Chromosoma (Berl.) 23, 1—9 (1967)

Diploid-Tetraploid Relationship among Old-World Members of the Fish Family *Cyprinidae**

Susumu Ohno, Junichi Muramoto and Lawrence Christian
Department of Biology, City of Hope Medical Center, Duarte, California, U.S.A.

Niels B. Atkin
Department of Cancer Research, Mount Vernon Hospital, Northwood, Middlesex, England

Received June 24, 1967

Abstract. Evidence suggesting that the goldfish and the carp of the family *Cyprinidae* are tetraploid species in relation to other members of the same family were presented. The two barb species, *Barbus tetrazona* and *Barbus fasciatus*, were chosen as representatives of diploid members of the family *Cyprinidae*. These barbs had the diploid chromosome number of 50 and 52 and the DNA value 20—22% that of placental mammals, while the goldfish (*Carassius auratus*) and the carp (*Cyprinus carpio*) had the diploid chromosome number of about 104 and the DNA value 50—52% that of placental mammals.

Introduction

Speciation from an immediate ancestor has no doubt been accomplished by allelic mutations at already existing gene loci. When evolution from one vertebrate class to another is considered, however, allelic mutations are no longer sufficient to account for all the changes that have taken place. Gene duplication now emerges as a prime factor in evolution.

Our previous studies on chromosome complements and DNA values of members of different vertebrate classes indicated that various degrees of gene duplication both by regional duplication of chromosomal segments and by polyploidization took place while vertebrates were still aquatic nearly 300 million years ago. Subsequent development of the chromosomal sex-determining mechanism tended to stabilize various genome lineages at characteristic degrees of gene duplication. Hence, evolution of terrestrial vertebrates from aquatic forms was polyphyletic.

Among ray-finned fishes (*Neopterygii*) of today, many were found to have a diploid chromosome complement of 48 acrocentrics. However, diverse DNA values were found. Flatfish of the order *Heterosomata* and members of the order *Microcyprini* had the DNA value only 20% that

* Supported in part by a grant (CA-05138) from the National Cancer Institute, U.S. Public Health Service, and in part by a research fund established in honor of General James H. Doolittle at Duarte, and by the British Empire Cancer Campaign for Research at Northwood. Contribution No. 11—67, Department of Biology, City of Hope Medical Center. Dr. Junichi Muramoto is a fellow of the Institute for Advanced Learning of the City of Hope Medical Center.

of placental mammals, while certain members of the order *Percomorphi* had the value 31% that of mammals and members of the suborder *Clupeoidea* the value 42% that of mammals (Ohno and Atkin, 1966, unpublished data). This increase in DNA value without a noticeable change in the diploid chromosome complement can be taken as evidence of gene duplication accomplished exclusively by repeated regional duplication of chromosomal segments. Rothfels and his colleagues (1966) have shown the similar situation in species of *Anemone* and related genera.

Suggestive evidence of polyploid evolution was furnished by certain teleost fishes which possessed approximately double the DNA value and twice the chromosome number of their close relatives. For instance, salmonoid fish had the DNA value 80% that of mammals and diploid complements made of 100 to 104 chromosome arms (Ohno and Atkin, 1966), while the DNA value of clupeoid fish was approximately 40% that of mammals and their diploid complements as a rule contained 48 acrocentrics (unpublished data).

Within the family *Cyprinidae* of the order *Ostariophysi*, the goldfish (*Carassius auratus*) has been shown to contain about 100 chromosomes in its diploid complement (Ojima *et al.*, 1966; Ohno and Atkin, 1966), and its DNA value was 52% that of placental mammals (Ohno and Atkin, 1966). While the goldfish's closest relative, the carp (*Cyprinus carpio*) has been reported to have a similarly high diploid chromosome number of 104 (Makino, 1939), the diploid chromosome number reported on other members of the family *Cyprinidae* was approximately 50 (Nogusa, 1960; Post, 1965; Chen, 1966). It was felt that the carp and the goldfish might represent the tetraploid state in relation to other members of the family *Cyprinidae*. Accordingly, the chromosome complements and DNA values of the goldfish and the carp were compared with those of the two species of barbs, *Barbus tetrazona* and *Barbus fasciatus*.

Materials and Methods

Five specimens each of the tetrazona barb, the fasciata barb, the goldfish and the carp of colored variety were used for the present experiment. Both sexes were represented in each species.

