

Tempo and mode of recurrent polyploidization in the *Carassius auratus* species complex (Cypriniformes, Cyprinidae)

J Luo^{1,6}, Y Gao^{2,6}, W Ma¹, X-y Bi¹, S-y Wang^{2,3}, J Wang¹, Y-q Wang¹, J Chai¹, R Du¹, S-f Wu², A Meyer⁴, R-g Zan¹, H Xiao¹, RW Murphy^{2,5} and Y-p Zhang^{1,2}

Polyploidization is an evolutionarily rare but important mechanism in both plants and animals because it increases genetic diversity. Goldfish of the *Carassius auratus* species complex can be tetraploids, hexaploids and octaploids. Polyploidization events have occurred repeatedly in goldfish, yet the extent of this phenomenon and its phyletic history are poorly understood. We explore the origin, tempo and frequency of polyploidization in Chinese and Japanese goldfish using both mitochondrial (mtDNA) and nuclear DNA sequences from up to 1202 individuals including the outgroup taxon, *Cyprinus carpio*. Analyses of *de novo* nuclear gene data resolve two clusters of alleles and the pattern supports the prior hypothesis of an ancient allotetraploidization for *Carassius*. Alleles shared by tetraploid and hexaploid individuals indicate recent autopolyploidizations within the *C. auratus* complex. Sympatric tetraploids and hexaploids share mtDNA haplotypes and these frequently occur independently within six well-supported lineages and sublineages on a small spatial scale. Gene flow estimates (F_{st} values) indicate that hexaploids differ only slightly from sympatric tetraploids, if at all. In contrast, allopatric populations of tetraploids and hexaploids differ from one another to a far greater extent. Gene flow between sampled localities appears to be limited. Coalescence-based time estimations for hexaploids reveal that the oldest lineage within any sampled locality is around one million years old, which is very young. Sympatric, recurrent autopolyploidization occurs in all sampled populations of the *C. auratus* complex. Goldfish experience polyploidization events more frequently than any other vertebrate.

Keywords: goldfish; autopolyploidization; tetraploid; hexaploid

INTRODUCTION

The frequency and hence the evolutionary importance of polyploidization for diversification differs greatly between plants and animals. More than 70% of angiosperms experienced one or more episodes of polyploidization and about 95% of fern species are polyploids (Soltis and Soltis, 1999; Van de Peer *et al.*, 2009). Notwithstanding, whole genome duplications have contributed to the evolutionary success of vertebrates. Extensive comparative genomic work reveals three rounds of whole genome duplication early in teleost diversification (Ohno, 1970; Vandepoele *et al.*, 2004; Van de Peer *et al.*, 2009). The genomes of all 25 000 or so species in this lineage of vertebrates reflect this whole genome duplication event. In fact, whole genome duplication appears to be causally related to their evolutionary success (Wittbrodt *et al.*, 1998). Generally speaking, polyploidization occurs only rarely in animals, especially in vertebrates, in part because sex determination and development are disrupted by polyploidization (Mable, 2004; Mable *et al.*, 2011). Among animals, polyploidization is common only

in some lineages of invertebrates such as ostracods and insects. Some species of fishes, amphibians and reptiles are polyploids and in some evolutionary lineages of fishes polyploidization is not uncommon (Dawley, 1989; Otto and Whitton, 2000; Leggatt and Iwama, 2003). Cyprinid fishes are of special interest in this regard because they are unusually rich in polyploidy lineages, several of which are ancient (Otto and Whitton, 2000), including the subfamilies Barbinae (Tsigenopoulos *et al.*, 2002), Cyprininae and Schizothoracinae (Yu *et al.*, 1989; Buth *et al.*, 1991; Le Comber and Smith, 2004). Full genomic duplications have occurred in at least 40 cyprinid species (Yu *et al.*, 1989; Buth *et al.*, 1991).

Recurrent polyploidization events occur either by interspecific hybridization and the fusion of two or more genomes (allopolyploidy) or by genome doubling within a species or a population (autopolyploidy). In plants, most cases of polyploidization occur via allopolyploidization, and this process is thought to be an important mechanism for the rapid formation of species (Soltis and Soltis, 1999;

¹Laboratory of Conservation and Utilization of Bio resources and Key Laboratory for Animal Genetic Diversity and Evolution of High Education in Yunnan Province, School of Life Sciences, Yunnan University, Kunming, Yunnan, China; ²State Key Laboratory of Genetic Resources and Evolution and Yunnan Laboratory of Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan, China; ³School of life sciences, University of Science and Technology of China, Hefei, Anhui, China; ⁴Lehrstuhl für Zoologie und Evolutionsbiologie, Department of Biology, University of Konstanz, Konstanz, Baden Württemberg, Germany and ⁵Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, Toronto, ON, Canada

⁶These authors contributed equally to this work.

Correspondence: Professor Y p Zhang, State Key Laboratory of Genetic Resources and Evolution and Yunnan Laboratory of Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China.

E mail: zhangyp1@263.net.cn

Hegarty and Hiscock, 2008; Van de Peer *et al.*, 2009; Hu *et al.*, 2011). Polyploidization may be involved in about 2–4% of the speciation events in angiosperms and ferns, respectively (Otto and Whitton, 2000), but only a handful of these cases are autopolyploidization events (Segraves *et al.*, 1999; Parisod and Besnard, 2007). In contrast, only in a single case of multiple autopolyploid origins among invertebrates is known: the lepidopteron *Solenbia triquetrella* (Psychidae) (Lokki and Saura, 1980). Little is known about the extent of multiple autopolyploid origins in vertebrates, and some possible cases are now believed to represent allopolyploidization (for example, Holloway *et al.*, 2006).

The genus *Carassius* has a recent, allotetraploid origin (Buth, 1983; Risinger and Larhammar, 1993; Yang and Gui, 2004; Luo *et al.*, 2006). Within *Carassius*, at least two species are recognized: *Carassius carassius* (the crucian carp) and *C. auratus*, a complex of several lineages (Table 1) that includes the silver crucian (gibel) carp and

Japanese ginbuna (silver crucian carp), nagobuna, nigorobuna and gengorobuna; the latter is sometimes considered to be the species *Carassius cuvieri* (Takada *et al.*, 2010 and therein). *C. carassius* occurs from the Ertis River in China westwards to Central and Eastern Europe and the British Isles (Luo and Yue, 2000; Wheeler, 2000); *C. carassius* may no longer occur in the Ertis River in China, based on our attempts to locate the species. *C. auratus* (Cypriniformes, Cyprinidae) is a complex of tetraploid and hexaploid fishes. Individuals within each lineage are morphologically similar yet their chromosome numbers and reproductive strategies vary. Except for exclusively tetraploid *C. cuvieri*, bisexual tetraploid and gynogenetic hexaploid and occasional octaploid forms occur sympatrically in China, Russia and Japan (Golovinskaya *et al.*, 1965; Chen *et al.*, 1996; Murakami *et al.*, 2001; Brykov *et al.*, 2002). Multiple levels of polyploidy occur in Fangzheng, Puan and Lake Dianchi, China (Zan, 1982; Zan *et al.*, 1986 and therein; Chen *et al.*, 1996 and therein).

