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Authors: Ohara, Kenichi, Ariyoshi, Takahiro, Sumida, Eiji, and Taniguchi, Nobuhiko

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Clonal Diversity in the Japanese Silver Crucian Carp, *Carassius langsdorfii* Inferred from Genetic Markers

Kenichi Ohara[1†], Takahiro Ariyoshi[2], Eiji Sumida[2] and Nobuhiko Taniguchi[1*]

[1]*Laboratory of Applied Population Genetics, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, 981-8555 Japan*
[2]*Department of Aquaculture, Faculty of Agriculture, Kochi University, Monobe, Nankoku, Kochi, 783-8502 Japan*

ABSTRACT—The Japanese silver crucian carp, *Carassius langsdorfii*, is a naturally polyploid fish that reproduces by gynogenesis, resulting in a geographically clustered clonal population. To determine the amount of clonal diversity in this species, we used genetic markers to compare fish from several Japanese locations. Samples were collected from Lake Kasumigaura (Ibaraki Pref.), Lake Imba (Chiba Pref.), Lake Fukushimagata (Niigata Pref.), Lake Biwa (Shiga Pref.), Lake Koyama (Tottori Pref.), and the Niyodo and Monobe Rivers (Kochi Pref.). The genetic markers used were the microsatellite loci *GF1**, *GF17**, and *GF29**, as well as isozyme (creatinekinase *CK**) and mtDNA (D-loop region) RFLP. In the seven locations sampled, 61 clonal lines were identified by combining the genotypes of the three microsatellite loci. Populations at all locations consisted of six to 28 clonal lines. Fourteen common clonal lines were detected in two or more locations, and 13 of these were observed in Lake Biwa. We suggest that these clonal lines have been widely distributed in Japanese freshwaters through the intentional stocking of fish species such as "gengorobuna" (*C. cuvieri*) and "ayu" (*Plecoglossus altivelis altivelis*) obtained from Lake Biwa and its adjacent waters. The *CK** genotypes and mtDNA haplotypes of fish from Lake Kasumigaura differed from those of other locations.

Key words: *Carassius langsdorfii,* clonal diversity, microsatellite, silver crucian carp

INTRODUCTION

Unisexual fishes are usually polyploid, and they frequently reproduce by a non-bisexual reproductive mechanism such as gynogenesis or hybridogenesis (Dawley, 1989). The Japanese crucian carp is one such polyploid fish that reproduces by gynogenesis.

Studies of the various reproductive modes in unisexual fishes, including the cytological mechanisms that underlie them, occasionally make taxonomic revision necessary. It has until now been accepted that the Japanese silver crucian carp is represented by diploid (found only in western Japan), triploid and tetraploid forms (Kobayasi, 1971), the latter two forms reproducing gynogenetically. The diploid crucian carp "kinbuna", *Carassius buergeri* subsp. was thought to be distributed only in north-east Japan (Nakamura, 1969), although a western Japanese diploid bisexual form, known as "Okinbuna", *C. buergeri* subsp., (Ochiai *et al.* 1979), was found to have highly similar electrophoretic and morphological characteristics (Taniguchi and Sakata, 1977). The triploid silver crucian carp produces unreduced triploid eggs (Yamashita *et al*., 1993), meaning that the offspring are usually genetically identical to the parent (Dong and Taniguchi, 1996; Umino *et al*., 1996). The triploid silver crucian carp is thus genetically independent from the diploid form (Ohara *et al*., 2000). On the basis of these findings, we consider the Japanese silver crucian carp, *C. langsdorfii* (Matsubara and Ochiai, 1965), is a naturally polyploidy fish that reproduces gynogenetically (Yamashita *et al*., 1993; Dong and Taniguchi, 1996).

