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# Natural Hybridization between Diploid Crucian Carp Species and Genetic Independence of Triploid Crucian Carp Elucidated by DNA Markers

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**ABSTRACT**—The population structure of genus *Carassius* in Lake Koyama, southeast Japan, was analyzed by genetic markers as microsatellite DNA, mtDNA RFLP and isozymes. Based on the ploidy level and morphological analysis, four *Carassius* groups were detected. The triploid group was identified as Ginbuna (*C. langsdorfii*). In the diploid group, Nagabuna (*C. burugeri sp*) and Gengoroubuna, (*C. cuvieri*) were identified. Remaining diploid individuals had morphological traits that were intermediate between Nagabuna and Gengoroubuna. These were considered as hybrids and their descendants. From the results of mtDNA RFLP and isozyme patterns, the triploid population was considered to be independent from the gene pools of diploid. The hybrids had the mtDNA haplotypes which were common to Gengoroubuna and Nagabuna populations. Based on the three microsatellite loci, Ginbuna was classified into six clonal lines. In the diploid population, substitution of the major alleles of Nagabuna and Gengoroubuna were found. The hybrids had alleles that were common in Nagabuna and Gengoroubuna. The values of the hybrid index ($I_H$) which are ranged from 0.771 to 0.964 in Nagabuna, from 0.102 to 0.806 in the hybrids and from 0.068 to 0.157 in Gengoroubuna. The hybrid population was verified to be derived from crossbreeding between the Gengoroubuna and Nagabuna populations. Evidence of backcrossing in nature by microsatellite DNA markers was also obtained in the diploid populations.

## INTRODUCTION

Fish hybridization in nature presents many problems in conservation biology and genetic resource management. A major objective of fisheries management is to conserve indigenous fish populations from overexploitation, habitat degradation, and exotic species that may interact detrimentally with native species through predation, competition, or hybridization (Campton, 1987). The widespread stocking of fishes outside their native geographic regions for fishery enhancement or other management purposes, has frequently resulted in hybridization between the native and introduced species and races (Campton, 1987).

Introduction of Gengoroubuna, *Carassius cuvieri* may involve some problems. Gengoroubuna was endemic only in Lake Biwa. It is diploid and reproduces bisexually (sex ratio 1:1) (Nakamura, 1969). Gengoroubuna has quite different morphological characters from the other crucian carp species. In particular, the number of gill rakers in Gengoroubuna ranges from 92 to 108, and the body depth is the highest among the Japanese crucian carp (Nakamura, 1969; Hosoya, 1993). Gengoroubuna has been improved by selective breeding in the pond culture around Osaka Prefecture, where the species is called “Kawachibuna or Herabuna” (Kawamura, 1964). Gengoroubuna and “Kawachibuna or Herabuna” are regarded to be identical genetically and morphologically (Nakamura, 1969; Matsui *et al*., 1993; Taniguchi 1974). “Kawachibuna or Herabuna” have been transplanted throughout Japan where they are reproducible (Nakamura, 1969; Hosoya, 1993).

Congeneric fish species are often interfertile, and hybrid swarms representing genetic admixtures of the two parental species may be produced, following introductions of non-native fishes (Hubbs, 1955; Schwartz, 1972). Individuals derived from hybridization between Gengoroubuna and Okinbuna (*C. burugeri burugeri*) have been reported based

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on genetic markers (Taniguchi, 1974). The genetic admixtures by introgressive hybridization may influence on the indigenous species of crucian carp. The genetic admixtures may be a reason of the taxonomic confusions of the Japanese crucian carp (Hosoya, 1993). Therefore, it is indispensable to solve these taxonomic problems in relation with conservation of the indigenous species.

In southeastern Japan, two different ploidy levels of *Carassius* are recognized (Hosoya, 1993). The first species, Japanese silver crucian carp, Ginbuna, *Carassius langsdorfii* (Matsubara and Ochiai, 1965) can be found throughout Japan. This species is a natural triploid fish (Dong and Taniguchi, 1996, Dong *et al.* 1997), and reproduces by gynogenesis (Kobayasi, 1971; Kobayasi and Ochi, 1972). The development of Ginbuna eggs is initiated by the sperm of another species (Kobayasi, 1971; Kobayasi and Ochi, 1972). However, the introduced sperm nucleus does not transform into a male pronucleus and makes no contribution to the zygotic genome (Nakakuki *et al.*, 1984; Yamashita *et al.*, 1990,1993). Thus, the silver crucian carp is found to be unisexual and produces unreduced polyploid eggs (Kobayasi, 1971). The offspring from the maternal fish are usually genetically identical to the maternal fish (Dong and Taniguchi, 1996, Umino *et al.*, 1996; Dong *et al.* 1997). Therefore, we assumed that Ginbuna has not been influenced genetically by the introduced Gengoroubuna. The second species, Nagabuna or Okinbuna (*C. burugeri sp.*) is diploid (sex ratio 1:1) which reproduces sexually and may hybridize with the introduced Gengoroubuna.