Table 1 Breed, distribution, chromosome number and karyotype of the genus *Carassius*

Species, subspecies or local types	Distribution	Reproductive strategy	Male/female	Chromosome no.	Ploidy	Karyotype formula	Source
<i>Carassius carassius</i> (Crucian carp)	Central & Eastern Europe, British Isles	Sexual	Male, female	100	4n	?	Vinogradov, 1998
<i>C. auratus auratus</i> (Goldfish)	Euro Asia Continent	Sexual	Male, female	100	4n	22 M + 30SM + 48 T. ST 24 M + 30SM + 46ST T	Zan <i>et al.</i> , 1986; Wang <i>et al.</i> , 1988
<i>C. auratus gibelio</i> (Gibel carp)	Euro Asia Continent	Gynogenesis	Female	156 ±	6n	42 M + 74SM + 40ST T	Shen <i>et al.</i> , 1983
<i>C. auratus gibelio</i> (Gibel carp)	Euro Asia Continent	\	Male, female	162 ±	?	48 M + 56SM + 18ST + 40 T	Shan <i>et al.</i> , 1988
<i>C. auratus</i> (back high type)	Yunnan	Gynogenesis	Female mainly	162 ±	6n	33 M + 53SM + 76ST T	Zan, 1982
<i>C. auratus</i> (Suogu breed)	Hunan	Sexual	Female	156	4n	36 M + 36SM + 84ST	Yu <i>et al.</i> , 1987
<i>C. auratus</i> (Qihe breed)	Henan	Gynogenesis	?	156 ±	6n	?	Lou <i>et al.</i> , 1989
<i>C. auratus</i> (Puan breed, Form A)	Guizhou	Gynogenesis	Female	156 ±	4n	38 M + 28SM + 18ST + 72 T	Yu <i>et al.</i> , 1992
<i>C. auratus</i> (Pengze breed)	Jiangxi	Gynogenesis	Male, female	166 ±	4n	32 M + 40SM + 18ST + 76 T	Chen <i>et al.</i> , 1996
<i>C. a. langsdorfi</i> (Ginbuna)	Japanese Islands	Gynogenesis	Female	152–158	6n	34 M + 62SM + 60 T	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980
<i>C. s. a. langsdorfi</i> (Ginbuna)	Japanese Islands	Sexual	Male, female	100	4n	12 M + 36SM + 52 T	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980
<i>C. cuvieri</i> (Gengorobuna)	Japanese Islands	Sexual	Male, female	100	4n	12 M + 36SM + 52 T	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980
<i>C. auratus buergeri</i> (Nagabuna)	Japanese Islands	Sexual	Male, female	100	4n	12 M + 36SM + 52 T	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980
<i>C. a. buergeri</i> (Nagabuna)	Japanese Islands	Gynogenesis	Female	150 ±	6n	?	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980
<i>C. a. grandoculis</i> (Nigorobuna)	Japanese Islands	Sexual	Male, female	100	4n	12 M + 36SM + 52 T	Kobayashi, 1978; Ueda and Ojima, 1978; Ojima and Yamano, 1980

Abbreviations: M, metacentric; SM, submetacentric; ST, subtelocentric; T, telocentric.

The occurrence of allopatric polyploids questions may owe to two phenomena: independent origins or a rather ancient, single origin followed by dispersal to different drainage systems within Eurasia (Table 1). For *Carassius*, preference for one alternative over the other involves biogeography as well as the tempo and mode of polyploidization. (Takada *et al.*, 2010) and (Gao *et al.*, 2012) detailed the biogeography of the complex. Risinger and Larhammar (1993) and (Buth *et al.*, 1991 and therein) documented the mode of polyploidization; allozymic and paralog information identified allo- and autopolyploidization events. The tempo of polyploidization remains unknown.

Analyses involving both maternally inherited mitochondrial DNA (mtDNA) and nuclear genes can identify the maternal progenitor (Evans *et al.*, 2004) of polyploids. If two paralogs cluster in different clades and with different species, then allopolyploidization provides the best explanation. When this pattern does not occur, we cannot reject the null hypothesis of autopolyploidization. The mtDNA gene tree can identify the maternal progenitor within one of the nuclear gene clusters (Evans *et al.*, 2004).

The origin hexaploids, their population genetic features and their relationship to tetraploids remains unclear. Although initial reports suggested that all hexaploid *C. auratus* are females, males have been recently discovered in several populations (Lu *et al.*, 2006; our unpublished data). Hexaploids might have originated from tetraploid *C. auratus auratus* (Allendorf and Thorgaard, 1984). Normally, bisexual tetraploid fish do not exclude the second polar cell until fertilization (Gui *et al.*, 1995), but hexaploids possibly originate when the second diploid polar cell is not excluded from a tetraploid egg as a consequence of either hydrostatic pressure shock (Gui *et al.*, 1995) or heat/cold shock (Pandian and Koteeswaran, 1998). Most hexaploid individuals are females and they reproduce gynogenetically as sexual parasites; females require exogenic sperm to stimulate egg development (Gui, 2007). Because hexaploids are closely related to tetraploids, they can serve as a model system to explore the origin, history and mechanisms of autopolyploidization within species.

We use our published sequence data (Gao *et al.*, 2012) and those for the Japanese goldfish from GenBank along with *de novo* ploidy information for Eurasian *Carassius* to investigate the history and possible multiple origins of polyploidy within the *C. auratus* complex. If hexaploidization is rare and originated only once or very few times in independent drainages, we would expect hexaploids to be restricted to one or a few matriline (Supplementary Figure S1). Alternatively, if hexaploidization is not rare but rather independently reoccurs often, then hexaploids should be widespread on the tree (Supplementary Figure S1). We track the maternal history and population structure of tetraploid and hexaploid populations within the *C. auratus* complex using mtDNA sequence data, and then map polyploidization events onto the tree. Subsequently, we investigate the origin of the hexaploids by calculating population coalescence times for each lineage. We sequence three nuclear genes to investigate the characteristics of duplicated genomes and autopolyploidy within each species complex.

MATERIALS AND METHODS

Samples

Sampling included all taxonomically recognized forms of *C. carassius* and the *C. auratus* complex, the latter from its native distribution in mainland China. We downloaded all available mtDNA sequences in GenBank (Supplementary Tables S1–S3) and combined them with six *de novo* sequences to form two mtDNA data sets for constructing a matrilineal genealogy: control region (CR) and CR + *cytb*. The first data set consisted of 1202 mitochondrial CR sequences that comprised 237 unique haplotypes, including the outgroup taxa. This data set consisted of seven parts: (1) 340 sequences from Gao *et al.* (2012) plus six

de novo sequences (total 346) that were accompanied by genome sizes and/or number of chromosomes (Supplementary Table S1, part 1 and 2); (2) 18 gibel carp studied by Li and Gui (2008) (Supplementary Table S1, part 1); (3) six Japanese sequences from the study by Gao *et al.* (2012) along with 725 sequences they used from GenBank that had ploidy information (Supplementary Table S2); (Murakami *et al.*, 2001; Iguchi *et al.*, 2003; Takada *et al.*, 2010); (4) three samples of *C. cuvieri* (Supplementary Table S3); (5) three samples of *C. carassius* from the study by Gao *et al.* (2012); (Supplementary Table S3); (6) 94 haplotypes downloaded from GenBank without ploidy information; and (7) seven sequences from carp (*C. carpio*) for the outgroup (Supplementary Table S3). The second data set contained complete sequences of the gene encoding cytochrome *b* (*cytb*) plus CR for 180 individuals taken from the study by Gao *et al.* (2012) including all taxa of *C. carassius* and the *C. auratus* complex (Supplementary Table S3). Among these sequences, 1095 had ploidy information and these were used for population analyses. The remaining 107 sequences were used for constructing matrilineal trees only.

We investigated whether polyploidization in the goldfish occurred by either auto or allopolyploidization at the nuclear genomic level (Woods and Buth, 1984). We sequenced three nuclear genes (see below) from 15 individuals as follows: four individuals of *C. carassius*, one silver crucian carp, one tetraploid and one hexaploid goldfish each from Lake Dianchi, Zhejiang and Heilongjiang (six total), three gonggongbuna (*C. cuvieri*) and one common carp (*C. carpio*).

Ploidy determination

We used two methods to newly determine ploidy levels for 340 individuals from the study by Gao *et al.* (2012) plus six new individuals: karyotyping, following the study by Yu *et al.* (1989), and flow cytometry, as in the study by Luo *et al.* (2006). The chromosomal numbers of 167 individuals were visually determined from karyotypes either using photographs or under a microscope (Olympus, Tokyo, Japan). Flow cytometry used a fluorescence activated cell sorter (FACS Vantage SE System, BD Biosciences, San Jose, CA, USA) to estimate ploidy levels of 179 individuals. We modified the method of Luo *et al.* (2006) by staining blood cells for 10 min and using 1:10 concentration of domesticated goldfish blood cells: target specimen cells to standardize genome size estimates.

DNA extraction, PCR amplification and nucleotide sequencing

MtDNA fragment amplification and sequencing for six individuals with known ploidy levels were performed as in the study by Gao *et al.* (2012). We targeted the CR because of its high level of variability.