Populations of some unisexual fishes have been shown to consist of several clonal lines (Moore, 1984). Wild Japanese silver crucian carp also consist of several genetically divergent clonal lines (Dong *et al.,* 1996; Umino *et al*., 1997; Ohara *et al.,* 1998). Large geographically-determined genetic divergences have also been reported (Taniguchi and Sakata, 1977; Shimizu *et al.,* 1993); however, some clonal lines have been found to be present at distant locations in Japan (Ohara *et al.,* 2000). A comprehensive study of the

* Corresponding author: Tel. +81-22-717-8740;
FAX. +81-22-717-8743.
E-mail: nobuhiko@bios.tohoku.ac.jp
† Present address: Lake Biwa Museum, 1091 Oroshimo, Kusatsu, Shiga, 525-0001 Japan

distribution of clones throughout Japan has not yet been carried out.

Microsatellite DNA polymorphism might be the most sensitive marker available for the identification of clonal lines by determining the zygotic condition and genotype of each locus (Ohara *et al.,* 1998). Mitochondrial DNA analysis is not very useful for identifying clones, but it can be effective for demonstrating maternal origins (Murakami *et al.,* 2001); therefore mtDNA analysis might be applicable in studies of clonal evolution. It is also known that creatinekinase (*CK**) polymorphism is an effective marker for detecting genetic variation among different geographic locations in the Japanese silver crucian carp (Taniguchi and Sakata, 1977; Dong and Taniguchi, 1996).

In this study, we investigated the clonal component, the distribution of clones, of samples collected from six locations around Japan using microsatellite DNA polymorphism, RFLP of mitochondrial DNA and electrophoretic patterns of *CK*.*

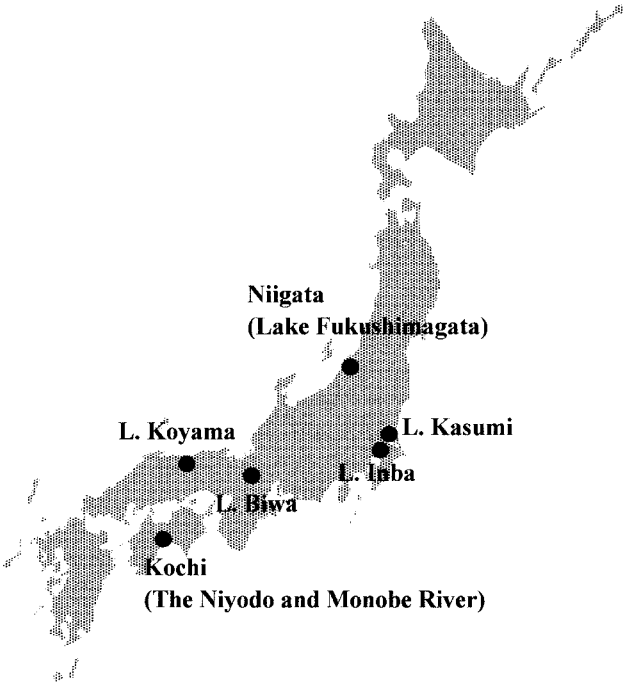

Fig. 1. Sampling locations of the Japanese silver crucian carp, *C. langsdorfii*.

MATERIALS AND METHODS

Sample collection and ploidy determination: We collected 74 fish samples from Lake Biwa in the Shiga Pref. (L. Biwa), 31 samples from Lake Fukushimagata in the Niigata Pref. (L. Fuku), 71 samples from Lake Kasumigaura in the Ibaraki Pref. (L. Kasumi), and 45 samples from Lake Imba in the Chiba Pref. (L. Imba) (Fig. 1). We also used previously published data to compare clonal components with each other (Ohara *et al*., 1999; 2000). From these earlier reports, we quote sixteen clonal lines (KOC-001~–015, –017) in 237 samples from two rivers, the Niyodo and Monobe Rivers in the Kochi pref. (Kochi) (Ohara *et al*., 1999), and six clonal lines (KOY-001~006) in 29 samples from Lake Koyama (L. Koyama) in the Tottori Pref. (Ohara *et al*., 2000). Because the distance between the two rivers in the Kochi prefecture was relatively close, we pooled the data from them, denoting them by the name Kochi. We determined the ploidy level of each sample by analyzing the major diameter of the erythrocyte (up to 15 μm) using the methods of Sezaki *et al.* (1977) and Onozato *et al.* (1983). As individuals of crucian carp verified as triploid were defined to be Japanese silver crucian carp, *Carassius langsdorfii* (Dong *et al.,* 1997), we used only the

