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Genetic Introgression by the Rose Bitterling, *Rhodeus ocellatus ocellatus*, into the Japanese Rose Bitterling, *R. o. kurumeus* (Teleostei: Cyprinidae)

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ABSTRCT—The Japanese rose bitterling, *Rhodeus ocellatus kurumeus*, is an endangered cyprinid species. The main reason of drastic diminution of *R. o. kurumeus* has been suggested to be due to hybridization between this subspecies and *R. o. ocellatus* introduced from China. Both RFLP analysis of mitochondrial DNA and RAPD-PCR analysis of genomic DNA were performed to elucidate the genetic introgression of *R. o. ocellatus* into *R. o. kurumeus*. The two subspecies were distinguished in the D-loop region of mtDNA by six restriction endonucleases. *Rhodeus o. kurumeus* had eleven subspecies-specific RAPD markers and *R. o. ocellatus* had two. Except some *R. o. kurumeus* populations, most of the populations of *R. ocellatus* in Japan were hybrids, equipped with subspecies-specific RAPD markers for the two subspecies, respectively. The genetic constitution of these markers in hybrids, however, greatly differed among populations. The *R. o. ocellatus* mtDNA was predominantly observed in hybrid populations, except two populations with mtDNAs of the two subspecies. Judging from the genetic dominance of morphological and physiological characters of *R. o. ocellatus* against *R. o. kurumeus*, hybrids probably have the same ecological dominance as *R. o. ocellatus* against *R. o. kurumeus*. Therefore, it is considered that *R. o. kurumeus* not only has its genetic property spoiled by hybridization with *R. o. ocellatus*, but also is expelled by *R. o. ocellatus* and hybrids. The replacement of mtDNA and genomes of *R. o. kurumeus* with those of *R. o. ocellatus* in hybridization might be accelerated by the backcross between hybrids and *R. o. ocellatus.*

INTRODUCTION

Genetic introgression has been known in many fish groups (Hubbs, 1955). It occurs when two species, in which reproductive isolation is not complete, meet and hybridize and their hybrids keep fertility, which is not inferior to those of parental species. Incomplete reproductive isolation is often observed especially in incipient species (Vrijenhoek *et al.*, 1989). In the wild, these species are usually isolated by geographical barriers. They, however, seem to easily hybridize with each other in sympatric situation (Dawley, 1987; Echelle and Connor, 1989). Hybridization between an endemic species and its exotic related species spoils the genetic identity of the endemic species. Through the hybridization, inherent genes of an endemic species are likely to be replaced by those of an exotic species, which eventually leads to the extinction of the

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endemic species (Allendorf and Leary, 1998). Recently, decrease of the populations of endemic fish species, caused by artificial transplantation of exotic species, has been reported in many groups such as *Salvelinus* (Allendorf and Leary, 1998), *Cyprinodon* (Echelle and Connor, 1989) and *Micropterus* (Whitmore, 1983).

The Japanese rose bitterling, *Rhodeus ocellatus kurumeus* Jordan and Thompson, is an endemic cyprinid species in Japan. Before World War II, it was widely distributed in small ponds and creeks in the western part of Japan (Nakamura, 1969; Miyadi *et al.*, 1976; Hosoya, 1982). The rose bitterling, *Rhodeus o. ocellatus* (Kner), is considered to have been introduced accidentally from mainland China into Japan, contaminated in the seedlings of grass carp *Ctenopharyngodon idellus* (Cuvier and Valenciennes) and silver carp *Hypophthalmichthys molitrix* (Cuvier and Valenciennes), during the 1940s (Nakamura, 1955). During the last five decades after introduction, *R. o. ocellatus* has drastically expanded its distribution all over Japan, which has given rise to hybrids between the two subspecies in the original distribution of *R. o. kurumeus*. Owing to hybridization with *R. o. ocellatus*, *R. o. kurumeus* has been suggested to have disappeared on a large scale (Nagata, 1980, 1997). *Rhodeus o. kurumeus* is now considered to be on the verge of extinction and listed as a critically endangered species in the Red List of brackish and freshwater fishes of the Environmental Agency of Japan (Hosoya, 2000).

Rhodeus o. kurumeus and *R. o. ocellatus* are very similar in external morphology one another (Nakamura, 1955, 1969; Kimura and Nagata, 1992). Nakamura (1969) found that *R. o. ocellatus* is primarily distinguished from *R. o. kurumeus* by the presence of white coloration (guanine layer) along the anterior margin of pelvic fins. Nagata (1980) experimentally demonstrated that the white coloration in pelvic fins was dominantly inherited in hybrids between the two subspecies and reported that many populations within the former distribution of *R. o. kurumeus* were already hybrids, more or less equipped with this character. Ueno (1987) found that *R. o. kurumeus* and *R. o. ocellatus* can be distinguished by two enzymes (LDH and PGDH) in the allozyme analysis. With two loci (*Ldh*-2 and *Pgdh*) in the two enzymes, Nagata *et al.* (1996) reconfirmed that some populations of the rose bitterling in Japan were genetically hybrids of the two subspecies. Only two loci, however, are not sufficient for a detailed analysis of the genetic situation of hybridization. In this study, using RFLP analysis of mtDNA and RAPD-PCR analysis of genomic DNA, the authors first tried the genetic distinction between *R. o. kurumeus* and *R. o. ocellatus* and then made a search for multiple RAPD markers specific to each subspecies. With haplotypes of mtDNA and subspecies-specific RAPD markers, the present situation of genetic introgression by *R. o. ocellatus* into *R. o. kurumeus* in Japan will be discussed.