We used the following primers for amplifying and sequencing three nuclear genes: a partial fragment of the gene encoding macrophage migration inhibitory factor (*MIF*), 4MIF 5' RCGCCAAAATAARCAATACT 3', 4MIF 5' FAAGATGTTGTCTGCTGTAAG 3'; a partial fragment of the gene encoding steroidogenic acute regulatory protein (*StAR*), 2StAR 5' RGTGATGCTGGAA CAGAAGAC 3', 2StAR 5' FGCACAACGGCACTTACAAA 3'; and a partial fragment of the gene for growth hormone (*GH*) 5' GH U2TGCTGGTTAGTT TGTGGTG 3', GH 5' L2GCTCYTCTGYGTTTCATCTTT 3'. Amplifications were performed using the Gene Amp PCR system 9700 (ABI, Foster City, CA, USA). The total PCR volume was 25 μ l with final concentrations of 1x buffer containing 0.15 mM MgCl₂ (TaKaRa, Dalian, China), 0.25 mM dNTPs (TaKaRa), 1U Taq DNA polymerase (TaKaRa) and 25 50 ng total DNA. Following an initial 5 min denaturing step at 94 °C, PCR comprised 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C 30 s, followed by a final extension at 72 °C for 7 min. The corresponding PCR products were purified on agarose gels and extracted using a kit (Watson BioMedical Inc., Shanghai, China). Subsequently, the purified products were ligated and cloned with the pUC18 DNA (TaKaRa). Each clone was picked individually from a Luria broth (LB) plate and cultured in LB liquid medium. Plasmid DNA was manually isolated via the standard alkaline lysis miniprep method. The plasmid DNA was sequenced using an ABI 3730 with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions. The following universal primers were used for sequencing the plasmids: BOO12 5' CGCCAGGGTTTTCCAGTCACGAC 3' and BOO13 5' AACAGCTATGACCATG 3'.

Data analysis

We added 21 CR haplotypes from downloaded sequences to the 216 haplotypes of Gao *et al.* (2012) and constructed genealogies. Ploidy levels were then mapped onto the trees, and the proportions of ploidy were also shown.

MtDNA variation and divergence within and between populations of different ploidy levels were explored using several indices as implemented in ARLEQUIN 3.5 (Excoffier *et al.*, 2005). Between population F_{st} (Reynolds *et al.*, 1983; Slatkin, 1995) and within population haplotype diversity (H) and nucleotide diversity (π) (Nei, 1987) were assessed. Neutrality tests estimated population stability, expansion and bottlenecks (Tajima, 1989; Fu, 1997). Coalescence time (T_{co}) and the maternal effective population size (N_{ef}) (Ruvolo, 1997) were calculated using a generation time (t) of 2y because the sexual maturity of the wild goldfish was shown to be 1 2y (Lorenzoni *et al.*, 2007). The frequency of polyploidy events was estimated by evaluating the number of shared haplotypes and the coalescence time (T_{co}) between the most divergent haplotypes.

The three nuclear gene sequences were aligned with DNASTAR 5.0 (DNASTAR Inc. Madison, WI, USA) and refined by eye. DAMBE 4.1.19 (Xia and Xie, 2001) was used to identify shared haplotypes, and MEGA 4.1 (Tamura *et al.*, 2007) was used to extract information on variable sites. These fragments were used to reconstruct polyploidization events in *Carassius*. Maximum parsimony (MP) analyses were implemented in PAUP* 4.0b10 (Swofford, 2002), maximum likelihood (ML) used RAxML (Stamatakis *et al.*, 2008) and Bayesian inference (BI) was implemented in MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). Best fitting models for the BI and ML analyses used likelihood ratio tests (Goldman, 1993; Huelsenbeck and Crandall, 1997) as implemented in the jMODELTEST 0.0.1 (Posada, 2008). For BI, four independent MCMC chains were simultaneously run for 5 000 000 generations while sampling one tree per 500 replicates, Burnin 0, and Burninfrac 0.5 (0.1 or 0.2 and so on), with two runs conducted independently. Sampled trees were used to construct a 50% majority rule consensus tree. Frequency of nodal resolution was termed a Bayesian posterior probability (BPP) and these values were mapped onto the consensus tree. Nodal support in the MP and ML analyses was assessed using nonparametric bootstraps (BS) (Felsenstein, 1985) calculated for MP in PAUP* and for ML in RAxML with 1000 replicates each. We assessed the robustness of our genealogy by comparing trees obtained from MP, ML and BI analyses.

RESULTS

Ploidy level and sequence variation

We successfully determined the ploidy levels of 346 fishes from mainland China and to this we added 18 samples from Li and Gui (2008) (total 364; Supplementary Table S1 part 1). *C. auratus* complex had 57 tetraploids and 146 hexaploids from Yunnan (Southwest China), 27 hexaploids from Guizhou (Southwest China), 23 tetraploids and 21 hexaploids from Zhejiang (East China), 5 tetraploids and 6 hexaploids from Hunan (East China), 14 hexaploids from Guangdong (East China) and 29 tetraploids and 36 hexaploids from Heilongjiang (Northeast China). In total, these included 114 tetraploids and 250 hexaploids (Supplementary Table S1). Further, the ploidy levels of 725 Japanese fish were previously determined (Supplementary Table S2; Murakami *et al.*, 2001; Iguchi *et al.*, 2003; Takada *et al.*, 2010). For six Japanese individuals whose CR sequences were used by (Gao *et al.*, 2012), we determined their ploidy levels.

For mitochondrial regions, the 320 426 nucleotide sites of partial CR sequences from 1202 fishes yielded 237 haplotypes (Supplementary Table S3). In the CR sequences, 130 nucleotide positions varied of which 106 were potentially parsimony informative and 24 were invariant. Uncorrected pairwise p distances between haplotypes ranged from 0.24 to 11.52%. Within *Carassius*, p distances averaged 3.84%. The majority of substitutions were transitions and

among ingroup representatives the transition/transversion ratio averaged 6.57. Inclusion of the outgroup dropped the transition/transversion ratio to 5.01. Pairwise p distances between *Carassius* and *Cyprinus* ranged from 10.53 to 17.63%, averaging 13.72%. Both genera were distinct genetically.

Gao *et al.* (2012) sequenced *cytb* for 104 selected samples (accession no. GU135503 GU135605; NC 010768) to resolve deeper level relationships than those revealed from CR data. We mapped our ploidy information onto their matrilineal genealogy.

Sequencing of the three nuclear genes used a total of 15 individuals including one common carp as the outgroup. Among the 269 clones sequenced for *GH*, 43 alleles were identified (accession no. JX406582 406620, KC462749 KC462762), including 2 6 alleles belonging to two copies in each individual. Thirty two alleles were identified from 272 clones of *StAR* (accession no. JX406621 406650, KC462770 KC462782), including 2 5 alleles belonging to two copies in each individual. Finally, for *MIF*, 32 alleles were identified from 269 sequenced clones (accession no. JX406651 JX406681, KC462763 KC462769), including 1 8 alleles belonging to at least two copies in each individual (Supplementary Table S4). There were 75/65, 62/62 and 92/23 potentially parsimony informative/uninformative nucleotide positions in *GH*, *MIF* and *StAR*, respectively. Phylogenetic analyses recovered two major groups each for *GH* and *StAR* (Figure 1). In *GH*, the maximum and minimum distances within group I were 4.5% and 0.3%, respectively, and within group II 4.1% and 0.3%, respectively. In *StAR*, the maximum and minimum distances within group I were 5.3% and 0.3%, respectively, and within group II 4.6% and 0.3%, respectively. Only one group was recognized for *MIF*, and the maximum and minimum distances were 5.2% and 0.3%, respectively. Distances for *GH* between ingroup sequences and carp sequences varied from 13.7 to 11.3% in group I, and 13.2 to 6.1% in group II. For *StAR*, distances ranged from 12.2% to 5.0% in group I and from 13.4 to 3.5% in the group II. Finally, within from *MIF* distances ranged from 23.2 to 9.1%.

Autopolyploidization or allopolyploidization based on nuclear genes?

Analyses of *GH* and *StAR* obtained very similar sets of relationships. Both trees depicted two primary clusters and one or two outgroup alleles occurred in at least one of the ingroup clusters (Figure 1). Further, within one cluster (Figure 1), the topology was similar to that derived from the mtDNA data: *C. carassius* diverged first, and the Japanese gengorobuna, the gibel carp and the sympatric Chinese tetraploids and hexaploids followed this in sequence. Finally, for all sampling localities, alleles of hexaploid individuals did not cluster together, not even within the collecting sites including Yunnan (Lake Dianchi), Zhejiang and Heilongjiang (Figures 1a and b). Unlike for *GH* and *StAR*, the tree derived from sequences of *MIF* did not resolve two distinct groups (Figure 1c) even after either including or deleting gaps in the phylogenetic analyses. Within each locality, hexaploids independently nested with sympatric tetraploids.