Table 1. The genotypes in the three microsatellite loci, genotypes of *CK** and haplotype of mtDNA of 14 common clonal lines in Japanese silver crucian carp collected from six locations

	Microsatellite locus					Locality code[ii]					
	*GF1**	*GF17**	*GF29**	*CK**	mtDNA	Biw	Koc	Koy	Nii	Kas	Imb
COM-001	**297/301/303*	**186/(186or194)/194*	**194/196/202*	**bbb*	#13	2	0	0	0	0	1
COM-002	**297/303/305*	**186/(186or194)/194*	**194/196/202*	**aab*	#6	1	0	0	0	0	2
COM-003 (KOC-001[i])	**307/(307or311)/311*	**185/192/193*	**188/(188or194)/194*	**abc*	#1	0	10	0	1	0	2
COM-004 (KOC-002[i])	**299/303/335*	**186/192/198*	**190/198/202*	**bbc*	#2	1	16	0	0	0	0
COM-005 (KOC-004[i])	**303/(303or305)/305*	**185/187/216*	**188/(188or194)/194*	**abc*	#1	3	26	0	3	1	2
COM-006 (KOC-005[i])	**299/(299or303)/303*	**188/189/198*	**186/188/194*	**abc*	#4	4	4	0	0	0	0
COM-007 (KOC-006[i])	**303/307/311*	**191/192/216*	**186/188/194*	**abc*	#1	1	20	1	5	0	2
COM-008 (KOC-008[i])	**297/(297or303)/303*	**194/(194or204)/204*	**192/(192or200)/200*	**abc*	#5	2	36	0	0	0	3
COM-009 (KOC-010[i])	**293/309/311*	**184/188/216*	**194/(194or196)/196*	**abc*	#6	1	10	4	0	0	1
COM-010 (KOC-013[i])	**303/(303or305)/305*	**194/(194or200)/200*	**196/(196or202)/202*	**abc*	#8	2	11	0	0	0	0
COM-011 (KOC-014[i])	**303/(303or311)/311*	**182/184/216*	**188/192/196*	**abc*	#6	2	11	0	0	0	0
COM-012 (KOC-015[i])	**303/311/335*	**186/192/216*	**190/194/196*	**abc*	#6	3	2	0	0	1	0
COM-013 (KOC-017[i])	**299/303/311*	**188/192/216*	**194/(194or196)/196*	**abc*	#6	2	7	15	1	0	0
COM-014 (KOY-003[i])	**297/307/315*	**186/(186or192)/192*	**192/(192or194)194*	**abc*	#6	2	0	6	1	0	0

[i] These designations come from Ohara *et al*., (1999) and Ohara *et al*., (2000)
[ii] Locality code: Biw, L. Biwa; Koc, Kochi; Koy, L. Koyama; Imb, L. Imba; Kas, L. Kasumi

Table 2. The genotypes in the three microsatellite loci, genotypes of *CK** and haplotype of mtDNA of 47 unique clonal lines in Japanese silver crucian carp collected from six locations