MATERIALS AND METHODS

Specimens examined

The total 172 fish samples of *R. ocellatus* used in this study were collected from seven localities in China (n=25), two in Korea (n=8) and 13 in Japan (n=139) from 1998 to 1999 (Fig. 1). All the fishes collected were fixed by 70% ethanol and identified, based on their external morphology and osteological characters by soft X-ray photographs (Uchida, 1939; Woo, 1964; Nakamura, 1969; Choi *et al.*, 1990; Kimura and Nagata, 1992), after removing some muscle tissue for DNA extraction. Samples of *R. o. kurumeus* were collected from five populations (Ushizu, Yanagawa, Okawa, Sanda and Yao [A]), whose genetic identity had been confirmed by allozyme analysis in the preceding studies (Nagata *et al.*, 1996; Tabe and Fukuhara, 1997). In all specimens, white coloration in pelvic fins was checked, which is a diagnostic morphological character of *R. o. ocellatus* (Nakamura, 1969).

Mitochondrial DNA analysis

Total genomic DNA was extracted from skeletal muscle, by using phenol-chloroform extraction (Lansman *et al.*, 1981). About 2.05-kb fragments in length from the cytochrome *b* to the 12S rRNA gene, including the entire noncoding region (D-loop), were amplified via PCR (Saiki *et al.*, 1988) from the whole genomic DNA, using a pair of the following primers: CB3R-L (5'-CAYATYMARCCMGAATGRTATTT-3') and 12SAR-H (5'-ATARTRGGGTATCTAATCCYAGTT-3') (Palumbi *et al.*, 1991). The conditions of amplification were 2 min at 94°C for preamplification denaturation; 30 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min; and then 72°C for 5 min for final elongation in a DNA thermal cycler (Takara, Kyoto). The length of all PCR products was confirmed as equal length by electrophoresis, prior to digestion with restriction endonucleases.

A total of 15 restriction endonucleases were used to digest the amplified products of PCR: *Acc* II, *Afa* I, *Alu* I, *Cfr*13 I, *Dde* I, *Eco*R I, *Hae* III, *Hha* I, *Hinf* I, *Hsp*92 II, *Mbo* I, *Mse* I, *Msp* I, *Scr*F I, *Taq* I (Table 1; from Life Technologies, Rockville, MD; Promega, Madison, WI; Takara Shuzo, Kyoto; Toyobo; Tokyo). For each sample, 1–4 μl of the PCR product containing the amplified DNA was digested following the recommendations of the enzyme manufacturers. The digested fragments of the D-loop segment were electrophoretically separated on horizontal 3% agarose gels. The bands of digested fragments were photographed with UV illumination, after staining with ethidium bromide.

Nucleotide sequence divergence in mtDNA was calculated by the length-difference method (Nei and Li, 1979), after comparing the electrophoretic patterns in each of the two haplotypes of mtDNA, digested by a restriction endonuclease recognizing four nucleotides except *Eco*R I, respectively. A dendrogram was generated by neighbor-joining method (NJ; Saitoh and Nei, 1987) and the reliability of each clade was checked by 1,000 bootstrap resampling of fragments with PHYLIP version 3.572 (Felsenstein, 1996).

RAPD-PCR analysis

Each RAPD-PCR reaction mixture consisted of 2.5 μl of 10×PCR buffer, 2 μl of each dNTP (2.5 mM), 1 μl of oligonucleotide primer (25 μM), 0.5 μg of genomic DNA, 0.1 U of Taq polymerase (Takara, Kyoto) in 25 μl final volume. Two kits (designated A and F), each containing 20 decamer RAPD primers (Operon Technologies, Alameda, CA) were used in this study. Amplifications were carried out with the following cycle program: 2 min at 94°C for preamplification denaturation; 35 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 2 min; and then 72°C for 5 min for final elongation. After amplification, all PCR products were subjected to electrophoresis in a 1.2% agarose gel. Band patterns were analyzed visually from photographs taken of the ethidium bromide stained gels.

RAPD markers, specific to *R. o. kurumeus* or *R. o. ocellatus*, were examined using the specimens of *R. o. kurumeus* from Yanagawa, Okawa, Sanda and Yao (A), and those of *R. o. ocellatus* from Qingpu (St. 6) and Sano (St. 21). The haplotype of mtDNA of all the specimens from Qingpu closely accorded with the predominant haplotype of *R. o. ocellatus* in Japan. The inheritance of RAPD mark-