In Lake Koyama of Tottori prefecture, some groups of genus *Carassius* with different morphological and physiological traits were also found (Sitizyo *et al.*, 1994). They were classified into three groups by morphological traits, and into two groups by their red blood cell size. Gengoroubuna was introduced into this lake for stock enhancement. Hence, there is a possibility that the indigenous diploid species, Nagabuna has been genetically influenced by introduced Gengoroubuna. In this study, we investigated the genetic influence of the introduced Gengoroubuna on native crucian carp using DNA and isozyme markers. We also studied the genetic relationship between diploid and triploid crucian carp.

## MATERIALS AND METHODS

### Determination of ploidy level and morphological trait analysis

**Sample collection and determination of ploidy level:** We collected 78 individuals consisting of three species (Ginbuna, Gengoroubuna and Nagabuna) from Lake Koyama of Tottori prefecture, Japan. We determined the ploidy level of samples by analyzing the red blood cell size using the methods detailed in Sezaki *et al.* (1977) and Onozato *et al.* (1983). Scientific names and common names of these crucian carp were given as in Hosoya (1993) and Matsubara and Ochiai (1965), respectively.

**Morphological analysis:** For morphological traits, we measured standard length (SL), body depth (BD), head length (HL), snout length (SNL), orbital length (OL), Caudal peduncle depth (CPD), length of dorsal fin base (DBL) and length of anal fin base (FBL). BD, HL, SNL, OL, CP, DBL and FBL were calculated as percentages of SL. Gill raker (GR) were also counted. The number of gill rakers was used as an important trait to identify the species of crucian carp. The mean values of two groups were statistically compared with each other using t-test (BD, HL, SNL, CPD, DBL and FBL). In case of non-equal variance, these values were analyzed using Dunn's method to test the difference between two groups (OL and GR).

### Determination of genotypes

**Isozyme marker:** The fish samples were preserved in a freezer at –20°C. Creatine kinase (CK; EC:2.7.3.2) from skeletal muscle were detected by horizontal starch-gel electrophoresis (Dong and Taniguchi, 1996). Identification of locus and alleles were performed according to Dong and Taniguchi (1996).

**mtDNA RFLP:** DNA samples were extracted according to Takagi *et al.* (1997). The region of approximately 2.1 kilo base pair (kbp) containing the D-loop region of the mtDNA was amplified by PCR. This region contains a part of the cytochrome *b* gene and the 12SrRNA gene region. Amplification of the specific region, thermal cycling parameters, digestion by restriction endonucleases, and the electrophoresis methods were identical to those described by Ohara *et al.* (1998). Primer sequences used (Martin *et al.*, 1992) were; L-15530 (25mer, ATATTAAACCCGAATGATATTT) and H1067 (25mer, ATATATGGGTATCTAATCCTAGTTT). The endonucleases used were *Hinf*I, *Rsa*I, *Mbo*I and *Taq*I. A composite mtDNA haplotypes, consisting of four letters, representing the fragment pattern generated by each of the restriction endonucleases, were compiled for each individual. The haplotype divergence $h=2n\,(1-\Sigma x_{i2})/(2n-1)$ was calculated (Nei, 1990).

**Microsatellite markers:** Three microsatellite primers *GF1**, *GF17** and *GF29**, developed by Zheng *et al.* (1995), were analyzed to measure the loci. Nomenclature of loci and alleles were according to Shaklee *et al.* (1990). The PCR was programmed for 7 cycles of 1 min at 94°C, 30 sec at 53°C, 30 sec at 72°C, 33 cycles of 30 sec at 90°C, 30 sec at 53°C, and 30 sec at 72°C, respectively. Following amplification, PCR products were mixed with a denaturing stop dye, heated at 95°C for 15 min, and electrophoresed on 6% polyacrylamide gel. Chemiluminescence detection of microsatellite loci was performed according to Perez-Enriquez *et al.* (1998). The reverse primer was end-labeled with biotin. The PCR and electrophoresis were performed according to Takagi *et al.* (1997). After the electrophoresis, DNA was transferred to a nylon membrane by blotting, then the membrane was dried and UV crosslinked. DNA on the membrane was detected by using Phototope™-Star Detection Kit (New England Biolabs). The sequence ladder obtained from the pUC19 plasmid was used as a size marker, and was prepared with the CircumVent Phototope™ Kit (New England Biolabs).

**Evaluation of genetic diversity and hybrid index:** The genetic diversity for each diploid group was estimated by the number of alleles, the effective number of alleles, and the observed ($H_o$) and expected heterozygosity ($H_e$). The significance of departure from Hardy-Weinberg equilibrium (HWE) for each locus at of each diploid group was tested by the exact test using the Markov Chain procedure as performed by ARLEQUIN soft ver 1.1 (Schneider *et al.* 1997).