	Microsatellite locus					
L. Biwa	*GF1**	*GF17**	*GF29**	*CK**	mtDNA	N
BIW-001	**293/297/301*	**184/(184or190)/190*	**194/(194or198)/198*	**abc*	#6	3
BIW-002	**297/(297or303)/303*	**186/188/196*	**192/194/196*	**abc*	#6	2
BIW-003	**293/301/313*	**186/(186or194)/194*	**194/(194or202)/202*	**abc*	#6	3
BIW-004	**297/303/305*	**184/(184or194)/194*	**192/194/200*	**abc*	#6	4
BIW-005	**297/303/311*	**194/198/200*	**194/(194or196)/196*	**abc*	#14	6
BIW-006	**303/(303or305)/305*	**184/186/194*	**186/194/196*	**abc*	#15	10
BIW-007	**297/299/303*	**186/(186or194)/194*	**190/192/196*	**abc*	#17	1
BIW-008	**301/303/317*	**194/(194or204)/204*	**202/202/202*	**abc*	#14	1
BIW-009	**303/(303or311)/311*	**184/188/189*	**184/188/194*	**abc*	#15	1
BIW-010	**297/307/313*	**184/186/192*	**192/194/202*	**abc*	#6	1
BIW-011	**293/297/301*	**184/(184or194)/194*	**194/(194or196)/196*	**abc*	#6	10
BIW-012	**301/(301or307)/307*	**188/(188or192)/192*	**186/(186or194)/194*	**aab*	#16	3
BIW-013	**297/301/303*	**184/(184or194)/194*	**194/196/202*	**abc*	#6	1
BIW-014	**295/297/303*	**186/188/194*	**194/196/202*	**abb*	#13	1
BIW-015	**295/301/303*	**184/(184or194)/194*	**192/194/200*	**bcc*	#14	1
Kochi						
KOC-003[i]	**303/307/311*	**188/(188or192)/192*	**188/(188or194)/194*	**abc*	#3	5
KOC-007[i]	**303/(303or311)/311*	**194/(194or204)/204*	**188/(188or194)/194*	**abc*	#3	4
KOC-009[i]	**303/(303or311)/311*	**184/(184or216)/216*	**188/(188or194)/194*	**aab*	#1	6
KOC-011-1[i]	**307/(307or311)/311*	**182/192/194*	**188/(188or194)/194*	**abc*	#3	65
KOC-011-2[i]	**307/(307or311)/311*	**182/(182or194)/194*	**188/(188or194)/194*	**abc*	#3	1
KOC-012[i]	**303/(303or305)/305*	**185/186/187*	**188/(188or194)/194*	**abc*	#7	3
L. Koyama						
KOY-005[i]	**303/(303or307)/307*	**184/186/202*	**188/(188or194)/194*	**abc*	#6	1
KOY-006[i]	**297/303/311*	**182/184/186*	**192/(192or194)/194*	**abc*	#6	2
Niigata						
NII-001	**301/303/307*	**194/(194or200)/200*	**188/194/196*	**abc*	#6	4
NII-002	**297/301/303*	**184/(184or186)/186*	**194/(194or202)/202*	**abc*	#6	8
NII-003	**303/309/311*	**193/193/193*	**194/(194or200)/200*	**aab*	#15	1
NII-004	**295/307/311*	**184/184/184*	**188/(188or204)/204*	–	#6	1
NII-005	**297/309/311*	**195/(195or197)/197*	**188/194/196*	–	#4	1
NII-006	**303/(303or307)/307*	**184/185/216*	**188/(188or194)/194*	–	#15	1
NII-007	**299/303/305*	**188/(188or190)/190*	**194/(194or204)/204*	–	#6	3
NII-008	**295/(295or303)/303*	**185/193/194*	**186/194/196*	**abb*	#16	1
L. Kasumi						
KAS-001	**309/313/343*	**192/194/204*	**194/(194or204)/204*	**bbb*	#13	45
KAS-002	**297/301/303*	**194/194/194*	**188/(188or194)/194*	**abc*	#4	2
KAS-003	**297/(297or301)/301*	**184/190/194*	**192/196/202*	**abc*	#6	1
KAS-004	**295/(295or297)/297*	**184/(184or186)/186*	**196/198/202*	**abc*	#6	1
KAS-005	**303/(303or305)/305*	**188/193/194*	**188/(188or194)/194*	**abc*	#1	1
KAS-006	**295/301/309*	**194/(194or196)/196*	**186/(186or194)/194*	**aab*	#16	1
KAS-007	**293/293/293*	**188/190/216*	**188/194/196*	**abc*	#15	1
KAS-008	**297/(297or317)/317*	**188/(188or194)/194*	**186/194/196*	**abc*	#16	1
KAS-009	**303/307/309*	**194/194/194*	**186/194/196*	**aab*	#16	15
KAS-010	**301/303/307*	**186/(186or194)/194*	**188/194/196*	**abb*	#16	1
L. Inba						
IMB-001	**303/309/311*	**194/194/194*	**184/186/194*	**aab*	#16	18
IMB-002	**293/307/311*	**184/(184or216)/216*	**184/186/194*	**aab*	#16	3
IMB-003	**303/(303or311)/311*	**188/192/216*	**194/194/194*	**aab*	#13	2
IMB-004	**297/303/309*	**194/194/194*	**186/194/196*	**aab*	#16	3
IMB-005	**297/303/311*	**184/(184or188)/188*	**188/(188or194)/194*	**aab*	#16	1
IMB-006	**303/305/311*	**182/194/216*	**188/192/196*	**abc*	#6	1