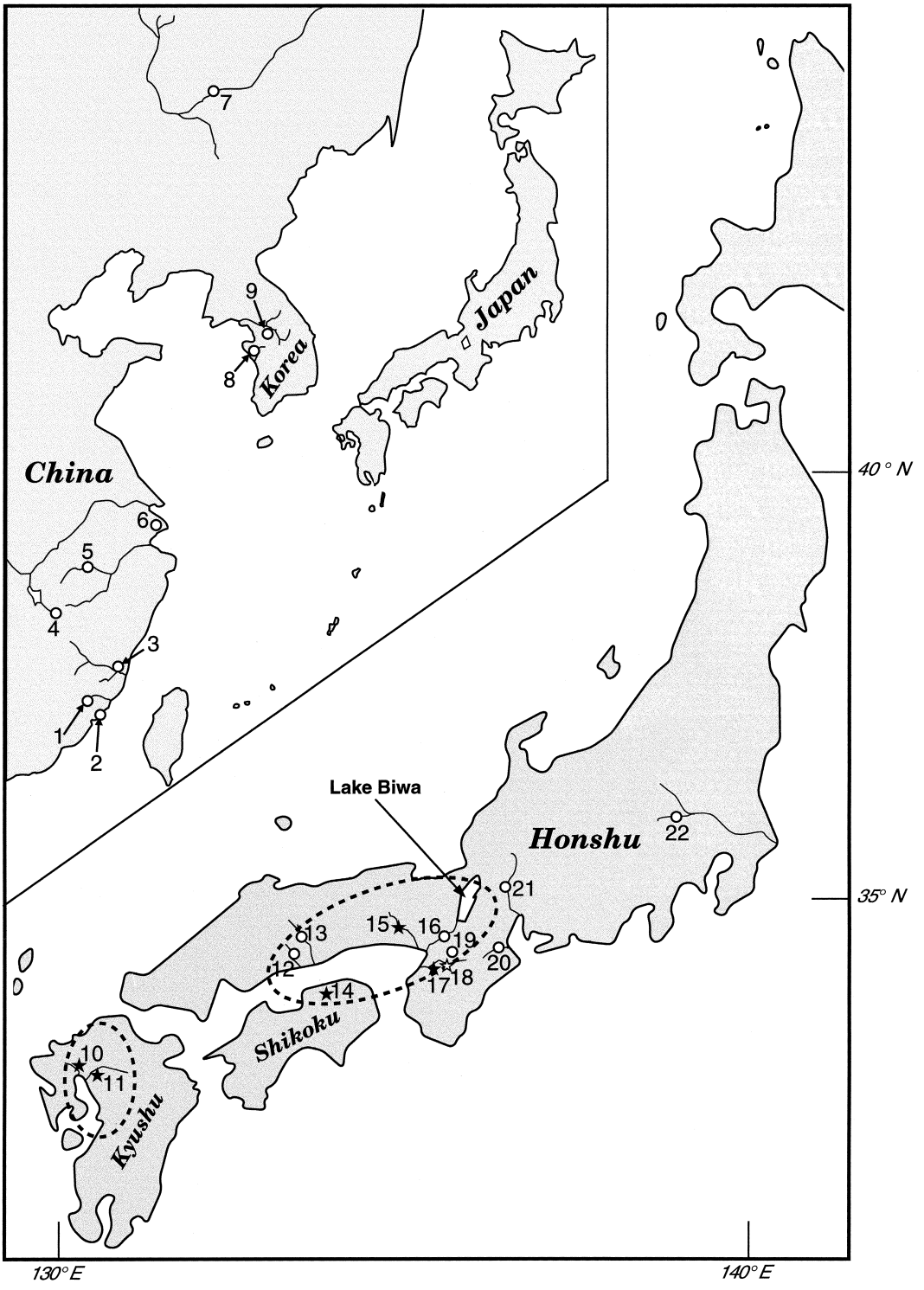


Fig. 1. Map showing sample localities of the rose bitterling used in this study. (1) Xianyou, Fujian (Mulan R. system); (2) Putian, Fujian (small river); (3) Fuzhou, Fujian (Min R. system); (4) Yujiang, Jiangxi (Changjiang R. system, Xin R.); (5) Xiuning, Anhui (Qiantang R. system, Xinan R.); (6) Qingpu, Shanghai (Changjiang R. system); (7) Haerbin, Heilongjiang (Amur R. system, Sungari R.); (8) Chuksan-ri, Kyonggi-do (Namhan R. system, Tuwol R.); (9) Kwangchon, Chungchongnam-do (Kwangchon R.); (10) Ushizu, Saga (Ushizu R. system); (11) Yanagawa, Fukuoka (Okinohata R.); (12) Souja, Okayama (Takahashi R. system); (13) Okayama, Okayama (Asahi R. system); (14) Okawa, Kagawa (Tsuda R. system, pond); (15) Sanda, Hyogo (Muko R. system, pond); (16) Yawata, Kyoto (Yodo R.); (17) Yao (A), Osaka (Yamato R. system, 9 ponds); (18) Yao (B), Osaka (Yamato R. system, 2 ponds); (19) Ikoma, Nara (Yodo R. system, 1 pond); (20) Ise, Mie (Kushida R. system, Ohori R.); (21) Ogaki, Gifu (Ibi R. system, 1 pond); (22) Sano, Tochigi (Tone R. system, Sai R.). Broken lines indicate the original distribution of *Rhodeus ocellatus kurumeus* (Nakamura, 1969; Miyadi *et al.*, 1980; Hosoya, 1982; Nagata, 1989). Localities of *R. o. kurumeus* and hybrids between *R. o. kurumeus* and *R. o. ocellatus*, identified from allozyme analysis in the preceding studies, are shown with ★ and ☆, respectively (Nagata *et al.*, 1996; Tabe *et al.*, 1997).

ers in F_1 was checked with artificially produced hybrids between *R. o. kurumeus* from Yanagawa and Yao (A) and *R. o. ocellatus* from Sano. The situation of hybridization of the two subspecies in the wild populations of the rose bitterling was examined from the expression of subspecies-specific RAPD markers for *R. o. kurumeus* and *R. o. ocellatus*. In addition, the extent of genetic introgression of *R. o. ocellatus* into *R. o. kurumeus* in each population was also assessed by calculating the averaged number of these subspecies-specific RAPD markers per specimen.

RESULTS

Mitochondrial DNA analysis

Genetic polymorphism in D-loop of mtDNA was observed in all 15 restriction endonucleases used in this study. Fragment patterns digested by restriction endonucleases were arranged into 20 haplotypes (Tables 1, 2). Twelve haplotypes

Table 1. Approximate size in base pairs (bp) of all fragment patterns observed on the D-loop region in the rosy bitterling