Maternal genealogy of the *C. auratus* complex

Matrilineal relationships of *Carassius* reconstructed using BI from 237 haplotypes of CR were used to visualize the distribution of the tetraploid and hexaploid lineages (Figure 2). In comparison, MP and ML analyses produced very similar topologies for the main lineages except for the relative positions of *C. carassius* and the Japanese gengorobuna. In the BI and ML trees, *C. carassius* formed the sister group of *C. auratus*, yet in the MP tree, the Japanese gengorobuna diverged first followed by *C. carassius* and then the other forms of

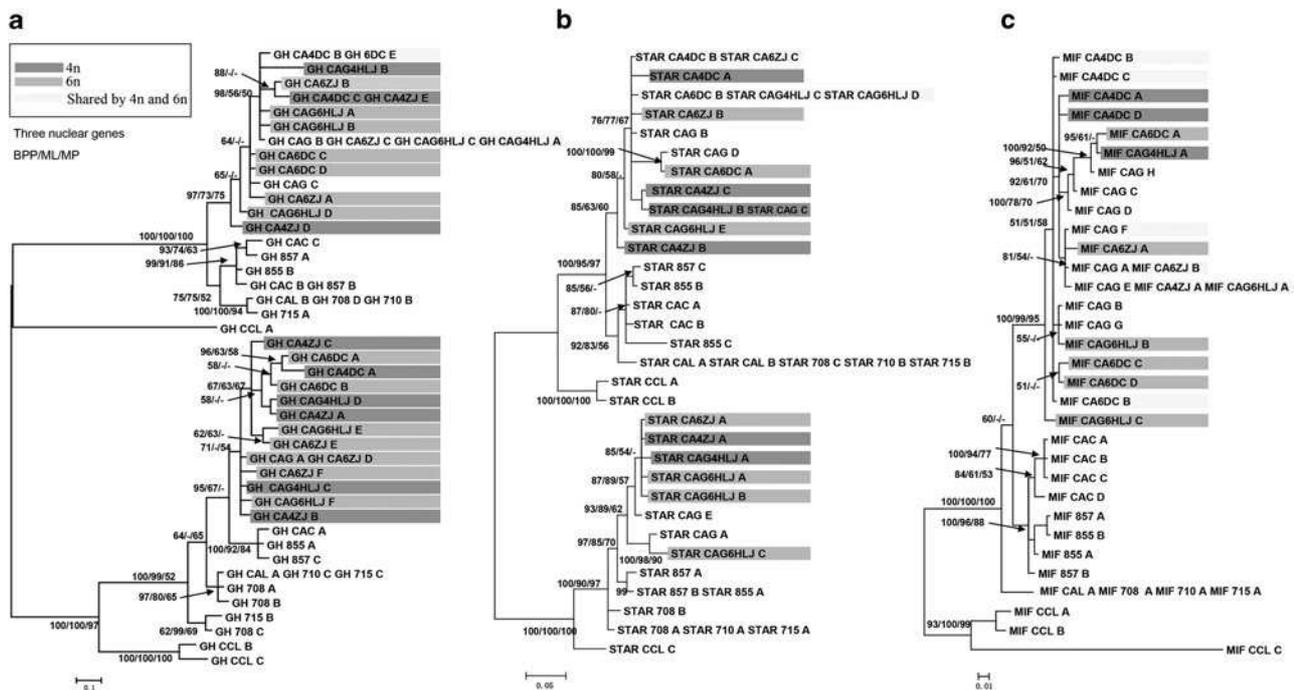


Figure 1 BI 50% majority rule consensus trees for three nuclear genes, *GH* (a) *StAR* (b) and *MIF* (c) indicate autopolyploidization within the genus *Carassius*. Numbers represent nodal support inferred from BPPs, ML bootstrapping and MP bootstrapping, respectively. In all genes, most sequences from sympatric tetraploid and hexaploid fishes clustered together. *GH* and *StAR* had two major groups, which nested with carp alleles and indicated an earlier round of allotetraploidization in the ancestor of goldfish and carp. Within each cluster of alleles for *GH* and *StAR*, sympatric tetraploid and hexaploid individuals share the same alleles (in yellow), a result of autopolyploidization. A full color version of this figure is available at the *Heredity* journal on line.

C. auratus. The conflicting nodes were not well supported. The Japanese gengorobuna rooted at the base of the BI tree with strong support (Figure 2; BPP = 100%; BS = 100%). Next, *C. carassius* from Central Europe and Russia branched off (Figure 2; BS = 93 100%). The remaining samples of the *C. auratus* complex formed five highly supported groups.

Analysis of the concatenated (CR + *cytb*) sequences resolved the main nodes of the CR tree and with stronger support (Figure 3). *C. carassius* split first and with high support (Figure 3; BPP = 100%; BS = 100%). Sequentially, the next lineages included the Japanese gengorobuna and ginbuna (lineages A and B, Figure 3), both of which received high bootstrap support. Compared with the CR tree, six sublineages were revealed in lineage C (sublineages C1–C6, Figure 3), excluding the Japanese ginbuna.

Hexaploid lineages in the *C. auratus* complex

Hexaploids were scattered on the maternal genealogies based on both CR (Figure 2) and the concatenated data (CR + *cytb*) (Figure 3). In the Japanese goldfish, the hexaploids mapped onto two highly supported branches. Similarly, hexaploid *C. auratus* were scattered among the six sublineages. This distribution of hexaploids received high nodal support and various estimated dates of origin. Thus, hexaploids had multiple independent origins in both the *C. auratus* complex and the Japanese ginbuna; at least six polyploidization events were identified in well supported matrilineages.

The proportion of tetraploids/hexaploids was summarized on sublineages C2, C5, C6, Japanese ginbuna and goldfishes from Anhui, Fujian and south central Ryukyus (Figure 2). The mapping depicted two clear features. First, tetraploids and hexaploids were intermixed among lineages. Hexaploidy occurred repeatedly. Second, tetraploids

and hexaploids, and even octaploids, shared the same haplotypes. In sublineages C2, C5 and C6, 18 haplotypes (marked in yellow in Figure 2; Supplementary Tables S1 and S2) were shared among tetraploid and hexaploids and these different haplotypes were substantially diverged from one another. The most divergent haplotypes, h39 and h41, differed by 12 substitutions yet both contained tetraploid and hexaploid individuals. In total, 15.38% (18/117) of the haplotypes were shared by tetraploid and hexaploid samples in sublineages C2, C5 and C6 (Figure 2; Supplementary Tables S1 and S2). In Yunnan, China, 12 of 20 (60%) haplotypes of the hexaploids were shared with tetraploids, and 93.84% (137/146) of the hexaploid individuals shared one of the 12 haplotypes. In the Japanese samples, haplotype CR58 was shared between tetraploids and hexaploids in sublineages C5 and C6. Goldfish from Japan and the Kyusyu Islands exhibited a similar pattern; 22 of 72 haplotypes (30.56%) were shared by tetraploids and hexaploids, including one haplotype, ht2 occurred in tetraploid, hexaploid and octaploid individuals (Figure 2; Supplementary Table S2).