[i] These designations come from Ohara *et al*., (1999) and Ohara *et al*., (2000)

Japanese silver crucian carp (triploid) to genetic analysis in this study.

Microsatellite DNA marker loci: Extraction of DNA was performed according to the methods of Takagi *et al.* (1997). Microsatellite primers, which were developed by Zheng *et al.* (1995), were used to detect the loci *GF1**, *GF17**, and *GF29** in this experiment. The reverse primer had the 5`-end labeled with biotin. The PCR was programmed for seven cycles of 1 min at 94°C, 30 sec at 53°C, and 30 sec at 72°C, and 33 cycles of 30 sec at 90°C, 30 sec at 53°C, and 30 sec at 72°C. Following amplification, the PCR product was mixed with denaturing stop dye, heated at 95°C for 15 min, and electrophoresis was performed using a 6% denatured polyacrylamide gel. Chemiluminescence detection was carried out according to Perez-Enriquez *et al.* (1998). After electrophoresis, DNA was transferred to a nylon membrane by blotting, and the membrane was then dried and UV cross-linked. DNA on the membrane was detected using a Phototope™ – Star Detection Kit (New England Biolabs). The sequence ladder obtained from the M13 was used as a size marker, and was prepared using a Phototope™ CircumVent Kit (New England Biolabs). The nomenclature of loci and alleles fol-

Table 3. Genetic diversity in three microsatellite loci, *CK** and mtDNA RFLP in Japanese silver crucian carp collected from six locations

	Biw	Koc	Koy	Nii	Kas	Imb
Sample size	74	237	29	31	71	42
No. of clonal lines	28	17	6	13	14	13
Presumed no. of clonal lines	40.53	17.33	ND.	ND.	ND.	18.05
Clonal diversity	0.949	0.871	0.687	0.892	0.559	0.824
Microsatellite DNA marker						
Mean no. of alleles	13.3	11.0	7.0	10.7	11.3	10.0
Mean no. of genotypes	21.0	11.3	5.0	12.7	10.0	11.3
Heterozygosity						
Triallelic	0.536	0.588	0.586	0.440	0.640	0.587
Diallelic	0.459	0.412	0.414	0.583	0.338	0.246
Homozygosity	0.005	0	0	0.022	0.022	0.167
Genotype frequency of *CK**						
**aab*	0.054	0.042	0	0.040	0.239	0.578
**abb*	0.014	0	0	0.040	0	0
**abc*	0.878	0.890	1.000	0.920	0.127	0.289
**bbb*	0.027	0	0	0	0.625	0.133
**bbc*	0.014	0.068	0	0	0	0
**bcc*	0.014	0	0	0	0	0
Haplotype frequency						
#1 (aaaa)	0.054	0.262	0.034	0.290	0.028	0.133
#2 (badb)	0.014	0.068	0	0	0	0
#3 (cacb)	0	0.316	0	0	0	0
#4 (babb)	0.054	0.017	0	0.032	0.028	0
#5 (bbbb)	0.027	0.152	0	0	0	0.067
#6 (bacb)	0.437	0.127	0.966	0.581	0.042	0.133
#7 (baeb)	0	0.013	0	0	0	0
#8 (aacb)	0.027	0.046	0	0	0	0
#13 (fcad)	0.041	0	0	0	0.634	0.089
#14 (bbab)	0.108	0	0	0	0	0
#15 (baca)	0.149	0	0	0.065	0	0
#16 (daea)	0.041	0	0	0.032	0.245	0.578
#17 (bagb)	0.014	0	0	0	0	0
No. of haplotypes	11	8	2	5	5	5
Haplotype diversity	0.737	0.787	0.067	0.582	0.534	0.625

*Hin*f I, d, 470bp, 420bp, 250bp, 210bp; f, 650bp, 470bp, 420bp, 160bp
Rsa I, c, 560bp, 440bp, 390bp, 260bp
Taq I, g, 1200bp, 600bp
Mbo I, d, 1610bp, 380bp

low that of Shaklee *et al.* (1990).