Restriction enzyme	Fragment patterns (bp)							
Acc II								
A	1030	570	250	200				
B	1030	310	260	250	200			
Afa I								
A	940	420	360	140	110			
B	1260	530	140					
C	1260	420	140	110				
D	940	360	250	170	140	110		
E	940	420	360	110	100			
F	940	530	360	100				
G	940	630	360					
H	1070	420	250	140	110			
Alu I								
A	1160	350	170	110				
B	1160	320	210	120				
C	1160	340	320					
D	1160	350	240	110				
E	1160	320	170	110				
F	1060	320	300	100				
*Cfr*13 I								
A	690	640	220	180	110	110		
B	640	610	220	180	110	110		
C	610	540	220	180	110	110		
D	800	640	220	180	110			
E	640	640	220	180	150	120		
F	910	640	220	180				
Dde I								
A	560	440	400	370	210			
B	770	560	440	210				
C	440	400	370	360	210	200		
D	400	380	360	210	200	190	180	140
E	1050	210	190	180	160	130		
F	560	400	260	210	190	180	140	
*Eco*R I								
A	1310	450	290					
B	830	480	450	290				
Hae III								
A	920	560	270	120	80			
B	720	560	270	200	120	80		
C	920	660	270	120	80			
D	720	660	270	200	120	80		
Hha I								
A	840	430	410	250	100			
B	1000	430	250	250	100			
C	1060	430	250	160	100			
D	1220	430	250	100				
E	680	430	410	250	160	100		
F	1120	430	250	100	90			

Restriction enzyme	Fragment patterns (bp)									
*Hin*f I										
A	900	360	300	250	150	90				
B	770	450	450	250	130					
C	770	450	410	250	130					
D	900	450	300	250	150					
E	460	450	440	300	250	150				
F	810	450	300	250	150	90				
*Hsp*92 II										
A	680	390	260	220	170	150	80			
B	400	390	280	260	220	170	150	80		
C	460	390	300	260	200	170	150	80		
D	680	390	260	220	190	150	80			
E	460	390	260	240	200	170	150	80		
Mbo I										
A	1280	390	340							
B	1280	350	340							
C	1280	340	290	140						
D	720	600	350	340						
E	1320	340	250	140						
Mse I										
A	420	220	220	200	110	90	90	60	50	
B	340	220	220	210	110	90	90	60	50	
C	340	300	220	220	210	120	90	90	60	50
D	340	220	220	170	110	90	90	60	50	
E	360	220	220	200	110	90	90	60	50	
F	340	220	220	210	150	110	90	90	60	50
G	420	220	220	210	110	100	90	60	50	
H	420	220	220	110	90	90	60	50		
I	340	340	220	210	110	100	60	60	50	
Msp I										
A	1000	350	280	140	100	60				
B	1000	420	350	100	60					
*Scr*F I										
A	600	460	290	270	240	100				
B	600	290	270	240	200	150	110	100		
C	600	430	290	270	240	100				
D	770	290	290	270	240	100				
E	600	560	460	240	100					
Taq I										
A	840	400	320	250	240					
B	650	430	320	260	250	140				
C	1080	400	320	250						
D	1080	320	260	250	140					

Table 2. Composite haplotypes for restriction enzyme polymorphisms in the rose bitterling

Haplotype	Composite fragment pattern														
	Acc II	*Afa* I	*Alu* I	*Cfr*13 I	*Dde* I	*Eco*R I	*Hae* III	*Hha* I	*Hinf* I	*Hsp*92 II	*Mbo* I	*Mse* I	*Msp* I	*Scr*F I	*Taq* I
mt1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
mt2	A	D	A	A	A	A	A	A	A	A	A	A	A	A	A
mt3	A	A	A	A	B	A	A	A	A	A	A	E	A	A	A
mt4	A	A	A	D	A	A	A	A	D	A	A	A	A	A	A
mt5	A	A	A	A	A	A	A	E	A	A	A	A	A	A	A
mt6	A	A	A	A	A	A	A	A	A	A	A	H	A	A	A
mt7	A	B	B	B	D	A	B	B	B	C	B	B	A	C	B
mt8	A	B	C	C	D	A	B	C	C	C	B	C	A	C	B
mt9	A	B	B	B	D	A	B	C	B	C	B	B	A	C	B
mt10	A	B	B	B	D	A	B	D	B	C	B	B	A	C	B
mt11	A	H	C	D	F	A	D	F	D	E	E	I	A	C	D
mt12	B	G	F	F	E	A	C	C	F	D	D	G	A	D	C
mt13	A	C	D	A	C	A	A	D	D	A	C	D	A	A	C
mt14	A	C	A	A	C	A	A	D	D	A	C	D	A	A	C
mt15	A	E	E	E	A	B	A	C	D	B	B	F	A	B	A
mt16	A	E	E	E	A	B	A	C	E	B	B	F	A	B	A
mt17	A	E	E	E	A	B	A	C	D	B	B	F	A	A	A
mt18	A	A	E	E	A	B	A	C	D	B	B	F	A	A	A
mt19	A	F	E	E	A	B	A	C	D	B	B	F	A	A	A
mt20	A	A	E	E	A	B	A	C	D	B	B	F	B	E	A
Total number of fragment patterns	2	8	6	6	6	2	4	6	6	5	5	9	2	5	4

(mt1-6 and mt15–20) were found in Japan (Table 3). *Rhodeus o. kurumeus* (St. 10–11, 14–15 and 17) had six haplotypes (mt15-20). Haplotype groups, mt1-6 and mt15–20, were clearly distinguished by the following six restriction endonucleases: *Alu* I, *Cfr*13 I, *Eco*R I, *Hsp*92 II, *Mbo* I and *Mse* I (Table 2, Fig. 2). Nucleotide sequence divergence among mt1-6 or mt15–20 was very low, ranging from 0.06 to 0.64% (mean±SD= 0.34±0.16%, n=15) in mt1–6, and 0.11 to 0.85% (mean±SD= 0.40±0.21%, n=15) in mt15–20. However, it was high between mt1-6 and mt15–20, ranging from 1.40 to 2.47% (mean±SD =1.97±0.26%, n=36).