Hybrid index ($I_H$) was evaluated from allele frequencies of the three microsatellite loci following Campton and Utter (1985). The index ($I_H$) was defined as

$$I_H = \frac{\log_{10}(P_x)}{\log_{10}(P_x)+\log_{10}(P_y)},$$

where

$$P_x = \prod_{i=1}^{L} k_i \prod_{j=1}^{A_i} (\mathrm{X}_{ij})^{m_{ij}},$$

$$P_y = \prod_{i=1}^{L} k_i \prod_{j=1}^{A_i} (\mathrm{Y}_{ij})^{m_{ij}},$$

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$X_{ij}$ and $Y_{ij}$ are the average frequencies of the jth allele at the ith locus for species *X* and *Y*, respectively; $m_{ij}$ is the number of alleles of the *j*th type observed at the ith locus for each individual being evaluated; $A_i$ is the total number of known alleles at the ith locus for the two species combined; $k_i$ is the binomial sampling coefficient associated with the genotype of an individual at the ith locus; and *L* is the number of diagnostic loci used to distinguish the two species. We have substituted a frequency of 0.001 for alleles with a frequency of zero. In this study, species *X* is Nagabuna and species *Y* is Gengoroubuna. The index can assume any value between 0.0 and 1.0 and will be close to one of these two values when individuals have a very high relative probability to belonging of species *X* (Nagabuna) or *Y* (Gengoroubuna), respectively.

**Identification of clonal lines in Ginbuna:** When at least the two individuals belong to one combined genotype on the three microsatellite loci, we recognized it as a independent clones. The three loci combined genotype which include only one individual was also regarded as a independent clones tentatively in this paper according to Ohara *et al*. (1999) in which the microsatellite marker was effective for identifiing all the clonal lines detected by combination of genetic markers of isozymes, mtDNA and DNA fingerprinting.

## RESULTS

### Determination of ploidy level and morphological traits

The results of the ploidy analysis showed that 28 individuals were triploid, and 50 individuals were diploid. The 28 triploid individuals were identified as Ginbuna (*C. langsdorfii*) according to Dong *et al*. (1996). The diploid individuals were classified into three groups using the number of gill rakers (Hosoya, 1993). The first group (28 individuals), with number of gill rakers ranging from 45 to 57, was identified as Nagabuna, *C. burugeri sp*.. The second group (12 individuals), with gill rakers ranging from 92 to 108, was identified as Gengoroubuna, *C. cuvieri*. The third group (10 individuals), with gill rakers ranging from 64 to 89, could not be identified as any *Carassius* species recorded in Japan. Sitizyo *et al*. (1994) reported that individuals with average number of gill rakers of 76.5±17.5, may be derived from hybridization between Gengoroubuna and another crucian carp species. Therefore, these fishes were designated "Hybrids" in this study. The Ginbuna (28 individuals) had the number of gill rakers ranging from 46 to 56. We analyzed these two ploidies and three gill rakers groups.

The results of morphological analysis were summarized in Table 1. BD and DBL of Nagabuna were significantly shorter than the other groups ($P$<0.05). HL and SNL were not significantly different among these four groups. The values of Hybrids were intermediate between Nagabuna and Gengoroubuna in BD, CPD, SNL, OL, DBL, ABL and GR. BD, CPD, OL, DBL and ABL of Ginbun were significantly different from Nagabuna ($P$<0.05).

### Genetic differentiation in diploid species

In the mtDNA RFLP analysis, the numbers of detected fragment Patterns were three in *Hinf*I, two in *Rsa*I, five in *Taq*I, three in *Mbo*I, respectively (Table 2). The haplotypes were decided by combining these fragments patterns. The haplotpe designations are the same with those described by Ohara *et al*. (1998). The seven haplotypes were found (#1, #6, #7, #9, #10, #11 and #12). Hybrids had the mtDNA haplotypes which are also common to the Gengoroubuna and Nagabuna populations (Table 2). The haplotype diversity in each of the three diploid groups ranged widely from 0.082 to 0.779 with the greatest in Hybrids.

The allele frequency of the three microsatellite DNA loci in the three diploid groups are summarized in Table 3, and banding patterns of microsatellite DNA are shown in Fig. 1. In *GF1** locus, the major alleles were **305* (0.805) in Nagabuna, **295* (0.583) in Hybrids and **295* (0.850) in Gengoroubuna. In *GF17** locus, the major allele was **184* in Nagabuna and Hybrids (0.692 and 0.458). For *GF29**, the major alleles were **188* in Nagabuna and Hybrids (0.962 and 0.458), and **202* in Gengoroubuna (0.750). Substitutions of the major alleles of Nagabuna and Gengoroubuna were found in *GF1** and *GF29**.