For chromosome analysis, each specimen received an intramuscular injection of 0.1 to 0.5 cc of 0.5% colchicine solution depending upon its body size 50 minutes prior to the time of sacrifice. For recovery of mitotic metaphase figures, gills and spleen of each fish were cut into small cubes of about 3 mm^3 in size. These cubes underwent hypotonic pretreatment in pH 7.0 distilled water for 15 minutes at room temperature. They were then transferred to a 50% acetic acid fixative. After 30 minutes of fixation, a squash preparation was made from each cube. Each preparation underwent 15 minutes of hydrolysis in 1N HCl at 50° C. Giemsa solution was employed for staining the preparations. Meiotic figures of the male were recovered from sexually mature testes in the same manner.

Comparative DNA values of the four species were obtained by measuring the Feulgen stain content of erythrocyte nuclei using the Deeley integrating microden-

sitometer, incorporating a crushing condenser (DEELEY, 1955). The exact details of the technical problems associated with this method have been given earlier (ATKIN, MATTINSON, BEÇAK and OHNO, 1965). As before, the air-dried blood smears made on No. 1 coverslips were sent air mail from Duarte to Northwood. In Northwood, control cells (human lymphocytes from tonsillar tissue) were added to the smears before fixation by freeze-substitution.

Measurements on the goldfish have already been reported (OHNO and ATKIN 1966). Single smears of each of the 3 other species were stained together, and second smears of the fasciata barb and the carp were also stained together. Measurements were made on both smears of each of the two latter species and the results were pooled. Measurements were also made on human lymphocytes on air-dried smears made immediately on receipt of the tonsillar material, which were compared with lymphocytes subsequently added to the same smears before fixation by freeze-substitution. No significant difference was found, and it was concluded that no change in the stainability of the lymphocytes had occurred during the short period of storage before freeze-substitution. The ratios obtained between the fish erythrocytes and the control cells were therefore taken as the DNA value of the species relative to the human female lymphocyte value.

Results

Differences in the diploid chromosome complements of the four species are summarized in Table 1, while the relative DNA values of the four species are given in Table 2. The two barb species demonstrated the

Table 1

Species	Diploid chromosome number	Definitely two-armed chromosomes	Smaller subterminals	Acrocentrics	Minutes
Barbus tetrazona	50	34	6	10	0
Barbus fasciatus	52	30	4	16	2
Carassius auratus	104 or less	46	16	42	0
Cyprinus carpio	104 or less	46	18	36	4

Table 2

Species	Sex	Number of cells measured[a]	DNA value relative to human female leukocyte value and standard error
Barbus tetrazona	♂	30	0.204 ± 0.004
Barbus fasciatus	♂	30	0.221 ± 0.003
Carassius auratus	♂	40	0.525 ± 0.014[b]
Cyprinus carpio	♂	50	0.498 ± 0.014

[a] Approximately the same number of control cells were also measured; the control cells were human small lymphocytes from tonsillar tissue removed by tonsillectomy from female patients.

[b] based on previous measurements (OHNO and ATKIN, 1966).

diploid chromosome number of 50 and 52 while the diploid complements of the goldfish and the carp, at the most, contained 104 chromosomes. The DNA value of the two species of barbs was 20—22% that of man, while the DNA value of the goldfish and the carp was 50—52% that of man. Thus, it appeared that within the family *Cyprinidae*, the diploid-tetraploid relationship indeed exists.

1. The Tetrazona Barb (Barbus tetrazona)

The karyotype of this species prepared from a male splenic metaphase figure is shown in Fig. 1. The diploid chromosome number was 50. In

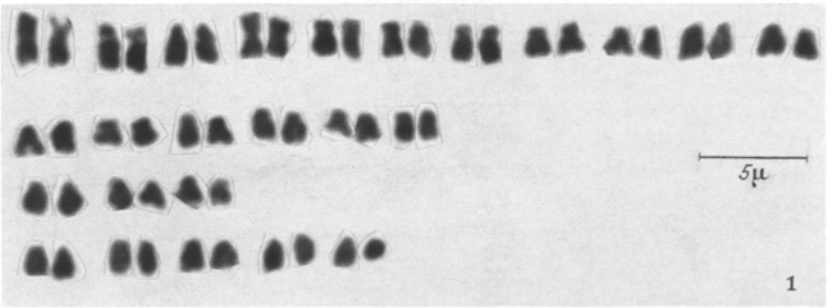

Fig. 1. Serial alignment of the 50 chromosomes of *Barbus tetrazona*. 17 pairs of definitely two-armed chromosomes are aligned in order of declining size in the first and second rows. Three pairs of smaller subterminals constitute the third row, and five pairs of acrocentrics make up the fourth row.