Genetic variation within/between populations of differing ploidy

Levels of variation in CR for tetraploid and hexaploid individuals were estimated after assigning populations to China, Kyusyu and Japan (Table 2). Among Chinese *C. auratus*, hexaploid haplotypes (40) were more common than tetraploid haplotypes (35) (Table 2). Similarly, hexaploid haplotypes (5) outnumbered tetraploid haplotypes (41) in Japan, including the Kyusyu Islands (Table 2; Supplementary Table S2). Similarly, hexaploid populations showed higher levels of polymorphism than did the tetraploids within China ($H_{4n} = 0.9360 \pm 0.0109$; $H_{6n} = 0.9413 \pm 0.0048$), Japan ($H_{4n} = 0.9252 \pm 0.0097$; $H_{6n} = 0.9417 \pm 0.0074$) and the Kyusyu

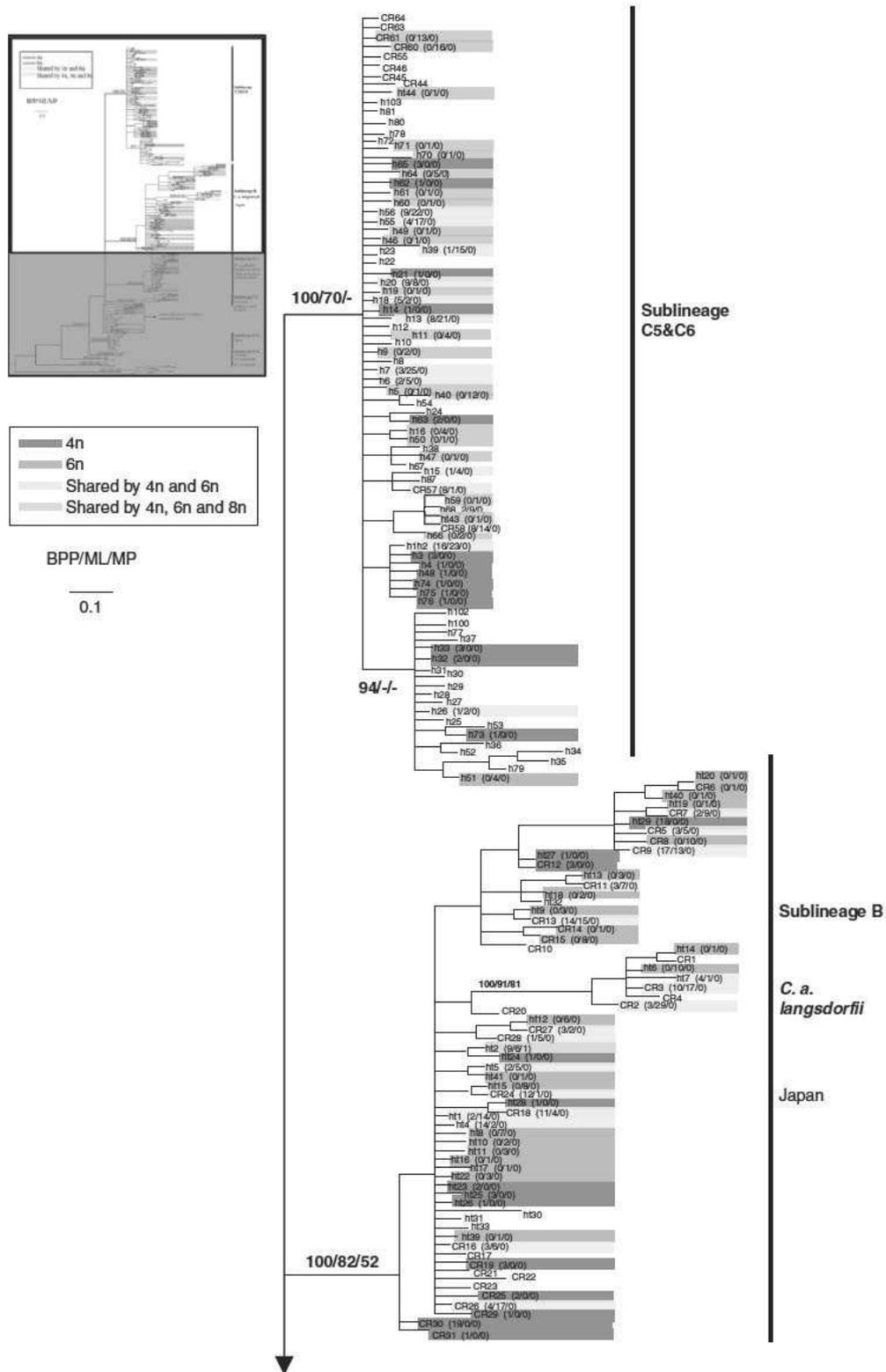


Figure 2 BI 50% majority rule consensus tree depicting the matrilineal relationships within *Carassius* based on 320 426 nucleotides positions of mtDNA CR sequences. Numbers represent nodal support inferred from BPPs, MLBS and MPBS, respectively. Branch support values are given for major lineages only. Colors indicate ploidy levels. Haplotype name is followed by the number of tetraploid, hexaploid and octaploid individuals. *C. carpio* formed the outgroup. A full color version of this figure is available at the *Heredity* journal online.

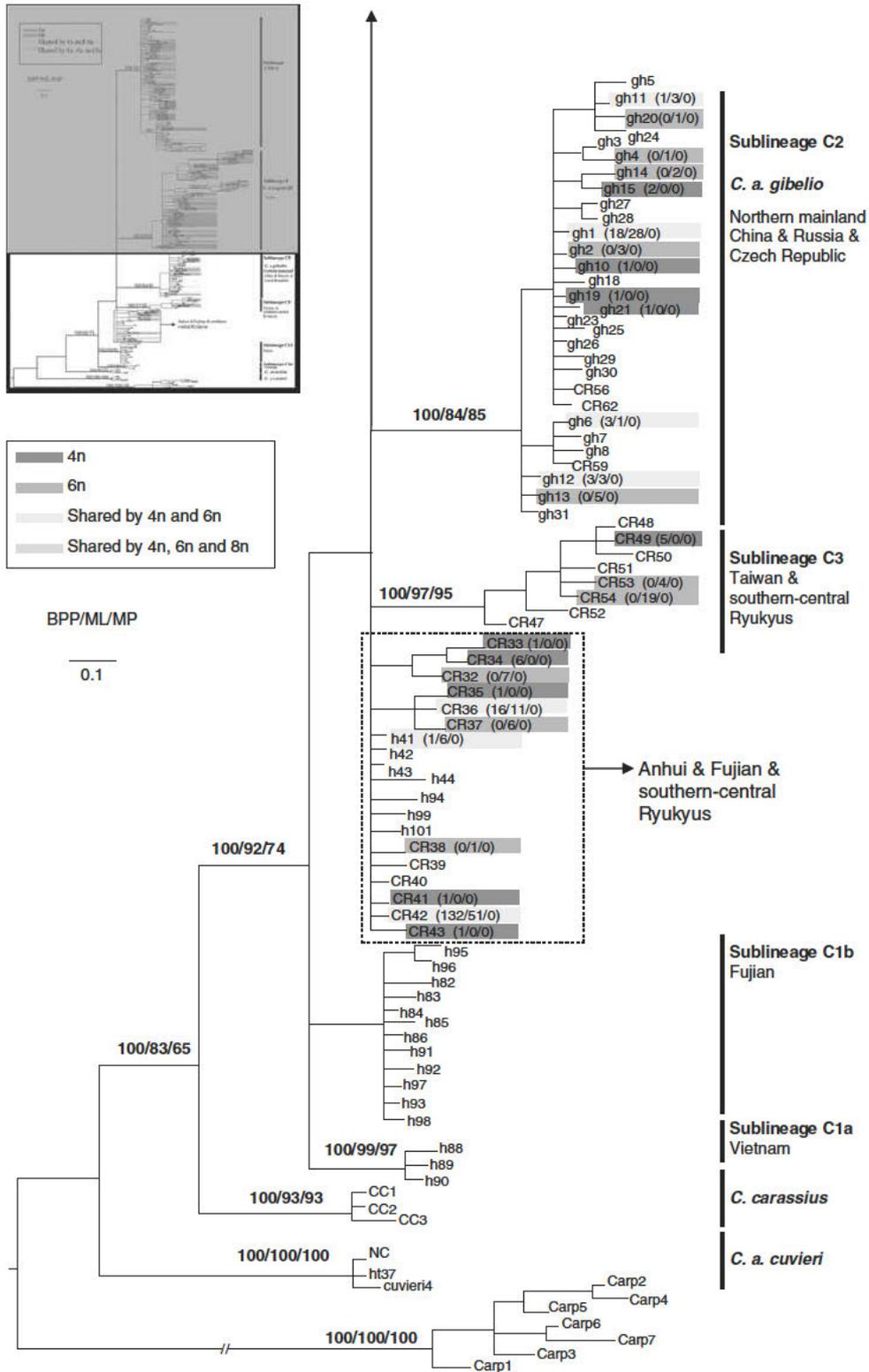


Figure 2 Continued.

Islands ($H_{4n} = 0.6720 \pm 0.0325$; $H_{6n} = 0.8896 \pm 0.0134$). The same patterns occurred when all data were combined.

Estimated coalescence times for Chinese hexaploids were slightly younger than their sympatric tetraploids and the inverse pattern occurred for populations from the Kyusyu Islands

(Table 2). Estimated coalescence times for tetraploids and hexaploids from Japan were the same as were those for all three groups combined. Thus, female effective population size (Table 2) was concordant with the estimated coalescence times.