The number of alleles, genotypes, observed heterozygosity ($H_o$), and expected heterozygosity ($H_e$) in three diploid groups are summarized in Table 4. Observed heterozygosity (mean; 0.750) of the Hybrids population was higher than those of the other two diploid groups. The ratio of observed heterozygosity over expected heterozygosity ($H_o/H_e$) of Hybrids population indicated heterozygote excess as 1.278 and 1.270 for *GF1** and *GF29**, respectively. Genotypic frequencies for each of the three loci which were detected for the three diploid populations confirmed Hardy-Weinberg expectations ($P$<0.05) (Table 4).

The values of hybrid index ($I_H$) were ranged from 0.771 to 0.906 (mean 0.907) in Nagabuna from 0.102 to 0.806 (mean 0.476) in Hybrids, and from 0.068 to 0.157 (mean 0.110) in Gengoroubuna (Fig. 2).

**Table 1.** Morphological characters of Nagabuna, Hybrids, Gengoroubuna and Ginnbuna.

| | SL | SL/BD*1 | SL/HL*1 | SL/CPD*1 | SL/SNL*1 | SL/OL*2 | SL/DBL*1 | SL/ABL*1 | GR*2 |
|---|---|---|---|---|---|---|---|---|---|
| Nagabuna | 189.5±37.0 | 34.1±1.7 | 31.3±1.2$^a$ | 14.0±0.6$^a$ | 7.3±0.7$^a$ | 11.8±0.8$^a$ | 32.8±1.9 | 8.0±1.1 | 49.3±1.7$^a$ |
| Hybrids | 211.9±88.4 | 38.8±2.2$^a$ | 31.4±1.5$^a$ | 14.1±0.8$^a$ | 7.1±0.7$^a$ | 12.1±1.4$^a$ | 35.9±1.1$^a$ | 8.8±0.7$^a$ | 75.6±6.4$^b$ |
| Gengoroubuna | 242.6±55.9 | 44.4±2.5 | 29.9±1.4$^a$ | 15.5±0.9$^b$ | 6.9±1.0$^a$ | 12.2±1.5$^a$ | 37.9±1.9$^a$ | 9.1±0.5$^{ab}$ | 100.1±4.5$^b$ |
| Ginbuna | 172.0±38.1 | 37.4±2.1$^a$ | 30.2±1.7$^a$ | 14.9±0.9$^b$ | 7.2±0.8$^a$ | 10.4±0.9 | 36.8±2.4$^a$ | 10.5±1.0$^b$ | 45.9±1.8$^a$ |

*1 Value with the same superscripts are not significant difference in t-test ($P$<0.05) for compared with two groups

*2 Value with the same superscripts are not significant difference in Dann's Test ($P$<0.05) for compared two groups

SL; Standard Length, BW; Body Weight, BD; Body Depth, HL; Head Length, CPD; Caudal Peduncle Depth, SNL; Snout Length, OL; Orbital Length, DBL; Dosal Fin Base Length, ABL; Anal Fin Base Length, GR;Gill Raker, DFS; Dosal Fin Spine, AFS; Anal Fin Spine, LLS; Pored Scale on Lateral lines

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**Table 2.** Frequency distribution of seven haplotypes and haplotypic diversity (h) in Nagabuna (Naga), Hybrids (Hyb), Gengoroubuna (Gen) and Ginbuna (Gin) for mtDNA RFLP analysis.

| Haplotype | Naga | Hyb | Gen | Gin |
|---|---|---|---|---|
| # 1 (aaaa)[*1*2] | 0 | 0 | 0 | 0.111 |
| # 6 (bacb) | 0 | 0 | 0.100 | 0.889 |
| # 7 (babb) | 0 | 0 | 0.100 | 0 |
| # 9 (baaa) | 0.958 | 0.200 | 0 | 0 |
| #10 (dbfc) | 0 | 0.200 | 0.100 | 0 |
| #11 (dbcc) | 0 | 0.300 | 0.600 | 0 |
| #12 (dbdc) | 0.042 | 0.300 | 0.100 | 0 |
| n[*3] | 24 (4) | 10 | 10 (2) | 27 (1) |
| h | 0.000 | 0.779 | 0.000 | 0.000 |

[*1] Designation of haplotypes is the same with those described by Ohara (1998).
[*2] The letter in parethesis for each haplotypes denote restriction patterns for *Hinf*I, *Rsa*I, *Taq*I, *Mbo*I from left to right.
[*3] Numbers in parenthesis is unidentified.

### Genetic difference of triploids from diploids

In the isozyme analysis, the genotypes of Ginbuna indicated as **abc* in *CK** locus. Nagabuna and Gengoroubuna had the genotypes of **aa*, and Hybrids had the genotypes of **aa* and **ab* (one individual), while no individuals with allele of **c* in *CK** being specific in Ginbuna of western Japan (Taniguchi and Sakata 1977; Dong *et al*., 1996) was not observed. Haplotype #1 was limited to Ginbuna, and #7 to Gengoroubuna (Table 2). The Ginbuna population had an allele in the three microsatellite loci being uncommon to the diploid population.