All the photomicrographs were taken by Leitz-Panphot 100 × 10. Actual magnification of chromosomes used for the karyotypes of Figs. 1, 3, 4 and 5 are indicated by the 5 micron scale added to Fig. 1. Figs. 2 and 6 are slightly more enlarged

making the karyotype, the chromosomes were classified into three groups: 1. definitely two-armed chromosomes which included mediocentrics and submediocentrics of all sizes and larger subterminals which can not be confused with acrocentrics, 2. smaller subterminals which at times can be confused with acrocentrics, 3. acrocentrics. This practice was followed in the study of the other three species as well, for due to smallness of individual chromosomes, smaller subterminals were often difficult to distinguish from acrocentrics.

Observing Fig. 1, it can be seen that the diploid complement of this species consists of 17 pairs of two-armed chromosomes, 3 pairs of smaller subterminals and 5 pairs of acrocentrics. Meiotic figures of this species contain 25 bivalents (Fig. 2).

2. The Fasciata Barb (Barbus fasciatus)

As shown in Fig. 3, this species had the diploid chromosome number of 52. Fifteen pairs of two-armed chromosomes, two pairs of smaller subterminals and eight pairs of acrocentrics and a pair of minutes constituted the diploid complement. The DNA value of 22% that of man was almost identical with that of the tetrazona barb. It is of interest to note that while this presumptive diploid species is endowed with a pair of minutes, the carp possesses four minutes, as will be shown.

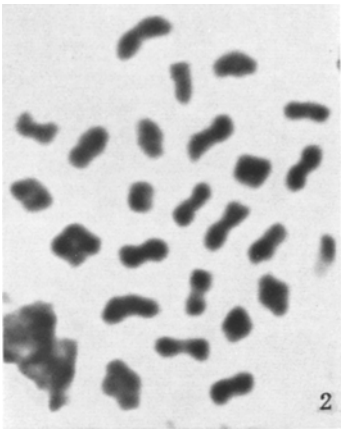

Fig. 2. First meiotic metaphase figure from the testis of a male *Barbus tetrazona*: 25 bivalents are seen

The fasciata barb cannot possibly be the direct diploid ancestor of the carp, but it is conceivable that this particular barb species has retained a pair of minutes which were in the ancestral diploid chromosome complement of an extinct species which evolved into the carp and the goldfish by tetraploidization. Similarly, after becoming the tetraploid species, these minutes were retained by the carp but not by the goldfish.

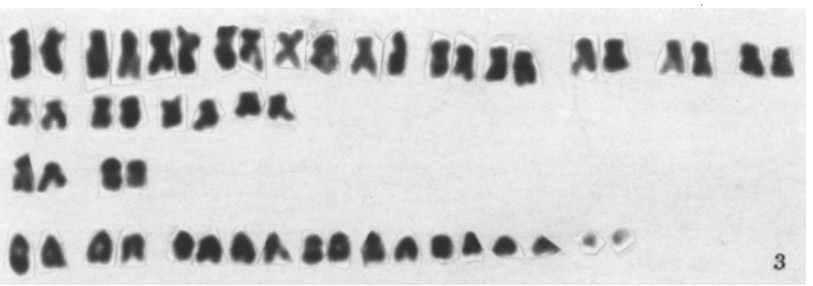

Fig. 3. Karyotype of *Barbus fasciata* ($2n = 52$) prepared from a male metaphase figure from the gill. 15 pairs of definitely two-armed chromosomes occupy the first and second rows. Two smaller subterminal elements make up the third row, and eight pairs of acrocentrics and a pair of minutes constitute the bottom row

3. The Goldfish (Carassius auratus)

In the previous paper (Ohno and Atkin, 1966), it was stated that the diploid chromosome number of the goldfish appears to vary from 96 to 104 between as well as within individual fish. While Ojima, Hito-

sumachi and Makino (1966) claimed that this species had the definite diploid chromosome number of 100, a male splenic metaphase figure from which the karyotype of Fig. 4 was prepared contained 104 chromosomes. There were 23 pairs of definitely two-armed chromosomes, 8 pairs of smaller subterminals and 21 pairs of acrocentrics. The DNA value 52% that of man is approximately twice the DNA value of the two barb species. Despite its apparent tetraploid nature, male diakinesis figures of