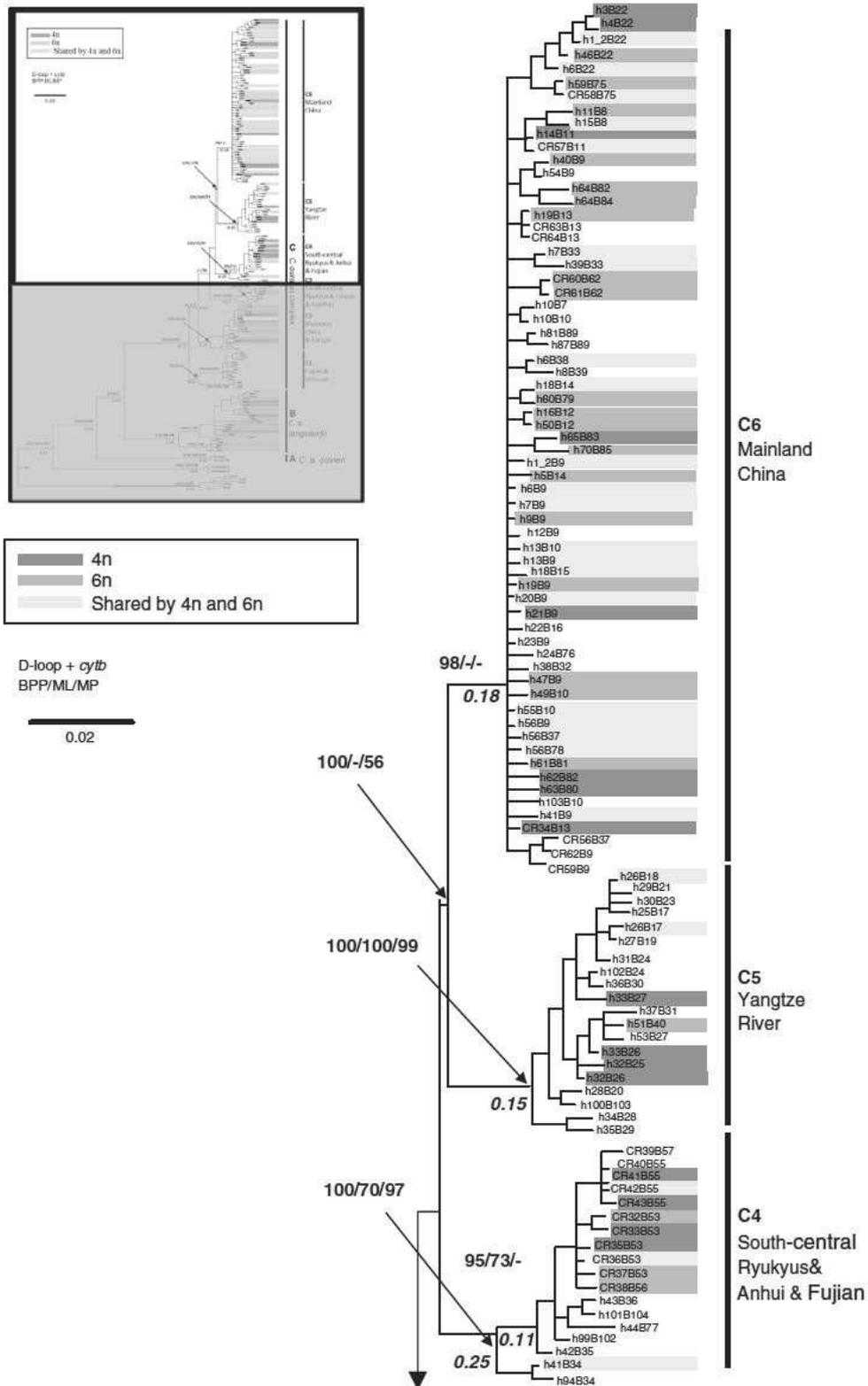


Figure 3 BI 50% majority rule consensus tree depicting the matrilineal relationships within *Carassius* based on 426 bp from the mitochondrial CRplus 1141 bp from the complete gene encoding cytochrome b. Numbers represent nodal support inferred from BPPs, MLBS and MPBS, respectively. Branch support values are given for major lineages only. Colors indicate ploidy levels. Haplotype name is followed by the number of tetraploid, hexaploid and octaploid individuals. *C. carpio* formed the outgroup. A full color version of this figure is available at the *Heredity* journal online.

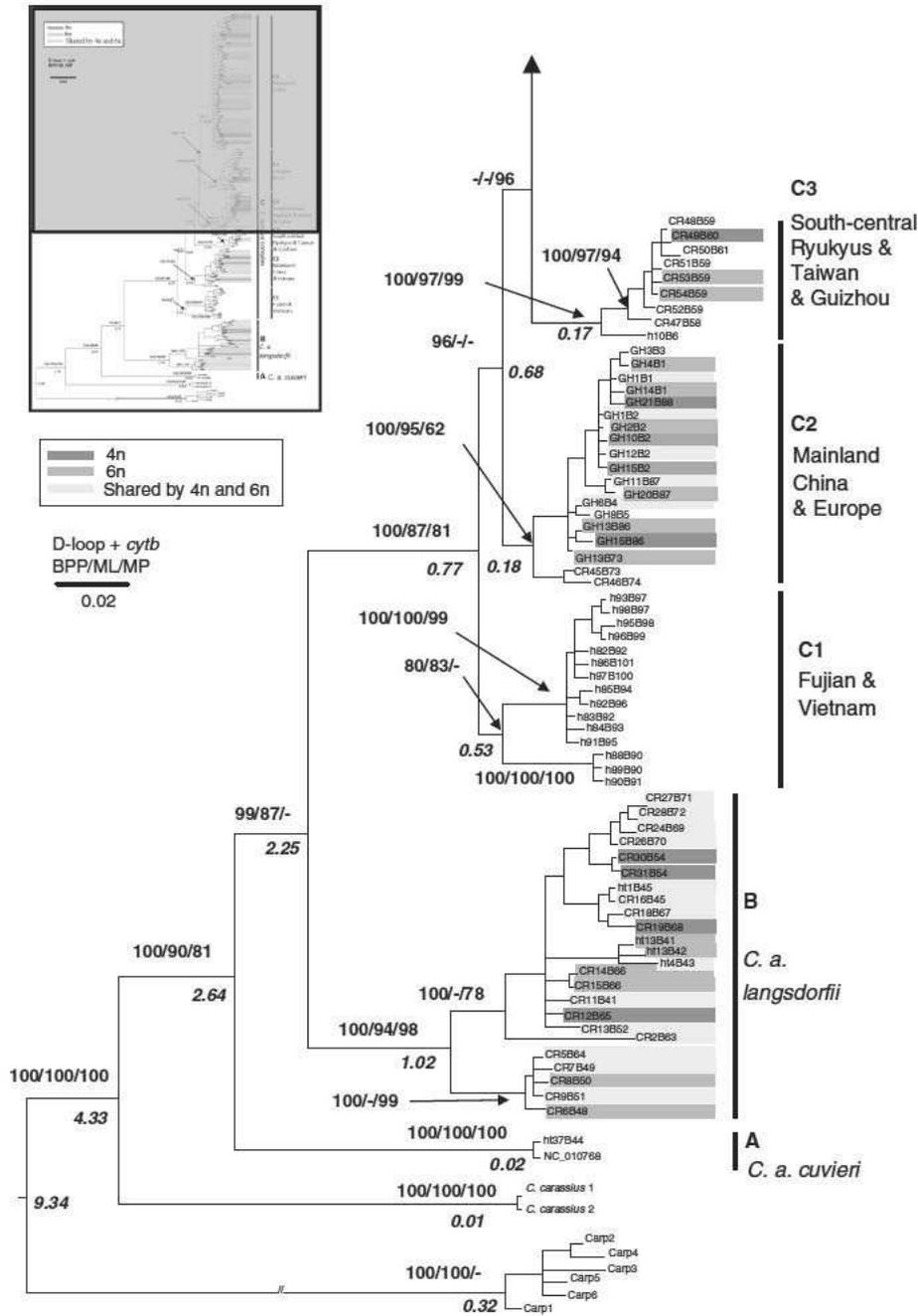


Figure 3 Continued.