### Clonal lines found in Ginbuna

In the microsatellite DNA analysis, the number of genotypes of Ginbuna were seven, six and four in the loci, *GF1**, *GF17** and *GF29**, respectively. All individuals in Ginbuna

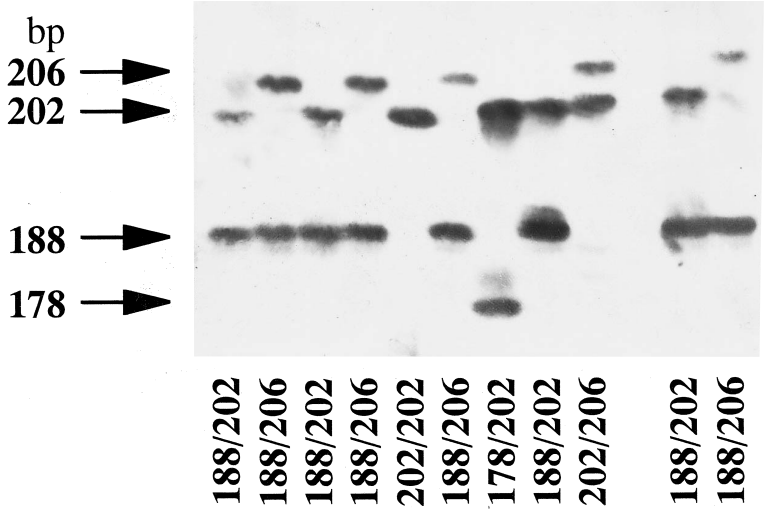


**Fig. 1.** Microsatellite banding patterns of eleven individuals of the Hybrids population in *GF29** microsatellite primer.

**Table 3.** Allele frequencies of the three microsatellite loci in diploid populations, Nagabuna, Hybrids and Gengoroubuna.

| *GF1** | Nagabuna | Hybrids | Gengoroubuna |
|---|---|---|---|
| **293* | 0.000 | 0.042 | 0.000 |
| **295* | 0.000 | 0.583 | 0.850 |
| **297* | 0.000 | 0.042 | 0.100 |
| **303* | 0.019 | 0.000 | 0.000 |
| **305* | 0.808 | 0.250 | 0.050 |
| **307* | 0.077 | 0.000 | 0.000 |
| **311* | 0.019 | 0.000 | 0.000 |
| **313* | 0.038 | 0.000 | 0.000 |
| **315* | 0.038 | 0.083 | 0.000 |
| **202* | 0.038 | 0.042 | 0.000 |

| *GF29** | Naga | Hybrids | Gengoroubuna |
|---|---|---|---|
| **172* | 0.000 | 0.000 | 0.100 |
| **178* | 0.000 | 0.042 | 0.000 |
| **188* | 0.962 | 0.458 | 0.000 |
| **194* | 0.019 | 0.000 | 0.000 |
| **195* | 0.019 | 0.000 | 0.000 |
| **200* | 0.000 | 0.000 | 0.100 |
| **202* | 0.000 | 0.292 | 0.750 |
| **206* | 0.000 | 0.208 | 0.050 |

| *GF17** | Nagabuna | Hybrids | Gengoroubuna |
|---|---|---|---|
| **174* | 0.000 | 0.000 | 0.150 |
| **182* | 0.000 | 0.042 | 0.000 |
| **183* | 0.019 | 0.000 | 0.000 |
| **184* | 0.692 | 0.458 | 0.000 |
| **188* | 0.000 | 0.292 | 0.150 |
| **190* | 0.000 | 0.042 | 0.000 |
| **192* | 0.000 | 0.042 | 0.250 |
| **196* | 0.000 | 0.000 | 0.200 |
| **198* | 0.000 | 0.000 | 0.250 |
| **204* | 0.096 | 0.042 | 0.000 |
| **207* | 0.019 | 0.000 | 0.000 |
| **208* | 0.019 | 0.000 | 0.000 |
| **215* | 0.019 | 0.000 | 0.000 |
| **216* | 0.038 | 0.000 | 0.000 |
| **218* | 0.019 | 0.000 | 0.000 |
| **220* | 0.019 | 0.042 | 0.000 |
| **224* | 0.019 | 0.000 | 0.000 |

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**Table 4.** Estimation of genetic diversity at the three microsatellite loci (*GF1**, *GF17** and *GF29**) in the three diploid populations (Ngabuna, Hybrids and Gengoroubuna).

