Characteristics of Chromosomes and Electrophoretic Patterns of Muscle Protein and Lactate Dehydrogenase in Fish of Genus *Carassius* (Cypriniformes)

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Fish of the genus *Carassius* with the Japanese common name of the funa, are widely distributed in the fresh water of Japan. However, there is considerable discrepancy of its systematic classification, as a result of its polymorphic characteristics. According to Nakamura 1969, the funa is classified into five subspecies, *C. auratus* subsp. "kinbuna", *C. a. langsdorfii* "ginbuna", *C. a. buergeri* "nagabuna", *C. a. grandoculis* "nigorobuna", and *C. a. cuvieri* "gengorobuna".

Ojima *et al.* 1966 reported that the diploid chromosome numbers of the funa and the goldfish were 100 in both sexes. Kobayashi *et al.* 1970, 1973 reported the occurrence of triploidy and tetraploidy in the ginbuna. These polyploid individuals consist of only females and propagate unisexually by gynogenesis (Kobayashi 1985, Kobayashi *et al.* 1977, Ojima and Asano 1977). Ueda and Ojima 1978 revealed that intensely C-banded short arms were shown in a pair of the second largest submetacentric chromosomes of the kinbuna (both members in the female, but only one in the male) and of the triploid ginbuna, but not in the other subspecies of the diploid type. Such C-banded marker chromosomes were found in most varieties of goldfish (Ojima *et al.* 1979) and the Chinese funa, *C. a. auratus* (Ojima and Takai 1979). The Chinese funa corresponded in the electrophoretic patterns of muscle protein as well as in the C-banding patterns with the goldfish, suggesting that the origin of the goldfish was the Chinese funa (Ojima and Takai 1979). Takai and Ojima 1983 reported on the artificial production of tetraploid individuals from the triploid ginbuna, and proposed a possible origin of the natural tetraploid ginbuna on the basis of the chromosomal and the electrophoretic studies of these tetraploidy. Thus cytogenetic analyses have provided several interesting findings to assist in understanding the speciation and the phylogenetic relationship of the funa. On the other hand, Taniguchi and Ishiwatari 1972 and Taniguchi and Sakata 1977 suggest that analysis of the electrophoretic patterns of muscle protein was very useful for the classification of the funa. However, the relationship between the classification by the muscle protein patterns and by the chromosomes is not clear.

In the present paper, we will report on the characteristics of the chromosomes and the electrophoretic patterns of muscle protein and lactate dehydrogenase (LDH), and the relationship between them, based on the specimens collected from eight rivers and three lakes, with special remarks on the speciation, the phylogenetic relation, and the classification of the funa.

Materials and methods

Specimens used for this study are given in Table 1. Chromosome preparations were made from directly prepared kidney cells (Takai and Ojima 1986), PHA cultured kidney cells (Yamamoto *et al.* 1973), and cultured fin cells (Ojima 1978), treated with colchicine, according

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to the conventional air-drying method and stained with Giemsa. After microscopic observation, the slides were destained with 70% alcohol. C-banding was done by a modification of the BSG method (Sumner 1972) as follows. The slides were immersed in saturated $\text{Ba}(\text{OH})_2$ solution at 60°C for 60–90 seconds, dipped in 0.01 N HCl for several seconds, rinsed well with distilled water, and air-dried. They were incubated in $2 \times \text{SSC}$ (0.3 M NaCl + 0.03 M trisodium citrate) solution at 50°C for 60 minutes, rinsed with distilled water, and stained with 4% Giemsa diluted with 1/15 M phosphate buffer, pH 7.0 for about 10 min. For electrophoretic analysis, specimens dissected for chromosome studies were stored frozen at -20°C until the time of use. Sampling of muscle tissue and the horizontal starch-gel electrophoresis for muscle protein were carried out according to the method of Ojima and Takai 1979, which is a modification of the method used by Taniguchi and Sakata 1977. The electrophoresis of LDH was carried out at $2\text{mA}/\text{cm}^2$ for 12 hr. using 12% starch-gel and the Buffer system I according to Show and Prasad 1970 (electrode: 0.155 M tris-0.043 M citric acid; gel: 66.7 ml of the buffer for electrode was diluted to 1 litre). The staining of LDH isozymes was made with the solution including 2 ml of 40% sodium lactate, 5 mg of NAD, 5 mg of NBT, and 1 mg of PMS in 20 ml of 0.2 M tris-HCl buffer (pH 8.6).