We evaluated the possibility of population expansion by assuming that the 39 (17 in China plus 22 in Japan) incidences of haplotypes being shared by tetraploids and hexaploids represented independent polyploidization events. Subsequently, we estimated the frequency of polyploidization by coalescence times. The suggested coalescence time for the 17 Chinese populations with hexaploid individuals was $T_{co} = 0.94 \times 10^6$ years (Table 2), that is, a rate of at least 0.55 polyploidization events per 10^5 years. For the 14 populations on the main islands of Japan, the coalescence time was $T_{co} = 1.18 \times 10^6$ years (Table 2), or a minimum rate of 0.84 polyploidization events per 10^5 years. Finally, the eight occurrences of hexaploids on the Ryukyu Islands had $T_{co} = 1.13 \times 10^6$ year (Table 2), or 1.41 events per 10^5

years. Thus, population expansion was unlikely to explain the pattern. Further, a recent population expansion was not identified by using either the Tajima's D or Fu's neutrality test among the different ploidy levels of either the Chinese or the Japanese populations. Populations in the species complex appeared to be established and stable.

When individuals were assigned to one of three populations (China, Kyusyu and Japan), F_{st} values indicated that most sympatric tetraploid and hexaploid populations were more similar to each other than to individuals in non sympatric populations (Table 3). The F_{st} values of sympatric tetraploid/hexaploid populations in China, Ryukyu Islands and Japan were less than 0.140. Except for Chinese tetraploids and hexaploids from the Kyusyu Islands, for which

Table 2 Genetic diversity of tetraploids and hexaploids in the *C. a. auratus* complex based on mitochondrial control region, defined by lineage (1) and sympatric and non-sympatric tetraploid and hexaploid populations (2)

	N	Haplotype (HN)	Polymorphic sites (PN)	Gene diversity (H)	Nucleotide diversity (π)%	T_{co} (years)	N_{ef}
(1)							
C5 and C6 4n	91	27	18	0.9316 ± 0.0122	0.8846 ± 0.5233	3.77 × 10 ⁵	9.43 × 10 ⁴
C5 and C6 6n	322	35	25	0.9044 ± 0.0102	0.8110 ± 0.4834	4.24 × 10 ⁵	1.06 × 10 ⁵
subB 4n	162	30	25	0.9357 ± 0.0065	1.9364 ± 1.0265	6.60 × 10 ⁵	1.65 × 10 ⁵
subB 6n	215	38	25	0.9506 ± 0.0054	2.0829 ± 1.0944	6.60 × 10 ⁵	1.65 × 10 ⁵
C2 4n	30	8	7	0.6322 ± 0.0959	0.2731 ± 0.2024	2.36 × 10 ⁵	5.89 × 10 ⁴
C2 6n	47	9	8	0.6318 ± 0.0766	0.2819 ± 0.2068	2.83 × 10 ⁵	7.08 × 10 ⁴
(2)							
<i>C. a. auratus</i> 4n (CH)	114	35	37	0.9360 ± 0.0109	1.9912 ± 1.0286	0.99 × 10 ⁶	2.48 × 10 ⁵
<i>C. a. auratus</i> 6n (CH)	250	40	39	0.9413 ± 0.0048	1.7456 ± 0.9069	0.94 × 10 ⁶	2.36 × 10 ⁵
<i>C. a. auratus</i> 4n (KY)	238	18	36	0.6720 ± 0.0325	2.0887 ± 1.0967	1.08 × 10 ⁶	2.70 × 10 ⁵
<i>C. a. auratus</i> 6n (KY)	193	18	34	0.8896 ± 0.0134	3.2436 ± 1.6543	1.13 × 10 ⁶	2.83 × 10 ⁵
<i>C. a. auratus</i> 4n (JAP)	114	23	24	0.9252 ± 0.0097	2.1181 ± 1.1159	1.18 × 10 ⁶	2.95 × 10 ⁵
<i>C. a. auratus</i> 6n (JAP)	185	38	39	0.9417 ± 0.0074	2.2277 ± 1.1642	1.18 × 10 ⁶	2.95 × 10 ⁵
<i>C. a. auratus</i> 4n (All)	466	76	53	0.9064 ± 0.0110	3.1954 ± 1.6195	1.13 × 10 ⁶	2.83 × 10 ⁵
<i>C. a. auratus</i> 6n (All)	628	93	57	0.9750 ± 0.0017	3.7081 ± 1.8612	1.13 × 10 ⁶	2.83 × 10 ⁵

Abbreviations: CH, China; JAP, Japan; KY, Kyusyu; N_{ef} , estimated effective population size; T_{co} , estimated time to coalescence.

Table 3 Pairwise F_{ST} between (1) different lineages and (2) sympatric and non-sympatric tetraploid and hexaploid populations

(1)	C5 and C6 4n	C5 and C6 6n	sub 4n	sub 6n	C2 4n	C2 6n
C5 and C6 4n	\					
C5 and C6 6n	0.05951**	\				
subB 4n	0.72781**	0.77716**	\			
subB 6n	0.70736**	0.75823**	0.03692**	\		
C2 4n	0.83776**	0.82471**	0.66209**	0.66848**	\	
C2 6n	0.84308**	0.82348**	0.67655**	0.67913**	0.00281	\
(2)						
	China 4n	China 6n	Kyusyu 4n	Kyusyu 6n	Japan 4n	Japan 6n
China 4n	\					
China 6n	0.01413	\				
Kyusyu 4n	0.39061**	0.41156**	\			
Kyusyu 6n	0.21249**	0.25333**	0.14008**	\		
Japan 4n	0.60135**	0.62542**	0.40157**	0.31834**	\	
Japan 6n	0.59791**	0.62181**	0.40037**	0.32612**	0.03397**	\

Note: ** denotes $P < 0.000$.

$F_{st} = 0.212$, all other values of F_{st} of non sympatric tetraploid/hexaploid exceeded 0.253, and most were greater than 0.318. Non sympatric tetraploid/hexaploid populations showed higher pairwise divergence than those in sympatry.

DISCUSSION

Genealogical relationships and recurrent polyploidy

C. carassius (Linnaeus 1758) and *C. auratus* (Linnaeus 1758) are clearly distinguished only in the total evidence mtDNA tree (Figure 3); they are resolved as polyphyletic (polygenealogical sensu Murphy and Méndez de la Cruz, 2010) in the CR trees because the Japanese gengorobuna splits earlier than *C. carassius* (Figure 2). Analyses of nuclear *GH* and *StAR* resolve the Japanese gengorobuna as a unique lineage following after *C. carassius* (Figures 1a and b). In contrast, analyses of *MIF* suggest that the Japanese gengorobuna diverged before *C. carassius* (Figure 1c). These inconsistent results suggest that these two lineages might have diverged near

simultaneously. Irrespective of the particular topologies of the trees, the Japanese gengorobuna has been treated as a species, *C. cuvieri*. *C. carassius* has 100 chromosomes (Vinogradov, 1998) as does the Japanese gengorobuna, along with its diagnostic C banding pattern (Ueda and Ojima, 1978). Considering that both species are tetraploids and sequentially root at the base of the tree (Figures 2 and 3), their common ancestor appears to be a tetraploid (Vinogradov, 1998).

The taxonomic classification of the *C. auratus* complex is complicated (Figures 2 and 3). Other than the Japanese gengorobuna, two main lineages exist: lineage B (Japanese ginbuna) and a lineage C (mainly from China and the Kyusyu Islands). Lineage C contains six sublineages (Gao *et al.*, 2012). Recurrent polyploidizations occur within both lineages and tetraploid and hexaploid individuals frequently share identical haplotypes. Every case implies the independent origin of a hexaploid. Several hexaploid lineages of *C. auratus* occur in China, for example, Lake Dianchi (Luo *et al.*, 1999), and in these cases they are most closely related to local tetraploids (Zan *et al.*, 1986 and

therein; Zhang *et al.*, 1998 and therein). Curiously, hexaploids from Guizhou cluster with both local tetraploid *C. auratus* and the gibel carp. This population contains mixed, divergent lineages. Regardless, at this locality the hexaploids are not monogenealogical (*sensu* Murphy and Méndez de la Cruz, 2010); the mtDNA data show that they do not share a single common maternal ancestor.