Each *R. o. kurumeus* haplotype was characteristically distributed in Kyushu, Shikoku and Honshu, respectively (mt15 and 16 in Kyushu, mt20 in Shikoku and mt17–19 in Honshu; Table 3). They were also found from populations in Souja and Okayama. The haplotype mt17 in Souja and Okayama accorded with that in Sanda and Yao (A). In Souja and Okayama, however, haplotypes other than mt15–20 were also found in the same populations (mt1–3 in Souja and mt1 in Okayama). Although *R. o. kurumeus* haplotypes (mt15–20) had a characteristic geographical distribution pattern, haplotypes of mt1-6 in Japan did not have such a pattern but a random distribution. Among the haplotypes of mt1–6, mt1 was predominantly observed in Honshu and also discovered in Fuzhou, Qingpu and Haerbin in China. The other haplotypes of mt2–6, observed in Japan, were found in neither China nor Korea in this study.

In NJ tree (Fig. 3), 20 haplotypes of *R. ocellatus* were clearly arranged into six clades. *Rhodeus o. kurumeus* haplotypes (mt15-20) constituted one major clade, while the other haplotypes (mt1–14) were grouped into five major clades: mt1–6, mt7–10, mt11, mt12 and mt13–14. Haplotypes of mt1, mt7–12 and mt13–14 were found only in *R. o. ocellatus* in China and Korea. Haplotypes mt2–6, found in Japan alone, constituted one clade of *R. o. ocellatus* with mt1. Namely, five clades of mt1–6, mt7–10, mt11, mt12 and mt13–14 are *R. o. ocellatus* clades. Among the five *R. o. ocellatus* clades, the Korean clade (mt13–14) was nearest to the *R. o. kurumeus* clade (range =1.81 to 2.57%, mean±SD = 2.22±0.23% in nucleotide sequence divergence, n=12) and mt1–6 clade was the second. The nucleotide sequence divergence among the five clades of *R. o. ocellatus* was extremely high, ranging from 1.42 to 5.17% (mean±SD =3.63±1.09%, n=69). Bootstrap values strongly supported the *R. o. kurumeus* clade at 94.0%.

RAPD analysis

Fourteen RAPD primers out of the 40 primers tested in this study showed RAPD bands specific to local populations or subspecies of *R. ocellatus* (Table 4). In *R. o. kurumeus*, population-specific RAPD markers were observed in eight primers (OPA-02, 07 and 12; OPF-02, 09, 15, 18 and 20). Yao (A), Sanda and Okawa populations of *R. o. kurumeus* shared many RAPD bands (OPA-07 and 12; OPF-02, 09, 15 and 20), but population-specific RAPD bands for these sampling sites were hardly found, except one band of OPA-02 for Yao (A). Yanagawa population was clearly distinguished from Yao, Sanda and Okawa populations by five primers (OPA-07, OPF-09, 15, 18 and 20). RAPD bands common to all the populations of *R. o. kurumeus* were observed in 11 primers (OPA-02, 05, 09 and 16; OPF-02, 07, 08, 09, 15, 17 and 18; Fig. 4A, 4B). Sano and Qingpu populations of *R. o. ocellatus* did not share any RAPD bands with *R. o. kurumeus*. These two popu-

Table 3. Distribution of mtDMA haplotypes in local populations of the rose bitterling

	St.[1]	Locality	N	Haplotype													
				mt1	mt2	mt3	mt4	mt5	mt6	mt7	mt8	mt9	mt10	mt11	mt12	mt13	mt14
China	1	Xianyou	2								2 (100.0)						
	2	Putian	4									2 (50.0)			2 (50.0)		
	3	Fuzhou	2	1 (50.0)						1 (50.0)							
	4	Yujiang	5								1 (20.0)	2 (40.0)	2 (40.0)				
	5	Xiuning	2											2 (100.0)			
	6	Qingpu	7	7 (100.0)													
	7	Haerbin	3	3 (100.0)													
Korea	8	Chukusan-ri	6													5 (83.3)	1 (16.7)
	9	Kwangchon	2													2 (100.0)	
Japan	10	Ushizu[2]	10														
	11	Yanagawa[2]	10														
	12	Souja	10	4 (40.0)	2 (20.0)	2 (20.0)											
	13	Okayama	10	3 (30.0)													
	14	Okawa[2]	14														
	15	Sanda[2]	5														
	16	Yawata	10	6 (60.0)		4 (40.0)											
	17	Yao (A)[2]	26														
	18	Yao (B)[3]	4	2 (50.0)		2 (50.0)											
	19	Ikoma	10				10 (100.0)										
	20	Ise	10	2 (20.0)		6 (60.0)			2 (20.0)								
	21	Ogaki	10	6 (60.0)		2 (20.0)		2 (20.0)									
	22	Sano	10	10 (100.0)													

[1] Sampling stations shown in Fig. 1.
[2] Identified as *Rhodeus ocellatus kurumeus* from allozyme analysis in the preceding studies (Nagata *et al.*, 1996; Tabe *et al.*, 1997).
[3] Identified as hybrid between *R. o. kurumeus* and *R. o. ocellatus* from allozyme analysis in the preceding studies (Nagata *et al.*, 1996; Tabe *et al.*, 1997).

lations showed a great variation in RAPD band patterns among individuals and only two RAPD bands (OPF-08 and 15), common to the populations of *R. o. ocellatus*, were found (Fig. 4A, 4B). In the distinction between *R. o. kurumeus* and *R. o. ocellatus*, eleven primers (OPA-02, 05, 09 and 16; OPF-02, 07, 08, 09, 15, 17 and 18) were found to be useful.

In F_1 of the two subspecies, expression of RAPD bands specific to *R. o. kurumeus* or *R. o. ocellatus* was observed to follow the mode of Mendelian inheritance, irrespective of sex (Fig. 4C). Such RAPD markers, which were inherited maternally like mtDNA, were not recognized in this study.