| | *GF1** | *GF17** | *GF29** | AVG[*3] |
|---|---|---|---|---|
| Nagabuna | | | | |
| No. of alleles | 6 | 11 | 3 | 7 |
| E. no. of alleles[*1] | 1.51 | 2.02 | 1.08 | 1.539 |
| No. of genotypes | 7 | 11 | 3 | 7 |
| $H_o$ | 0.423 | 0.462 | 0.077 | 0.321 |
| $H_e$ | 0.338 | 0.506 | 0.075 | 0.306 |
| $H_o/H_e$ | 1.251 | 0.913 | 1.030 | 1.065 |
| P[*2] | 0.674 | 0.404 | 1.000 | 0.693 |
| Hybrids | | | | |
| No. of alleles | 5 | 8 | 5 | 6 |
| E. no. of alleles | 2.42 | 3.27 | 3.05 | 2.913 |
| No. of genotypes | 5 | 5 | 6 | 5 |
| $H_o$ | 0.750 | 0.667 | 0.833 | 0.750 |
| $H_e$ | 0.587 | 0.694 | 0.672 | 0.651 |
| $H_o/H_e$ | 1.278 | 0.961 | 1.240 | 1.160 |
| P | 0.586 | 0.407 | 0.583 | 0.525 |
| Gengoroubuna | | | | |
| No. of alleles | 3 | 5 | 4 | 4 |
| E. no. of alleles | 1.36 | 4.76 | 1.71 | 2.611 |
| No. of genotypes | 3 | 8 | 4 | 5 |
| $H_o$ | 0.300 | 0.700 | 0.300 | 0.433 |
| $H_e$ | 0.265 | 0.790 | 0.415 | 0.490 |
| $H_o/H_e$ | 1.132 | 0.886 | 0.723 | 0.914 |
| P | 1.000 | 0.296 | 0.127 | 0.474 |

[*1] Effective number of alleles = 1 / (1–Expected heterozygosity)
[*2] Probability of Hardy-Weinberg expectations
[*3] Average

population were heterozygous for all loci (Table 5). By combining the genotypes from three loci, Ginbuna was classified into six clonal lines which were designated as KOY-001 to KOY-006. The genotypes of these clonal lines were compared from those of the clonal lines collected from Kochi prefecture of southwest Japan (Ohara *et al*., 1999), such as KOC-006, KOC-010 and KOC-017. Although only one individual was found in KOY-002 and KOY-006, these are the same with the clonal lines found in Kochi prefecture. Fig. 3 shows that KOY-001 and KOC-017, KOY-002 and KOC-010, KOY-004 and KOC-006 had the same genotypes for the three microsatellite loci, respectively.

## DISCUUSSION

The Hybrids showed intermediate morphological characters between Nagabuna and Gengoroubuna. Kobayasi (1972) produced hybrids between Gengoroubuna and Kinbuna, *Carassius burgeri* subsp., in which the morphological characters were intermediate between the two parental species. Sitizyo *et al*. (1994) reported that the average number of gill rakers was 76.5±17.5, and speculated that these individuals could be derived from hybrids between Gengoroubuna and the other crucian carp. Fish with hybrid index ($I_H$) ranged between 0.2 and 0.8 could be recognized as natural hybrids (Campton, 1987). From the genetical and morphological results, we suggest that the Hybrids are derived from cross breeding between Gengoroubuna and Nagabuna. This is the genetic influence of introduced Gengoroubuna to the native diploid population, Nagabuna.

Genetic differences between the Nagabuna and Gengoroubuna were observed in both microsatellite and mtDNA markers. These genetic markers were useful for identification of the two species, and they were effective in tracing the hybridization of the two species in Lake Koyama. The **305* allele in *GF1** may also originate from Nagabuna (common in Nagabuna and Gengoroubuna), since the allele is major in Nagabuna. We estimated that haplotype #12 (common in Nagabuna and Gengoroubuna) originated from Gengoroubuna because this haplotype had similar banding patterns to haplotype #10 and #11, which were common in Gengoroubuna. The maternal species can be identified or, at least speculated if the two parental species are characterized by different haplotypes of mtDNA. Based on mtDNA analysis, the maternal species of two Hybrids individuals originated from Nagabuna (haplotype #9), and eight of those originated from Gengoroubuna (haplotype #10, #11 and #12). This is evidence that both Nagabuna and Gengoroubuna species, were possible to be maternal species of Hybrids.

The genotypes of microsatellite loci of the Hybrids give the evidence of introgressive hybridization occurred by backcrossing with their parental species. However, the original populations of Gengoroubuna and Nagabuna appeared not to have frequently introgressed with each other, since only one allele **305* of *GF1** and one haplotype #12 are common in both Nagabuna and Gengoroubuna populations (Fig. 2). A small amount of introgression may be very difficult to detect. Campton (1987) suggested that introgressive hybridization could cause the genetic loss of an entire species or a unique population. In case of genus *Carassius* at the Lake Koyama, introgressive hybridization between Nagabuna and Gengoroubuna may have caused genetic loss of the Nagabuna population. At the same time, it may have resulted in the reduction of restocking effect on Gengoroubuna.