Hexaploid *C. a. auratus* show greater variation than tetraploids. Hexaploid individuals appear to be more common than tetraploids in nature, as suggested by previous collections (Xiao *et al.*, 2011). Kleptogenesis – the temperature dependent incorporation of sperm in otherwise gynogenetic polyploids (Bogart *et al.*, 2007) may be a contributing factor. Alternatively, unidirectional gene flow and recent expansion of the hexaploid populations of *C. auratus* might also promote higher levels of polymorphism in the hexaploid goldfish. These possibilities require further testing.

In Japan, both tetraploid and hexaploid forms exist in the ginbuna (Murakami *et al.*, 2001; Takada *et al.*, 2010 and therein). Because gene flow can only occur between tetraploids, the mixture of tetraploids and hexaploids indicates additional recurrences of polyploidization (Figures 2 and 3).

The tempo, mode and origin of tetraploid and hexaploid *Carassius*

The ancestor of *Carassius* is most likely a tetraploid carp (Buth *et al.*, 1991 and therein; our analyses). All *Carassius* have at least 100 chromosomes, twice as many than the standard cyprinid karyotype, and their genome sizes are also about twice as large as diploid cyprinids (Ohno and Atkin, 1966; Ohno, 1970; Yu *et al.*, 1989; Risinger and Larhammar, 1993). The initial tetraploidization might have been an allopolyploidization event that occurred about 14.2–14.5 Ma, as estimated from nuclear gene studies (Risinger and Larhammar, 1993; Yang and Gui, 2004; Luo *et al.*, 2006). Our analyses from both mitochondrial and nuclear data place tetraploid *C. carassius* and Japanese *C. cuvieri* at the base of the tree for *Carassius*. Analyses of *GH* and *StAR* also support a model of allopolyploidization of the genus because at least one allele of each gene clusters within the outgroup carp, and two clusters are indicated (Figures 1a and b). Hence, our results confirm the assumption of allotetraploidization of the genus *Carassius*. Analyses of the nuclear gene data cannot reject the hypothesis of recurrent polyploidization via autopolyploidization because we did not detect either *C. carassius* or *C. cuvieri* within the *C. auratus* complex. For example, tetraploid and hexaploid individuals share localized alleles within clusters of both *GH* and *StAR* (Figures 1a and b) only in the *C. auratus* complex. Thus, ancient allopolyploidization and recent autopolyploidization are indicated for *Carassius*.

The tempo indicates that recurrent autopolyploidization occurred after the origin of the *C. auratus* complex. Gao *et al.* (2012) estimated divergence time of *C. carassius* and the *C. auratus* complex to be 3.6–4.0 Ma. Hexaploids in different geographic regions are younger than the two species themselves (Table 2) and no recurrent event occurred in their common ancestor. Analyses suggest a rate of about 0.55–1.41 mutation events per 10^5 years.

The most intriguing discovery lies in the ubiquitous, recurrent hexaploidization. The mapping of polyploids on the matrilineal genealogy precludes the possibility of one polyploidization event (Supplementary Figure S1). The nuDNA allele tree, matrilineal genealogy, coalescence times and scale of divergence in sympatric/non sympatric tetraploid/hexaploid individuals indicate that the hexaploidization has several independent origins (Figures 2 and 3; Tables 2 and 3). Shared mtDNA haplotypes reject the hypothesis of a single origin for the hexaploids and in doing so they support the alternative hypothesis of multiple formations of polyploids.

Convergent mutations can explain a single origin but two lines of evidence reject this possibility. First, the absence of saturated substitutions precludes convergence as an explanation. Second, because these unisexual fishes are gynogenetic, divergent $4n$ and $6n$ individuals should occur within both sister lineages if the common ancestor was a hexaploid; this does not occur in any of the incidences of sympatry. Shared haplotypes occur in divergent lineages that differ by up to 13 polymorphic sites. Independent polyploidization events best explain the numerous co occurrences of tetraploids and hexaploids.

Laboratory experiments identify some possible mechanisms for hexaploidization. Hydrostatic pressure, cold and heat shock following fertilization results in the retention of the second polar cell. When this occurs, three nuclei combine yielding an additional set of chromosomes and a hexaploid individual (Gui *et al.*, 1995; Pandian and Koteeswaran, 1998). Thus, possible environmental perturbations may promote the formation of hexaploids. Variation in hydrostatic pressure or temperature is a potential factor and environmental data and future analyses may confirm this possibility. Regardless, after formation, hexaploids reproduce gynogenetically.

The frequent dispersal of historically isolated populations might facilitate mixture of the tetraploids and hexaploids. This scenario does not appear to be true for *Carassius*. Geographic structure exists in both Japan and mainland China (Takada *et al.*, 2010; Gao *et al.*, 2012). Migration does not seem to have blurred the geographic structure and, again, the evidence suggests site specific independent polyploidization events. This mechanism may explain elevated levels of variation in hexaploids; hexaploids would have two paternal contributions.

Gynogenesis is common in hexaploid *Carassius*, and this might significantly influence population structure. It occurs sporadically among invertebrates (Smith, 1978), and in vertebrates it is especially widespread in polyploid teleosts (Gui, 1997; Le Comber and Smith, 2004). Smith (1978) argued that short term selection can favor unisexuality. Gynogenetic reproduction forms a barrier to gene flow from hexaploid to tetraploid individuals. Hexaploid females only require sperm from tetraploid males to trigger egg development; egg and sperm nuclei do not fuse (Gui, 1997). Where tetraploids, hexaploids and, rarely, octaploids coexist in natural waters, hexaploid male *C. auratus* occur but they do not contribute genetically to tetraploid reproduction. A combination of hexaploid and tetraploid nuclei would form an irregular number of chromosomes (about 125) and no such individuals are known (Chen *et al.*, 1996; Xiao *et al.*, 2011). Thus, hexaploids depend on tetraploids even though the former do not fertilize eggs of the later and their syntopic occurrence is widespread.

Polyplod *Carassius*: a model system for investigations in vertebrates

With its large genome, *Carassius* possesses many duplicated genes. Ancestral carp appear to have about 50 chromosomes (Yu *et al.*, 1989) and a genome size of about 2.0 pg per cell. In contrast, tetraploid *Carassius* have from 3.52 pg to 3.84 pg per cell, almost twice that of diploid barbs (<http://www.genomesize.com/>; Luo *et al.*, 2006). Hexaploid *Carassius* have about 5.25 pg per cell (<http://www.genomesize.com/>). Our results indicate that tetraploids have 2–4 copies for each nuclear gene, and hexaploids normally have 3–6 copies (Supplementary Table S4). Thus, *Carassius* is likely to be a polyploid genus, and not a diploid/triploid system as sometimes claimed (for example, Takada *et al.*, 2010).

The existence of both allo and autopolyploidization events facilitates investigations into post polyploidization events at both genetic and epigenetic levels. The origin of the allopolyploidization in

Carassius is known (Yang and Gui, 2004; Luo *et al.*, 2006). The importance of allopolyploidization and how its evolutionary effects differ from those of autopolyploidization (Parisod and Besnard, 2007) is a topic of considerable interest (Soltis and Soltis, 1999; Vandepoele *et al.*, 2004; Van de Peer *et al.*, 2009). This level of interest differs from that for autopolyploidization (Parisod and Besnard, 2007). Autopolyploidization might be advantageous under conditions of environmental instability because of genic redundancy and polysomic inheritance (Parisod and Besnard, 2007). Thus, this genus is an ideal model system for investigating genetic and epigenetic changes soon after polyploidizations, either allo and autopolyploidy.

In summary, intraspecific polyploidization occurs multiple times in angiosperms, yet this phenomenon is less common and not yet fully understood in vertebrates. We document recurrent polyploidization events in the genus *Carassius* based on matrilineal history. The *C. auratus* complex has recurrent formations of hexaploids; tetraploids and hexaploids share identical haplotypes in three main lineages and within localities. The origin of the gibel carp likely owes to a single hexaploidization event in China within the *C. auratus* complex. The other two main lineages, the Chinese *C. auratus* complex and the ginbuna of Japan, have at least 22 independent hexaploidization events, plus one octaploidization. These events make the species of the genus *Carassius* an ideal model system for the study of recurrent polyploidization and the evolution of genomic complexity. How polyploidy contributes to adaptation and evolution, as well as speciation in vertebrates, is a very promising area of future research.

DATA ARCHIVING

Sequences were deposited in GenBank: Accession numbers: JX406582 JX406681 and KC462749 KC462782.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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