Identification of clonal lines and estimation of genetic diversity: When one or more individuals belonged to one combined genotype of the three microsatellite loci, we recognized it as an independent clonal line. In the two band types, one allele was recognized to be duplicated in one of the two bands (Ohara *et al.*, 1999). The clonal diversity (*D*) was estimated by the formula, $D = 1 - \sum n_i (n_i - 1) / N (N - 1)$ (Moore, 1984), where n_i is the number of individuals of a given clone and N is the total number of individuals. To estimate the number of different clonal lines at each location; the octave method (Ohtsuka and Tsuji, 1997) was used.

Isozymes: Muscle samples were preserved in a freezer at –20°C. For isozyme analysis, polymorphism of creatinekinase (CK; EC: 2.7.3.2) was detected by horizontal starch-gel electrophoresis. The designation of alleles and genotypes followed Dong and Taniguchi (1996).

mtDNA RFLP: Analysis of mtDNA RFLP followed Ohara *et al.* (1998). The region of approximately 2.1 kbp in length, containing the whole D-loop region of the mtDNA, was amplified by PCR. This region contains part of the cytochrome b gene and the 12SrRNA gene region. The primer sequences used were L15556 and H1067. The restriction endonucleases used in this study were *Hin*f I, *Rsa* I, *Mbo* I, and *Taq* I. A composite mtDNA haplotype, consisting of four letters and representing the fragment pattern generated by each of the endonucleases, was compiled for each individual. The haplotype diversity $h = 2n (1-\sum x_i^2) / (2n - 1)$ was calculated (Nei, 1987). Differences among the haplotypes were determined using the program FreeTree (Pavlíek *et al.*, 1999). Genetic distances (*d*) of haplotypes were computed from Nei-Li's coefficient of similarity (*s*), as $d = 1 - s$. The dendrogram was constructed by the UPGMA and bootstrap values (for 1000 resamplings) were computed for every node.

RESULTS

Microsatellite analysis: Combining the genotypes of the three microsatellite loci investigated, 61 clones were identified from the six locations (Tables 1 and 2). A total of 39 clonal lines were newly discovered. Fifteen unique clonal lines (designated as BIW-001 to -015) were detected in L. Biwa, eight (NII-001 to -008) in L. Fuku., ten (KAS-001 to -010) in L. Kasumi, and six (IMB-001 to -006) in L. Imba (Table 2). Fourteen common clonal lines were detected in two or more locations and designated as common clones COM-001 to -014. The common clonal lines KOC-001, -002, -004, -005, -006, -008, -010, -013, -014, -015, -017, and KOY-003, that had been recognized previously were renamed as COM-003 to -014, respectively (Table 1) (Ohara *et al.*, 1999; Ohara *et al.,* 2000). COM-005 and COM-007 were found in five locations, and COM-009 and COM-013 in four locations. The four clones had a common allele, **216* in *GF17** and genotype **abc* in **CK*. Thirteen of the 14 common clonal lines were observed in L. Biwa, and 11 in the Kochi prefecture samples.

The level of genetic variability observed in the microsatellite DNA is summarized in Table 3. The total numbers of clones differ by location, ranging from six in L. Koyama to 28 in L. Biwa. Genotype frequencies of triallelic and diallelic fish in all locations were almost the same, and homozygous genotypes were rarely found. The clonal diversity is distributed from 0.559 in L. Kasumi to 0.949 in L. Biwa (Table 3). The total number of clonal lines expected was 40.53 in L. Biwa, 17.33 in the Kochi pref. and 18.03 in L. Imba (Table 3); other locations were in disagreement with a log-normal distribution because of the insufficient number of samples.