The results of isozymes, mitochondria and microsatellite DNA markers verified that the three diploid and one triploid populations (Ginbuna) do not belong to the same gene pool. The Ginbuna population consisted of several clonal lines which were similar to the previous reports (Dong *et al*., 1996; Umino *et al*., 1997; Ohara *et al*., 1998). In this study, Ginbuna collected from Lake Koyama showed high proportion of heterozygotes in loci of isozymes and microsatellite DNA. This may mean that it is a general character of Ginbuna which may relate to its origin (Simizu *et al*., 1993). However, there is no possibility that Ginbuna was produced by hybridization between Gengoroubuna and Nagabuna, since Ginbuna has different alleles from the diploid populations such as gene **c* in *CK**. The same genome types from three clonal lines, KOY-001, KOY-002 and KOY-004 are found in the different place located in Kochi Pref. (KOC-017, KOC-010, and KOC-006). These fish may belong to the same clonal lines though the sampling locations were distributed over a long distance about 200 km across the mountain and the sea.

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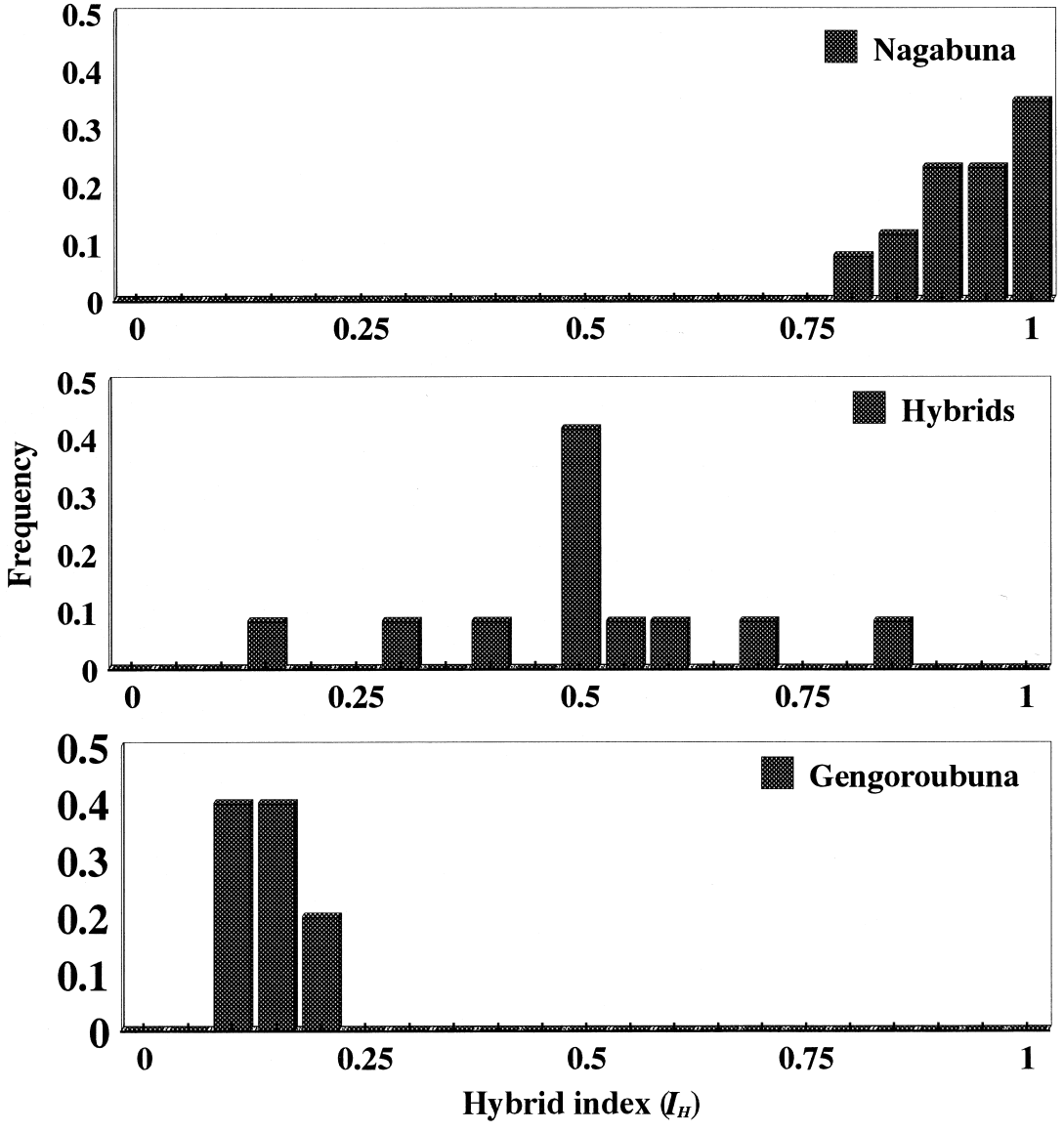


**Fig. 2.** Distribution of hybrid index score ($I_H$) for diploid crucian carp (Nagabuna, Hybrids and Gengoroubuna) from Lake Koyama. Hybrid indices were calculated from microsatellite genotypes.