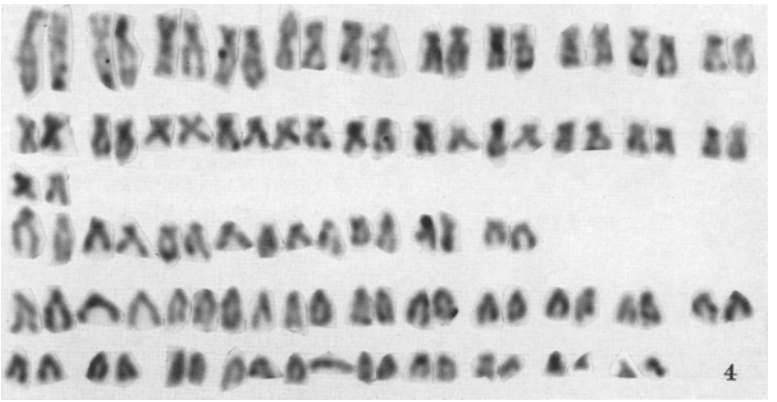

Fig. 4. Serial alignment of the 104 chromosomes of *Carassius auratus*. 23 pairs of definitely two-armed chromosomes are aligned on the first, second and third rows. Eight pairs of smaller subterminal chromosomes occupy the fourth row. The fifth and sixth rows are made of 21 pairs of acrocentrics

this species regularly contained 49 to 52 bivalents; no quadrivalents were encountered. Thus, it is conceivable that the goldfish arose from an allotetraploid ancestor which was produced as a result of hybridization between two different diploid species. Conversely, it is possible that an ancestral species to the goldfish arose as an autotetraploid yet four original homologues gradually diverged into two different pairs.

4. The Carp (Cyprinus carpio)

Matsui (1934) accumulated voluminous evidences showing the wild-type ancestor of the goldfish to be the funa (*Carassius carassius*) and not the carp. Both the funa and the carp inhabit lakes and streams of Japan and other parts of Northeastern Asia. They are closely related for they readily hybridize with each other and produce viable hybrids. The male sterility of these hybrids, on the other hand, furnishes a clear cut evidence that the two are different species.

Reflecting the close kinship to the goldfish, the carp had the DNA value nearly identical with that of the goldfish (Table 2); the highest

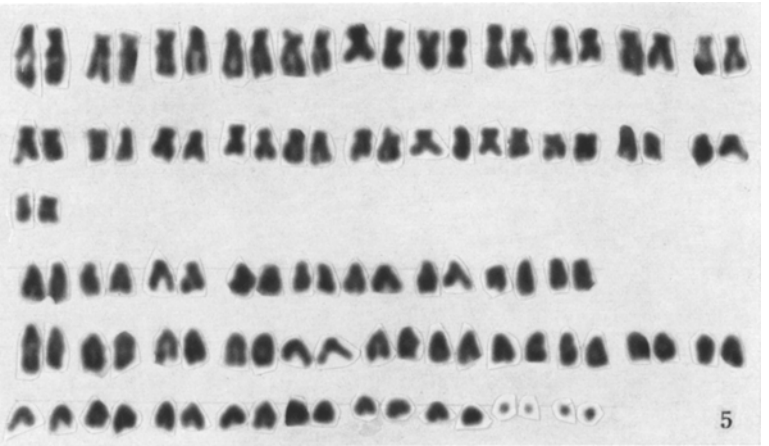

Fig. 5. Karyotype of *Cyprinus carpio* prepared from a male splenic cell. The first, second and third rows contain 23 pairs of definitely two-armed chromosomes. Nine pairs of smaller subterminals are aligned on the fourth row. 18 pairs of acrocentrics and four minutes occupy the fifth and sixth rows

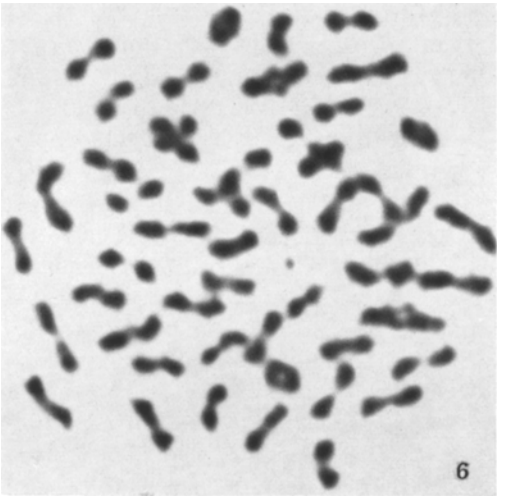

Fig. 6. First meiotic metaphase figure from the testis of a male *Cyprinus carpio*. 50 bivalents and one minute quadrivalent located in the center of the field are seen

diploid chromosome number obtained for the carp was also 104. The diploid chromosome complement of the carp was similar but readily distinguishable from that of the goldfish (Table 1), for it contained four minute chromosomes which were not included in the goldfish karyotype. The karyotype shown in Fig. 5 is also made of 104 chromosomes. It may

be noted that there are 23 pairs of definitely two-armed chromosomes, 9 pairs of smaller subterminals, 18 pairs of acrocentrics and two pairs of minutes. Male diakinesis figures of this species contained 49—51 apparent bivalents and a single minute body (Fig. 6). Two minute bivalents were never seen. It is most likely that four minutes were homologues and formed a single quadrivalent. It may be recalled that *Barbus fasciatus* had a pair of minutes.