Morphological and genetic characters of local populations

A total of 13 populations of *R. ocellatus* in Japan were surveyed concerning the morphological and genetic character distribution of *R. o. kurumeus* and *R. o. ocellatus* (Table 5). White coloration in pelvic fins (WCP) was present in all specimens from the populations, except those of St. 10, 11,

(%)					
mt15	mt16	mt17	mt18	mt19	mt20
10 (100.0)					
9 (90.0)	1 (10.0)				
		2 (20.0)			
		7 (70.0)			
					14 (100.0)
		5 (100.0)			
		22 (84.6)	2 (7.7)	2 (7.7)	

13-15 and 17. WCP was not recognized in specimens from populations of Ushizu, Yanagawa, Okawa, Sanda and Yao (A) at all, while it was slightly observed in three specimens of ten from Okayama population. Concerning mtDNA, Souja and Okayama populations had mtDNA of *R. o. kurumeus* and *R. o. ocellatus*, while all the other populations had only *R. o. kurumeus* or *R. o. ocellatus* mtDNA (Tables 3, 5).

All the eleven RAPD markers specific to *R. o. kurumeus* were also observed in Ushizu population (Table 5). Seven populations in St. 12–13, 16 and 18–21 owned both *R. o. kurumeus*-specific RAPD markers and *R. o. ocellatus*-specific ones (Fig. 4D). In these populations, however, the number of confirmed subspecies-specific RAPD markers per specimen greatly differed among populations. The averaged number of *R. o. kurumeus*-specific RAPD markers ranged from 1.0 to 6.5 and that of *R. o. ocellatus*-specific RAPD markers did from 0.3 to 1.8. Yao (B) population, adjacent to Yao (A) population of *R. o. kurumeus*, conspicuously had a large number of *R. o. kurumeus*-specific RAPD markers (mean±SD= 6.5±1.9, n=4), but the other six populations (St. 12–13, 16 and 19–21) had less than half of the eleven *R. o. kurumeus*-specific RAPD markers (mean±SD=1.0±1.0 to 4.6±1.5, n=10).

Ise and Ogaki, which are located out of the original distribution of *R. o. kurumeus* especially (Fig. 1), had a small number of *R. o. kurumeus*-specific RAPD markers (mean±SD= 1.8±0.4 in Ise and 1.0±1.0 in Ogaki, n=10), contrary to the prevalence of *R. o. ocellatus*-specific RAPD markers (mean±SD = 1.2±0.4 in Ise and 1.8±0.4 in Ogaki, n=10).

DISCUSSION

Genetic diversity of the rose bitterling

Rhodeus o. kurumeus and *R. o. ocellatus* in Japan differed markedly in the sequence of mtDNA (Fig. 3; Table 2). They were clearly distinguished by six restriction endonucleases (*Alu* I, *Cfr*13 I, *Eco*R I, *Hsp*92 II, *Mbo* I and *Mse* I) (Fig. 2). Nucleotide sequence divergence in D-loop between *R. o. kurumeus* (mt15–20) and *R. o. ocellatus* (mt1–6) was high (range =1.40–2.47%, mean±SD=1.97±0.26%, n=36) as the subspecies level, compared with that in the bagrid species, which ranges from 0.18 to 6.61% (Okazaki *et al*., 1999). The great divergence in nucleotide sequence between the two subspecies suggests that *R. o. kurumeus* and *R. o. ocellatus* in Japan are genetically distinct enough, although they are morphologically very similar (Nakamura, 1969; Nishiyama and Nagata, 1978). In Asia, *R. ocellatus* was composed of six clades in the NJ tree, including *R. o. kurumeus* clade (Fig. 3), where nucleotide sequence divergence among these clades was extremely high, ranging from 1.42 to 5.17%. Judging from the value of nucleotide sequence divergence between each clade in Fig. 3, *R. ocellatus* is considered to be genetically very heterogeneous. Accordingly, the taxonomy of all the populations of *R. ocellatus* should be reevaluated in the future.

The nucleotide sequence divergence in the six haplotypes (mt1–6) of *R. o. ocellatus* in Japan was comparatively low, ranging from 0.06 to 0.64% (mean±SD=0.34±0.16%, n=15), and constituted a single clade (Fig. 3) Therefore, although haplotypes mt2-6 were not discovered in China in this study, it is likely that they are distributed somewhere in China with mt1. Only the mt1 haplotype was found in Fuzhou, Qingpu and Haerbin in China, as well as Japan. The fact that the same haplotype of mt1 was found in both Qingpu, belonging to the Changjiang River system, and Japan agrees with the report of Nakamura (1955), in which he considered the original range of Japanese *R. o. ocellatus* as the Changjiang River system.