Fig. 1. Metaphase figures in (A) the diploid and (B) the triploid specimens.

Results and discussion

All the specimens were either of the diploid ($2n=100$) or the triploid types (Fig. 1). The diploid type, which had the chromosome number of 100, was found in both sexes. The triploid type, of which the chromosome numbers ranged between 154 and 156 mostly, appeared only in females. These two types were found together in most localities (Table 1).

Fig. 2 shows the starch-gel electrophoretic patterns of muscle protein (MP patterns) we obtained. These MP patterns were, as a whole, mostly the same as those according to Taniguchi and Sakata 1977. The bands 1 and 2 were found in all the patterns and a variety of patterns occurred by presence or absence of the bands 3, 4, 5 or 6. Some lightly stained thin bands were also seen in all the patterns but these will not be further discussed in this study. All the diploid specimens, with the exception of the gengorobuna, showed Type I. Type II was seen in the triploid specimens. Type III was observed in the gengorobuna. These three basic types corresponded with those classified by Taniguchi and Sakata 1977, making it clear that the specimens with Types I and II classified by Taniguchi and Sakata 1977 were the diploid

and triploid types, respectively. Type I was further divided into three subtypes, I-A, I-AB and I-B. According to Taniguchi and Ishiwatari 1972, these three subtypes are the variations controlled by the two co-dominant alleles, A and B. Type II was divided into three subtypes, II-1, II-2 and II-3. Taniguchi and Sakata 1977 reported four subtypes in Type II, but we did not found one of them. Type III existed in only one pattern.

Table 1. Electrophoretic patterns of muscle protein in the individuals collected from three lakes and eight rivers in Japan

Locality (Prefecture)	No. of specimens	Muscle protein pattern ¹								
		I(2n):	-A	-AB	-B	II(3n):	-1	-2	-3	III(2n)
L. Jonuma (Gunma)	32	16		1	15	16	16			
L. Suwa (Nagano)	10	10	10				16			
L. Biwa (Shiga)	42	15	4	9	2	19	14	1	4	8
Yasu R. (Shiga)	13	10	4	5	1	2		2		
Muko R. (Hyogo)	18	1	1			16	16		1	
Akashi R. (Hyogo)	8	3	2	1		5	4	1		
I R. (Hyogo)	25	3	2	1		20	20	1		
Ohgo R. (Hyogo)	9	5	5			4	2	2		
Asahi R. (Okayama)	5	4	4			1	1			
Niyodo R. (Kochi)	10	2			2	8	8			
Oyodo R. (Miyazaki)	10	9	9			1	1			

¹ Type I is found in the diploid specimens excepting the gengorobuna, Type II in the triploid specimens, and Type III in the gengorobuna.

Fig. 2. Electrophoretic patterns of muscle protein. Type I (1) is observed in the diploid specimens with the exception of the gengorobuna, Type II (2) in the triploid specimens and Type III (3) in the gengorobuna.

Type I had three common bands, bands 1, 2 and 6. In addition to these bands, Type I-A contained band 4, Type I-AB bands 3 and 4, and Type I-B band 3. In Type I-AB bands 3 and 4 showed the same density. Bands 3 and 4 were the bands controlled by two codominant alleles as described above. The frequency of these subtypes varied between each locality (Table 1). In the density of bands 2 and 6, two characteristics could be distinguished. One was that band 2 was a lightly stained thin band and band 6 was a deeply and widely stained band. The other was that band 2 was deeply stained, while band 6 was lightly stained. Typical patterns in the former case were found in the specimens from Lake Jonuma and Oyodo River.