Isozymes: Three alleles, **a, *b*, and **c*, and six genotypes, **aab*, **abb*, **abc*, **bbb*, **bbc*, and **bcc* were found in *CK** (Table 3). Individuals having identical genotypes in their microsatellite loci also possessed the same genotypes in *CK*,* and each of the six genotypes included many clones. The most common genotypes differed among L. Kasumi (**bbb*), L. Imba (**aab*) and the other three locations (**abc*) (Table 3). Eleven of the 14 most common clones had genotype **abc*.

mtDNA RFLP: In the mtDNA RFLP analysis, the numbers of detected fragment patterns were five in *Hin*f I, three in *Rsa* I, six in *Taq* I and three in *Mbo* I (Table 3). The haplotypes were determined by combining these fragment patterns. The eight haplotype designations, #1 to #8, were the same as those described by Ohara *et al.* (1998). Five new haplotypes, #13 to #17, were found in this study, and their fragment patterns are shown in Table 3. The haplotypes #9 to #12 were already listed in a previous publication (Ohara *et al.*, 2000). The individuals having identical genotypes in their microsatellite loci also have the same mtDNA haplotype, and each haplotype except for #2, #5, and #7 contained many clones. The haplotype diversities in six locations ranged widely, from 0.067 to 0.787 (Table 3), being extremely low in L. Koyama. The haplotype frequencies differed by location: haplotypes #1 and #6 were found in all locations, and the frequency of haplotype #6 was highest in L. Koyama (0.966) and L. Fuku (0.581). The frequencies of

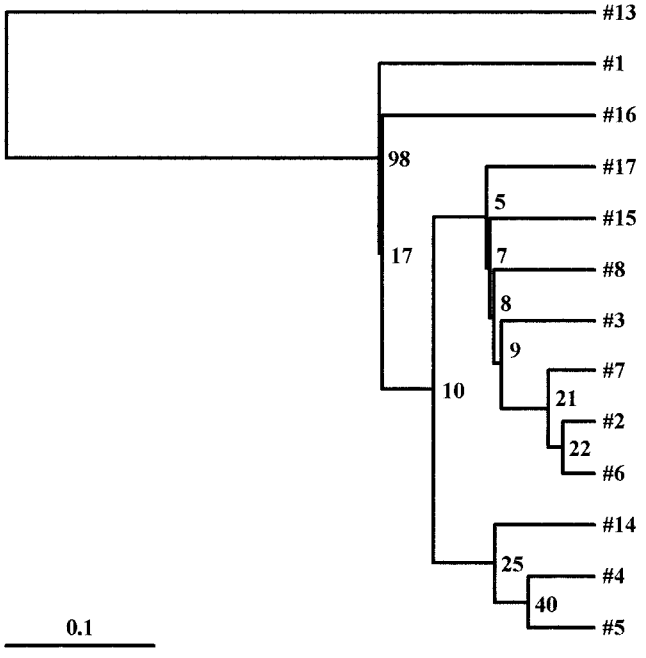

Fig. 2. Phylogenetic tree of haplotypes constructed on the basis of mtDNA RFLP using the UPGMA method and the FreeTree software application.

haplotypes #13 and #16 were highest in L. Kasumi (0.634) and L. Imba (0.518), respectively. Haplotype #13 was considerably different from the other eleven haplotypes, and this branch had again a bootstrap value of 100% (Fig. 2).

DISCUSSION

Efficiency of microsatellite DNA in the identification of clonal lines

Sixty-one clonal lines were detected by the combined genotypes of microsatellite DNA markers. The validity of their clonal nature and the effectiveness of these markers for the identification of clones were shown by the fact that the individuals having identical microsatellite genotypes belonged to the same *CK** genotypes and mtDNA haplotypes. The number of clonal lines observed in the Japanese silver crucian carp was rather high in comparison with other gynogenetic fishes, such as species of *Poeciliopsis, Menidia*, and *Phoxinus*, although the index of clonal diversity was similar to that of other unisexual species (Moore, 1984; Echelle *et al*., 1989; Elder and Schlosser, 1995). The observed numbers of clones in L. Biwa and L. Imba were 70% of the expected total number of clonal lines, whereas the observed and expected numbers of clones were almost identical in the Kochi prefecture. However, we used only three microsatellite loci in this study. If we were to use more loci, there is a possibility that we might identify many more clones.