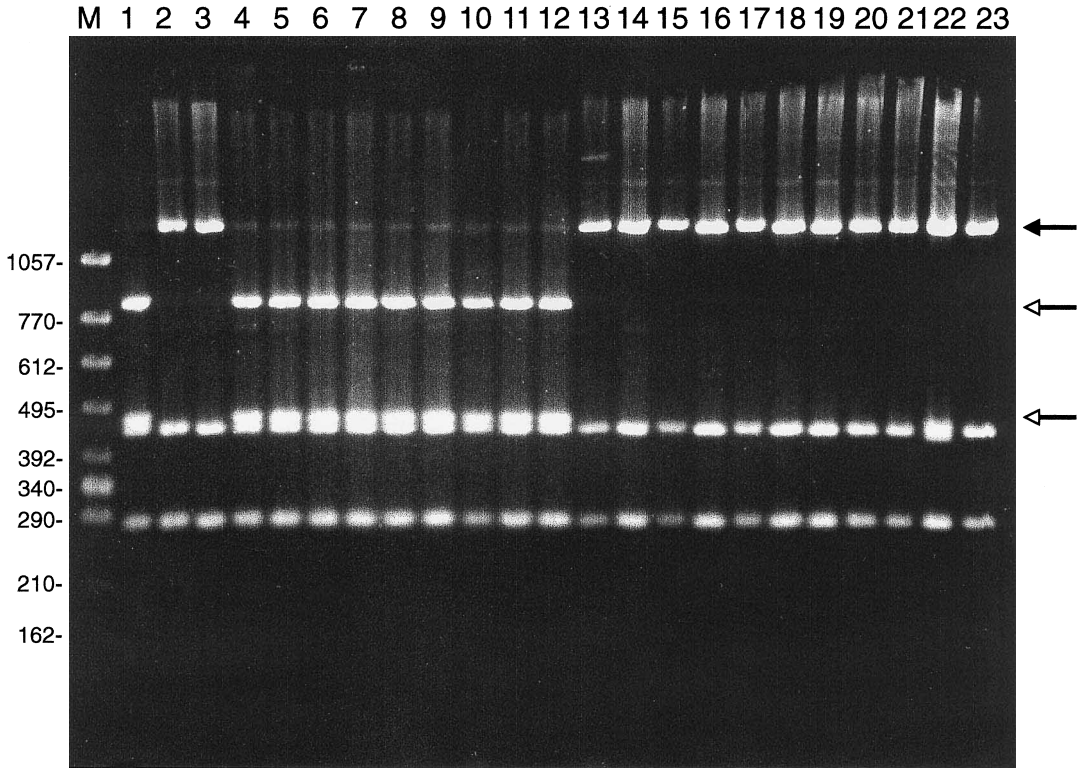


Fig. 2. Electrophoretic patterns of mtDNA digested by *Eco*R I in some local populations of the rose bitterling in Japan. Lanes 1–2, Souja; lanes 3–5, Okayama; lanes 6–8, Yawata; lanes 9–10, Ikoma; lanes 11–12, Yao (B); lanes 13–15, Yao (A); lanes 16–17, Sanda; lanes 18–19, Yanagawa; lanes 20–21, Ushizu; lanes 22–23, Okawa. Black and white arrows indicate specific bands to *Rhodeus ocellatus kurumeus* and *R. o. ocellatus*, respectively. M is a DNA size marker (ϕ X174 *Hin*c II digest).

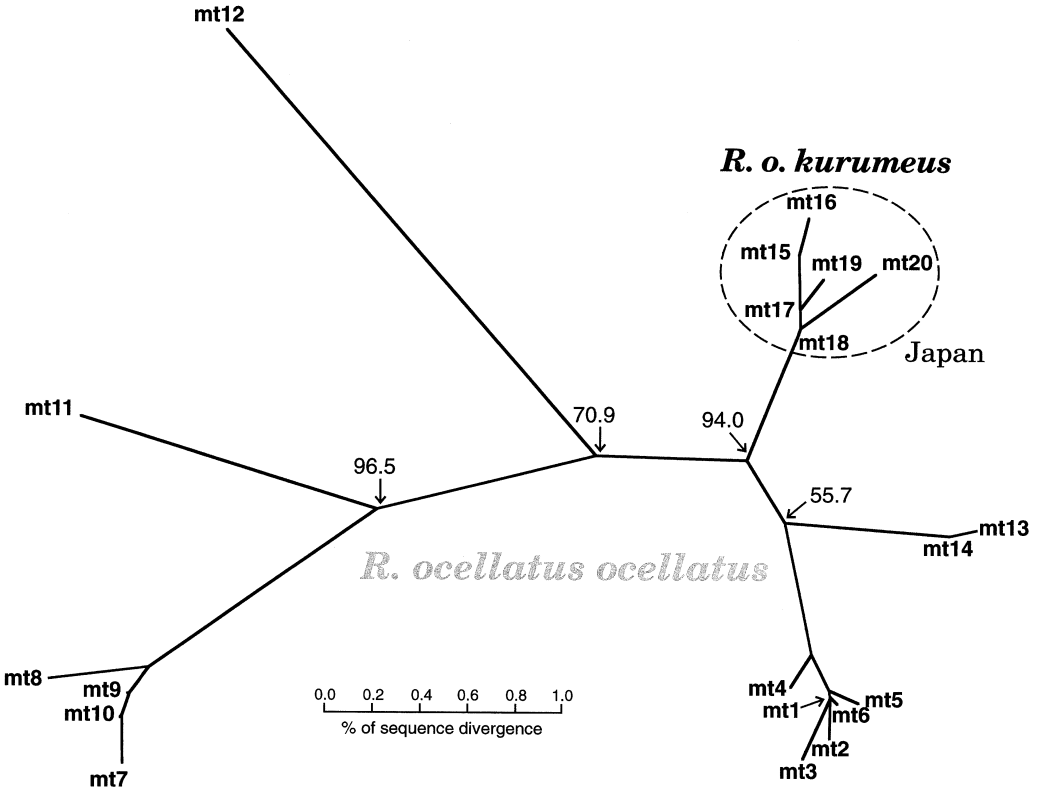


Fig. 3. Unrooted tree of composite haplotypes in the rose bitterling by the neighbor-joining method, based upon nucleotide sequence divergence (Nei and Li, 1979). Numbers at the forks show the bootstrap values (1,000 replicates). Symbols at the end of tree forks indicate the haplotypes, defined in Table 2. Broken lines mean a haplotype group of *R. o. kurumeus*.