Those in the latter case were observed in the specimens from Lake Suwa, Lake Biwa and Yasu River. The specimens of Type I included the subspecies classified as the kinbuna in Lake Jonuma, the nigorobuna in Lake Biwa, the nagabuna in Lake Suwa and the diploid ginbuna in the other localities. Some specimens from Lake Biwa showed the morphology of the ginbuna rather than the nigorobuna.

In Type II, band 2 was deeply stained. Type II-1 consisted of bands 1, 2, 3, 4, 5 and 6. Band 5 was characteristic and was not found in the other types. This type was further subdivided in relative density among bands 3, 4 and 5 into three patterns, which occurred at different frequencies between the localities. Type II-2 comprised of bands 1, 2, 3, 4 and 6. In this type, band 3 was stained to larger extent than band 4. Type II-3 contained bands 1, 2 and 4. The pattern of this type was the same as that of the Chinese funa, *C. a. auratus*, revealed by Ojima and Takai 1979. In the three subtypes, Type II-1 was found in the most

Fig. 3. Conventional and C-banding karyotypes of the diploid ginbuna (from Asahi River). The nagabuna, the nigorobuna and the gengorobuna show the same karyotypes and C-banding patterns. The kinbuna has deeply C-banded short arms in the second largest submetacentric chromosomes, although it remains the same karyotype. M: metacentric, SM: submetacentric, ST-A: subtelocentric and acrocentric

localities in large quantities but Type II-2 and Type II-3 were recognized in limited localities in small quantities (Table 1). The specimen with Type II-2 from Lake Biwa was morphologically similar to the nigorobuna rather than the ginbuna.

Type III consisted of bands 1, 2 and 3. Band 2 was deeply stained as was that of Type II. This type was found in only the gengorobuna. The two specimens from Lake Biwa had the pattern with band 6, in addition to bands 1, 2 and 3. These exceptional specimens, which are not shown in Table 1, may be hybrids between the gengorobuna and the nigorobuna. Taniguchi 1974 reported the same case.

The karyotypes of the specimens with Type I and Type III consisted of 6 pairs of metacentrics, 18 pairs of submetacentrics, and 26 pairs of subtelocentric and acrocentrics and no clear difference was recognized among the specimens (Fig. 3). Ueda and Ojima 1978 reported that the C-banded marker chromosomes appeared in the kinbuna but not in the nagabuna, the nigorobuna and the gengorobuna. The diploid ginbuna, however, was not researched.

In this study, C-banding patterns of the specimens from Yasu River, Akashi River, Ohgo River, I River and Asahi River, were examined. These specimens had no C-banded marker chromosome (Fig. 3). In Type II, some karyotypic difference was shown between the specimens with Type II-1, and Type II-2 and Type II-3. The karyotypes of the specimens with Type II-1 showed the diploid-like set of chromosomes rather than the triploid set as shown by Ueda and Ojima 1978 (Fig. 4). For example, it was apparent that the largest submetacentric chromosomes were two. However, those of Type II-2 and Type II-3 were characterized by the triploid set, as the largest submetacentric chromosomes clearly were three (Fig. 5). Ueda and Ojima 1978 reported that the two C-banded marker chromosomes occurred in the triploid gimbuna from Lake Biwa. We confirmed the same results in the specimens with Type II-1 from Lake Jonuma, Lake Biwa, Muko River, Akashi River, I River and Ohgo River. These specimens had several deeply stained supernumerary chromosomes as shown by Ueda and Ojima 1978 (Fig. 4). A noticeable result was that the specimen with Type II-3 from

Fig. 4. Conventional and C-banding karyotypes of the triploid gimbuna with Type II-1 of the muscle protein pattern (from Lake Jonuma). The karyotype shows a diploid-like set. There exist two C-banded marker chromosomes with the deeply stained short arms (arrows). An asterisk (*) shows the deeply C-banded supernumerary chromosome group.

Lake Biwa, whose karyotype showed the triploid set, had three C-banded marker chromosomes and several C-banded supernumerary elements (Fig. 5).