Discussion

In our previous paper (Ohno and Atkin, 1966), we proposed that the cumulative effect of gene duplication during the course of vertebrate evolution resulted in a progressive increase in DNA values which occurred not only by regional duplication of chromosomal segments but also by polyploidization. Gene duplication by polyploidization must have occurred at the initial stages of vertebrate evolution while vertebrates were still aquatic or amphibious, for once the chromosomal sex-determining mechanism was firmly established, further polyploidization became impossible.

Indeed, the first convincing evidence of polyploid evolution in vertebrates was presented by Beçak, Beçak and Rabello (1966) on South American frogs belonging to the family *Ceratophrydidae*. *Odontophrynus americanus* with 44 chromosomes was clearly a tetraploid species, for 44 chromosomes formed 11 quadrivalents instead of 22 bivalents during meiosis. Other members of this genus had the diploid chromosome number of 22.

The present findings on four fish species of the family *Cyprinidae* appear to furnish sufficient evidence that the goldfish and the carp are indeed tetraploid species in relation to other members of the family. Although the goldfish and the carp had nearly identical DNA values and diploid chromosome numbers, their karyotypes were slightly different, revealing that within the family *Cyprinidae*, a common tetraploid ancestor to both the goldfish and the carp arose sometime ago so that each had sufficient time to accumulate independent chromosomal rearrangements. It is not surprising, therefore, that about 104 chromosomes of both species form 50 odd bivalents rather than 25 or so quadrivalents during meiosis. If enough time is given, four original homologues of a tetraploid species would diverge into two different homologous pairs.

The two barb species presently studied cannot possibly be the direct descendants of an ancestral diploid species which, by becoming tetraploid, gave rise to the goldfish and the carp of today. Yet, from the present finding of these barb species, it can be inferred that such an ancestral diploid species had the diploid chromosome number of 50 or 52 and the DNA value of about 25% that of man as do the barb species of today.

References

ATKIN, N. B., G. MATTINSON, W. BEÇAK, and S. OHNO: The comparative DNA content of 19 species of placental mammals, reptiles, and birds. Chromosoma (Berl.) **17**, 1—10 (1965).

BEÇAK, M. L., W. BEÇAK, and M. N. RABELLO: Cytological evidence of constant tetraploidy in the bisexual South American frog, *Odontophrynus americanus*. Chromosoma (Berl.) **19**, 188—193 (1966).

CHEN, T.: Comparative karyology of selected deepsea and shallow water teleost fishes. Ph. D. dissertation, Biology, Yale University 1967.

DEELEY, E. M.: An integrating microdensitometer for biological cells. J. sci. Instrum. **32**, 263—267 (1955).

MAKINO, S.: The chromosomes of the carp, *Cyprinus carpio*, including those of some related species of *Cyprinidae* for comparison. Cytologia (Tokyo) **9**, 430—440 (1939).

MATSUI, Y.: Genetical studies on goldfish of Japan. J. Imperial Fisheries Inst. (Japan) **30**, 1—96 (1934).

NOGUSA, S.: A comparative study of the chromosomes in fishes with particular considerations on taxonomy and evolution. Mem. Hyogo Univ. Agric. **3**, 1—62 (1960).

OHNO, S., and N. B. ATKIN: Comparative DNA values and chromosome complements of eight species of fishes. Chromosoma (Berl.) **18**, 455—466 (1966).

OJIMA, Y., S. HITOSUMACHI, and S. MAKINO: Cytogenetic studies in lower vertebrates. I. A preliminary report on the chromosomes of the funa (*Carassius auratus*) and the goldfish (a revised study). Proc. Japan Acad. **24**, 62—66 (1966).

POST, A.: Vergleichende Untersuchungen der Chromosomenzahlen bei Süßwasser-Teleosteern. Z. Zool., Syst. Evolut.-forsch. **3**, 47—93 (1965).

ROTHFELS, K., E. SEXSMITH, M. HEIMBURGER, and M. O. KRAUSE: Chromosome size and DNA content of species of *Anemone* and related genera (*Ranunculaceae*). Chromosoma (Berl.) **20**, 54—74 (1966).

Dr. SUSUMU OHNO
Department of Biology, City of Hope Medical Center
Duarte, Californien 91010, U.S.A.