosis, caused by cyprinid herpesvirus-2, has affected many natural populations and commercial farms of gynogenetic *Carassius* BIOTYPES [103–107]. The Red Queen hypothesis predicts evolution towards equilibrium in populational composition of sexual and asexual BIOTYPES coexisting together and co-evolving with parasites. In this coexistence, gynogenetic nonrecombinant clones form the most common phenotype. A sexual recombinant rare phenotype is favored and expected more likely to escape infection by co-evolved parasites. This hypothesis was tested in sexually and gynogenetically coexisting populations of *Poecilia monacha* and *P. 2monacha-lucida*, respectively. Significantly higher accumulation of parasites was found in prevalent gynogenetic *P. 2monacha-lucida* [102]. Significantly higher parasite load was also found in asexual than in sexual *Carassius* BIOTYPES. Less common sexual BIOTYPES had higher immune reaction and resistance to parasites [108]. Coexistence of gynogenetic and sexual *Carassius* BIOTYPES might be a trustworthy instance of the Red Queen hypothesis as a driver of *Carassius* biodiversity but *C. gibelio* is still prevalent in natural waters and thus the equilibrium is not yet established by Red Queen hypothesis. We propose the current relationship between co-occurring invasive and native *Carassius* as the birth of existence the Red Queen hypothesis.

9. Dual mode of sex determination

Sex determination, i.e., whether an embryo develops into male or female individual, can be governed environmentally by temperature (environmental sex determination) or genetically by a cascade of influential sex-determining genes located on sex chromosomes. *Carassius* is an unique example of both possible ways of sex determination.

In *Carassius*, genetic sex determination system is male heterogametic XY (female homogametic XX). The XX/XY was evidenced in *C. auratus* using crossbreeding experiment of hormone-treated (sex-reversed) with normal individuals followed by analysis of the progeny sex ratio [109]. Male heterogametic sex of *C. auratus* was identified cytogenetically using C-banding method. Intensive heterochromatic blocks were found on the short arms of the second largest submetacentric chromosome pair in a diploid female and only one of these two heterochromatic blocks has been found in a diploid male. These cytogenetic findings confirmed XX/XY sex determination system [9]. Zan [29] found a heteromorphic chromosome pair in a diploid *C. auratus* male indicating male heterogamety and the far smaller Y chromosome than X. Additionally, Zan [29] identified three X chromosomes in a triploid *Carassius* female. But C-banding revealed additional ten chromosomes and there is no justified conclusion why three C-banded chromosomes were determined as three X chromosomes. Neither C-banding differences in number and position between sexes have been found in European *C. carassius* [50] nor in Japanese *Carassius auratus* COMPLEX [9]. Recently, Wen et al. [19] revealed a relatively large non-recombining region on *C. auratus* Y chromosome. This sex region of the length of ∼11.7 Mb mapped on linkage group 22 involves male-specific genetic markers. Some of these markers were found in all XY males and were absent in all but one XX females/sex reversed XX males. Even though sex-linked markers were tested on less diverse *C. auratus* artificial population [19] the results will help to study mechanisms of sex determination in diverse *C. auratus* COMPLEX.

The other mechanism of sex determination in *Carassius* is given by temperature of environment in which embryo develops [19,110,111]. Increasing of rearing temperature (usually 25°C and higher) promotes skewed sex ratio towards males. Sex reversal from female to male embryos has been detected after 12 days after fertilization [110] and sex reversed phenotypic males were genotypic XX females/neomales [19]. Moreover, environmental temperature together with oxygen concentration are associated with expression of *hypoxia-inducible transcription factor-1* gene and this gene may be involved in sexual differentiation and an ability to display an extreme anoxia tolerance of *C. carassius* and *C. auratus* [112]. Apart from male and female sex, hermaphroditic individuals (both testes and ovaries within an individual) of *C. auratus* were found in Western Siberia [113]. A natural occurrence of extremely atypical *Carassius* hermaphrodites has been probably caused by wide-ranged oscillation of water level, salinity and temperature. The Siberian area, where *C. auratus* hermaphrodites have been caught, is characterized by (1) long-lasting regression of water level, (2) high variability of water salinity and (3) water temperature in spring (spawning time of *Carassius*) fluctuates from 10°C at night to 30°C in the day time [113]. No molecular cytogenetic and genetic investigations have been done for finding out of an hermaphroditic origin. The other question that remains open is whether factors (1), (2) and (3) synergically collaborate together or whether only one of these three factors is epistatically responsible for natural production of abnormal hermaphroditic phenotype in *Carassius* SPECIES. It is clear that temperature can influence both sex and hermaphroditism of *Carassius* but no exact range of environmental values responsible for sex reversal and hermaphroditism has been tested.