The electrophoretic patterns of lactate dehydrogenase (LDH) isozymes in muscle tissue were divided into three types, Type L-1 with five bands, Type L-2 with nine bands, and Type L-3 with five bands (Fig. 6). Type L-1 was found in the kinbuna. Type L-2 was observed in the triploid specimens from Lake Jonuma, Lake Biwa, Yasu River, Muko River, Akashi River, and Niyodo River, and in the diploid specimens from Lake Suwa, Lake Biwa, Yasu River and Niyodo River. Type L-3 was recognized in the gengorobuna. Nearly all bony fishes have three distinct LDH loci, A, B and C. The LDH-A and LDH-B loci produce LDH-A and LDH-B subunits, respectively. Different combinations between these subunits form several molecular forms of tetrameric structure, in which the highest number is five. The LDH-A locus is expressed predominantly in muscle tissue and the LDH-B locus in heart tissue (Markert *et al.* 1975). It was decided that the bands appearing in the most cathodal side were the A₄

homotetramers because these bands showed the highest density. The A_4 bands in the three types showed the same mobility. This may represent that the LDH-A genes were homologous among the three types. In Type L-1 and L-3, it was decided that the bands appearing in the most anodal side were the B_4 homotetramers. In these two types, the B_4 bands were observed in different positions, suggesting that the LDH-B genes differed between the two types. It was clear that the pattern of Type L-2 occurred by three different genes.

According to Ohno 1970, the funa is an older tetraploid which completed the process of the diploidization. This may be a result of the fact that the funa was of such tetraploid origin. After the tetraploidization, a new LDH-B' gene would be differentiated from one of the duplicated LDH-B genes by DNA mutation. We considered that the B_4 band was the 5th band from the cathodal side since this band presented the highest activity in the heart

Fig. 5. Conventional and C-banding karyotypes of the triploid ginbuna with Type II-3 of the muscle protein pattern (from Lake Biwa). The karyotype shows a triploid set. There exist three C-banded marker chromosomes with the deeply stained short arms (arrows). An asterisk (*) shows the deeply C-banded supernumerary chromosome group.

tissue and the B'_4 band was the most anodal band. As causes that the expression of duplicated LDH-B genes was not detected in the kinbuna and the gengorobuna, two possible cases can be considered. In the first case, a new gene is not created from one of the duplicated genes. In Case two, although a new gene was created from one of the duplicated genes, either the original gene or the new gene was silenced. The occurrences of silenced genes after polyploidization, which have been often reported, can be recognized as a process of the diploidization of polyploidy and closely correlated with phenotypic evolution (Ferris and Whitt 1977). It can be thought that Case 1 is in more primitive condition and that Case 2 is in more advanced one. So far as the present results are concerned, it is difficult to decide whether Type L-1 and L-3 correspond to either of the two cases. Kafuku 1952 reported that the process of complication in the coiling of intestines is found in the order of the kinbuna, the ginbuna and the gengorobuna. If Type L-1 is in Case 1 and Type L-3 is in Case 2, the evolutionary

process of the duplicated LDH-B genes could be also found in the order of the kinbuna (L-1), the ginbuna (L-2), and the gengorobuna (L-3) as well as that of the coiling of intestines. The gengorobuna is thought to be the most advanced species amongst the funa. It is an interesting problem whether or not the kinbuna is the most primitive species as suggested by Kafuku 1952. Further investigation of more isozymes might clarify the evolutionary condition of the funa subspecies. At any rate, the three types of the LDH isozyme patterns seem to represent the different evolutionary stages.

This study, in addition to several previous studies (Kobayashi 1985, Onozato 1983), indicates that the diploid and the triploid individuals exist together mostly throughout Japan. The individuals with Type I, with exception of the kinbuna in the Kanto district, the nagabuna in Lake Suwa and the nigorobuna in Lake Biwa have been dealt with as diploid ginbuna in general, according to the classification by Nakamura 1969. Taniguchi and Sakata 1977 dealt with these as "kinbuna", which are called "ookinbuna" by Taniguchi 1982. According to them, "kinbuna" is an independent species and includes the kinbuna, the nagabuna and the

Fig. 6. Electrophoretic patterns of muscle LDH isozymes. L-1 is found in the kinbuna, L-2 in all the individuals excepting the kinbuna and the ginbuna, and L-3 in the gengorobuna.

nigorobuna as subspecies. This study indicates that the diploid and the triploid forms of the ginbuna differ genetically, although they are morphologically very similar. So far as the present results are concerned, the nagabuna and the nigorobuna do not differ genetically from the other diploid ginbuna. The kinbuna was the same in the MP patterns as the other diploid specimens, but differed from them in observations taken of the LDH and C-banding patterns. The gengorobuna was also distinguished from the others in MP and LDH pattern observations. These results suggest that the diploid funa was classified into three types, the kinbuna, the gengorobuna and the other diploid funa.