Genetic divergence of Japanese silver crucian carp

In an earlier study (Taniguchi and Sakata, 1977), the genotypes **bbb* of *CK** was highly prevalent in L. Kasumi, whereas most of the other locations except for the Kanto district consisted of **abc* genotypes. In this study, the **bbb* genotype was again mostly detected in L. Kasumi, and **abc* was mostly detected in western Japan. Shimizu *et al.* (1993) classified Japanese crucian carp into three groups, the “Pacific”, “Sea of Japan”, and “Western” types. These results support the hypothesis that Japanese silver crucian carp in the Kanto district are different from those of other locations.

It is believed that the Japanese silver crucian carp originated from hybridization between two unknown species (Taniguchi, 1974; Shimizu *et al*., 1993). Murakami *et al.* (2001) suggested that it consisted of two lineages with two different maternal ancestors. In this study, mtDNA haplotype #13 was divergent from the other haplotypes (Fig. 2). Because the four clonal lines with haplotype #13 did not have the allele **a* of *CK**, haplotype #13 must have originated from a different maternal ancestor.

Our findings also suggest that the Japanese silver crucian carp is at least diphyletic. The parental species have not been determined (Vrijenhoek *et al*., 1989; Shimizu *et al*., 1993). Allele **c* of *CK** has never been found in diploid crucian carp in Japan (Taniguchi and Sakata, 1977). Allele **B* of *AMY-2** (Shimizu *et al*., 1993) and the tandem repeated DNA sequence (Cal3nDr) (Murakami and Fujitani, 1997) are also specific to Japanese silver crucian carp. Therefore, it is possible that the silver crucian carp did not arise in Japan and that its roots might be traceable to other East Asian countries.

Historical distribution of the common clones

In this study, 14 common clonal lines were detected in two or more (up to five) locations. Echelle *et al.* (1989) reported that identical clonal lines of the unisexual genus Menidia were naturally distributed at several different locations along the shores of the Gulf of Mexico. In contrast, in the selfing hermaphroditic fish *Rivulus marmoratus*, identical clonal lines were not detected in two different years at the same location (Turner *et al*., 1992). There are at least two possible explanations for the presence in different locations of common clones of the Japanese silver crucian carp. The first is that common clones become naturally distributed over a long evolutionary period. The second possibility is that the common clones were distributed through anthropogenic means.

As to the second historical process, intentional stocking of freshwater fish species in the most recent 50 years could have accelerated the spread of distribution ranges. Fish species such as *Cyprinus carpio, Hypomesus transpacificus,* and *Plecoglossus altivelis altivelis* have been intentionally introduced to ponds and rivers in Japan to enhance fish resources over the last 50 years. This intentional stocking has caused spreads in the distributions of non-targeted species such as *Rhodeus ocellatus ocellatus* and *Zacco platypus* (Hosoya, 1993). In the genus *Carassius*, a strain called “kawachibuna” or “herabuna”, improved from “gengorobuna” (*C. cuvieri*), which was originally endemic to L. Biwa, has now been stocked throughout Japan (Hosoya, 1993). Eighteen million *C. cuvieri* individuals are released annually in Japan. We also found this species in the six locations described in this paper. In this study, 13 of the 14 common clonal lines were found in L. Biwa, and this result supports the notion that the common clones could have been accidentally introduced to locations through the stocking of *C. cuvieri* into irrigation ponds and dams throughout Japan. There is no clear evidence for the natural range expansion of common clones; however, they were spread with the stocking seeds of *C. cuvieri* from L. Biwa and its adjacent waters during the last 50 years.

In studies of the population structure of any Japanese freshwater fish species, it is necessary to take into account the possible effects of human-induced disturbance, both deliberate and accidental. The recognition in this study of both common and unique clones of Japanese crucian carp should provide insights that will be of undoubted value in further studies on the origins and mechanisms of clonal divergence of this species.

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