10. Perspective of a future research

Hybridization has often been viewed as a destructive force leading to hybrid sterility or non-viability but it is increasingly being recognized as a potential creative force in evolution because it can lead to an establishment of new genome combinations with the potential of clonal reproduction [114,115]. New HYBRIDS can be benefited by rapid adaptation to new environmental conditions and by a potential to occupy ecological niches where a primary reproductive barrier would be established between HYBRIDS and parental SPECIES [114,116]. Then HYBRIDS undergo hybrid speciation in which HYBRID SPECIES evolve. *Carassius* is in accordance with hybrid speciation hypothesis with several rounds of hybridization having occurred [65]. Cytogenetic analysis can reveal the exact ratio and identity of parental genomes within the hybrid genome but it is difficult to determine parental *Carassius* ancestors after several rounds of hybridization. Event though the determination of parental *Carassius* ancestors is under extensive investigation.

Genetic and environmental sex determination together with asexual and sexual mode of reproduction are natural phenomena which are hypothesized to be associated with transition between genetic and environmental sex determination, and asexual and sexual mode of reproductions [117]. For the study of transitions between sex determination mechanisms and modes of reproduction it will be needed to use cytogenetic, genomic, and gene editing framework. Next generation genomics (e.g., restriction site-associated DNA sequencing) and transcriptomics (e.g., RNA sequencing) are widely used as a valuable tool for identification of a non-recombining region, sex-specific single nucleotide polymorphism and/or differentially expressed genes [19,118–120]. In *Carassius* non-recombining region has been characterized in artificial population but additional analyses will be needed to determine differentially expressed genes in males/females and identify master sex determining gene which is on the top of the developmental cascade.

Then the FISH approach can map the sex determining genes on chromosomes [121]. Gene knockout using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) with Cas9 protein editing approach can reveal functionality respective non-functionality of master sex determining gene. There is the case of successful CRISPR/Cas9 knockout in *Carassius* [122]. The function of the *tyrosinase* gene was knocked out in *C. cuvieri* and *C. cuvieri* x *C. auratus* HYBRID and therefore melanin synthesis was suppressed. CRISPR/Cas9 knockout experiments were shown as a powerful tool for genetic engineering in aquaculture [122]. CRISPR/Cas9 could be used for knockout of a candidate gene for sex determination in *Carassius*. The one of candidate master sex determining genes has been proposed *anti-müllerian hormone* gene (*amh*). Eventually CRISPR/Cas9 could be a prospective tool for testing functionality of genetic mechanisms hidden beyond clonality and/or polyploidy as it has been similarly suggested in Yin et al. [123].

11. Conclusions

Carassius is a highly diverse COMPLEX involving several TAXA with different mitochondrial lineages [13], several ploidy levels [26], dual mode of reproduction, unisexual and bisexual populations [18,75], dual mode of sex determination [19], interspecies HYBRIDS [22] and obscure hermaphrodites [113]. In this review we highlighted *Carassius* as an experimental fish model to study evolution of above listed unusual and incredible phenomena using already well established molecular cytogenetics coupled with perspective genomics and genetic engineering.

Carassius COMPLEX involves two diametrically distinct entities: The first one is invasive and gynogenetic *C. gibelio* and the second one is vulnerable sexual *C. carassius*. The competition in ecological niche colonization favored *C. gibelio* owing to its quantital asexual reproduction mode avoiding mating costs. *Carassius carassius* is becoming endangered SPECIES in many regions [21,65,93,100]. To protect pure *C. carassius*, it is necessary to identify each individual at least using molecular cytogenetics (analysis of karyotype, in situ hybridization) and implement conservation programs of pure *Carassius* SPECIES [100,124]. Determination of the ploidy level tell us who is *Carassius* (Section 6) and we can reliably distinguish pure SPECIES from gynogenetic BIOTYPES/cryptic HYBRIDS. The proposed cytogenetic investigation would help to protect pure endangered *C. carassius* and understand evolution of the highly diverse *Carassius* COMPLEX.

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Abbreviations

The following abbreviations are used in this manuscript:

DAPI	4’,6-diamidino-2-phenylindole
FISH	Fluorescent in situ hybridization
GISH	Genomic in situ hybridization
NOR	Nucleolar organizer region
rDNA	Ribosomal DNA

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