In the triploid type, the specimens with Type II-1 of the MP pattern was found in large quantities in most localities. On the other hand, the specimens with type II-2 and II-3 were relatively few. In these three types, furthermore, the karyotypes were different between the specimens with Type II-1, Type II-2 and Type II-3. The C-banding patterns of the specimens with Type II-1 and II-3 were also different. On the basis of the findings on the karyotypic and electrophoretic studies, we are led to speculate about the speciation of the triploid funa

as follows. The triploid type characterized by Type II-1 originated from a common ancestor, and has spread the distribution widely throughout Japan. Band 5 in Type II-1 is not found in any other patterns. However, the specimens with Type II-2 might have been newly introduced at very low frequency in some localities. It is possible that the pattern of Type II-2 is induced by the cross between the individuals with Types I-A and I-B, I-A and I-AB, or I-AB and I-B. The specimens with Type II-2 from Lake Biwa were morphologically similar to the nigorobuna rather than the ginbuna. Taniguchi 1974 indicated that the specimens with Type II-2 from Lake Kasumigaura were morphologically intermediate between the kinbuna and the ginbuna. Type II-3 was the same pattern as that of Chinese funa, *C. a. auratus*. The specimens with type II-3 might be derived from the Chinese funa.

The results of the present study (Table 2) suggest that the Japanese funa may be classified into four groups, 1. the kinbuna, 2. the diploid ginbuna including the nagabuna and the nigorobuna, 3. the triploid ginbuna and 4. the gengorobuna. This classification seems to represent the condition of the speciation and evolution well. In this study, no occurrence of the tetraploid type was found.

Table 2. Relationships among chromosome numbers (CN), karyotypes (KTYP), C-banded marker chromosomes (C-MC), and electrophoretic patterns of muscle protein (MP) and LDH isozymes

Species	Japanese name	CN	KTYP ¹	C-MC	MP	LDH
<i>C. a. subsp.</i>	kinbuna	100	1	♀2, ♂1	I	1
<i>C. a. langsdorffii</i>	ginbuna	150+	2	2	II-1	2
		150+	3	—	II-2	2
		150+	3	3	II-3	2
		100	1	0	I	2
<i>C. a. buergeri</i>	nagabuna	100	1	0	I	2
<i>C. a. grandoculis</i>	nigorobuna	100	1	0	I	2
<i>C. a. cuvieri</i>	gengorobuna	100	1	0	III	3

¹ 1. 12M+36SM+52ST, A; 2. diploid like set; 3. triploid set.

Summary

Chromosomes and electrophoretic patterns of muscle protein and of LDH isozymes in the Japanese funa *Carassius auratus* were studied comparatively. All the specimens examined were either of diploid or triploid types. Electrophoretic patterns of muscle protein (MP patterns) were divided into three basic types. The specimens with Type I were the diploid type excepting the gengorobuna with Type III, and those with Type II were the triploid type. LDH isozyme patterns were divided into three types. Type L-1 was found in the kinbuna, and Type L-2 was in all the specimens excepting the gengorobuna with Type L-3 and the kinbuna. All the diploid specimens were the same karyotype constitution and the specimens with the exception of the kinbuna had no C-banded marker chromosomes. The triploid specimens with the different MP patterns showed different karyotypes and C-banding patterns, suggesting the different origins. As a result, the Japanese funa may be classified into four groups, the kinbuna, the diploid ginbuna, the triploid ginbuna, and the gengorobuna. This classification seems to represent well the condition of speciation and evolution.

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