**Table 5.** The genotypes, number of alleles, number of genotypes and allelic combination of the three microsa loci of six clonal lines of Ginbuna, Carassius langsdorfii elaburate on what *GF1**, *GF17** and *GF29** represen

| Types of clone (remarks) | *GF1** | *GF17** | *GF29** | Number of individuals | Haplotypes |
|---|---|---|---|---|---|
| KOY-001 (KOC-017) | *297/303/311* | *188/192/216* | *188/(188 or 194)/194* | 15 | #6 |
| KOY-002 (KOC-010) | *293/309/311* | *184/188/216* | *188/(188 or 194)/194* | 1 | #6 |
| KOY-003 | *297/307/315* | *186/(186 or 192)/192* | *192/(192 or 194)/194* | 6 | #6 |
| KOY-004 (KOC-006) | *303/307/311* | *191/192/216* | *186/188/194* | 3 | #1 |
| KOY-005 | *303/(303 or 307)/307* | *184/186/202* | *188/(188 or 194)/194* | 1 | #6 |
| KOY-006 | *297/303/311* | *182/184/186* | *192/(192 or 194)/194* | 2 | #6 |
| Number of alleles | 7 | 8 | 4 | | |
| Number of genotypes | 7 | 6 | 4 | | |
| Proportion of heterozygotes | 1.000 | 1.000 | 1.000 | | |
| Propotion of triallelic | 0.964 | 0.786 | 0.107 | | |
| Propotion of diallelic | 0.036 | 0.214 | 0.893 | | |

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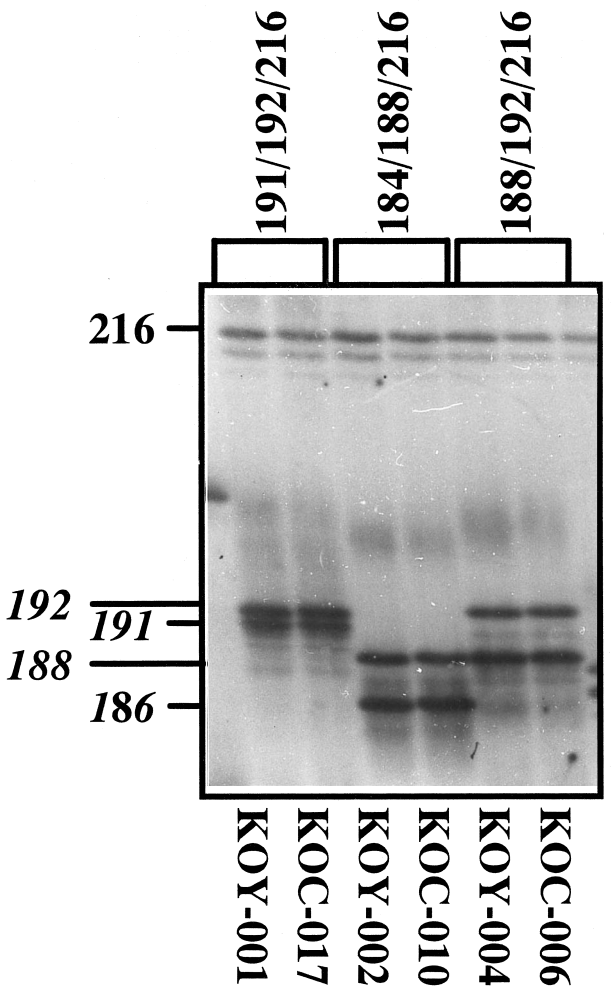


**Fig. 3.** Comparison of microsatellite banding patterns of Ginbuna collected from Lake Koyama (KOY) and Kochi (KOC), designated as KOY-001, KOC-017, KOY-002, KOC-010, KOY-004, KOC-006, from the *GF17** primer.

We found that a restocked Gengoroubuna habitat of indigenous diploid crucian carp led to the confusion of the genetic resources. In contrast, we suspected that one of the reasons of the taxonomic confusion of Japanese crucian carp was due to the hybridization caused by restocking of Gengoroubuna to the foreign waters. To solve the confusion in classification of Japanese crucian carp, we suggest that it is necessary to recognize both phenomena as the genetic independence of the diploid and triploid crucian carp and the hybridization of the introduced Gengoroubuna with the indigenous diploid crucian carp.

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