Table 4. Selected RAPD primers that identify local population- or subspecies-specific bands in the rose bitterling

RAPD primer	Sequence (5'-3')	*Rhodeus ocellatus kurumeus*				*R. o. ocellatus*-specific bands
		Yanagawa-specific bands	Yao (A), and Sanda-specific bands	Yao (A) Sanda- and Okawa-specific bands	Common bands in *R. o. kurumeus*	
OPA-02	TGCCGAGCTG		1		1	
OPA-05	AGGGGTCTTG				1	
OPA-07	GAAACGGGTG	1		1		
OPA-09	GGGTAACGCC				1	
OPA-12	TCGGCGATAG			1		
OPA-16	AGCCAGCGAA				2	
OPF-02	GAGGATCCCT			1	1	
OPF-07	CCGATATCCC				2	
OPF-08	GGGATATCGG				1	1
OPF-09	CCAAGCTTCC	1		2	1	
OPF-15	CCAGTACTCC	1		1	1	1
OPF-17	AACCCGGGAA				1	
OPF-18	TTCCCGGGTT	1			2	
OPF-20	GGTCTAGAGG	1		1		

Genetic introgression

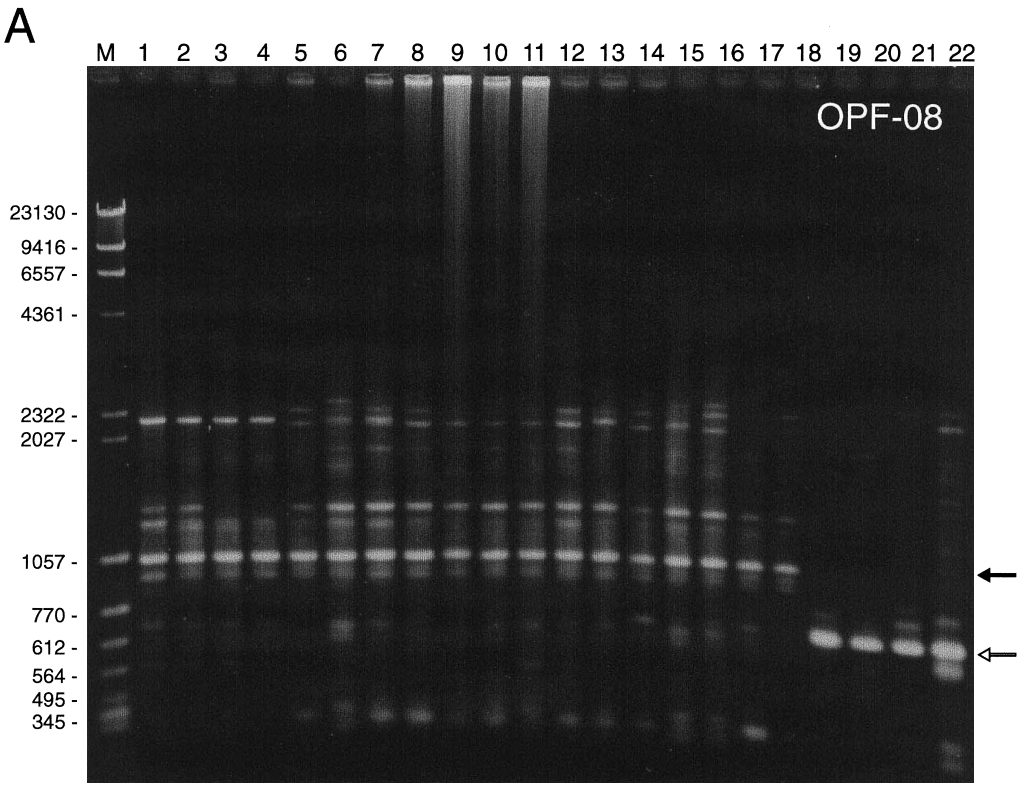
Two enzymes, LDH and PGDH, were found to be useful in the distinction between *R. o. kurumeus* and *R. o. ocellatus* (Ueno, 1987). As for later generations, however, distinction of hybrids by the two enzymes is difficult, due to random segregation and recombination of chromosomes. RAPD markers are also known to follow the mode of Mendelian inheritance (Foo *et al.*, 1995; Liu *et al.*, 1998). In this study, RAPD markers by the eleven RAPD primers were found to be specific to *R. o. kurumeus* or *R. o. ocellatus* (Table 4), and could show hybridization between the two subspecies at the genomic level (Table 5). Seven populations (St. 12, 13, 16 and 18–21) were hybrids, but the genetic status of hybrids differed among populations, judging from the appearance of RAPD markers specific to *R. o. kurumeus* or *R. o. ocellatus*, respectively. The averaged number of confirmed *R. o. kurumeus*-specific RAPD markers was relatively high in Okayama and Yao (B), compared with the remaining five hybrid populations (Table 5). Okayama population especially still kept other characteristics of *R. o. kurumeus* in mtDNA and the coloration of pelvic fins, in addition to the RAPD markers (Tables 3, 5). It inhabits an upper branch isolated by a dam from the main stream of Asahi River, and Yao (B) population did a small pond located in the foothills of Mt. Ikoma. In common, the two populations are completely isolated from other drainage systems in mountain areas. Probably, because of the small number of introduced *R. o. ocellatus* or the short elapsed time after introduction, the genetic introgression of *R. o. ocellatus* into *R. o. kurumeus* in Okayama and Yao (B) was not so high, compared with other populations. Contrary to Okayama and Yao (B) populations, Souja, Ise and Ogaki populations had a very small number of *R. o. kurumeus*-specific RAPD markers among the hybrid populations (Table 5). *Rhodeus o. kurumeus* was formerly distributed in the western part of Japan, that is, the drainage systems located westward of Lake Biwa (Nakamura, 1969; Fig. 1), not inhabiting Ise and Ogaki. Hybrids in Ise and Ogaki might not have originated there, but have been transplanted from other localities within the original distribution of *R. o. kurumeus*.

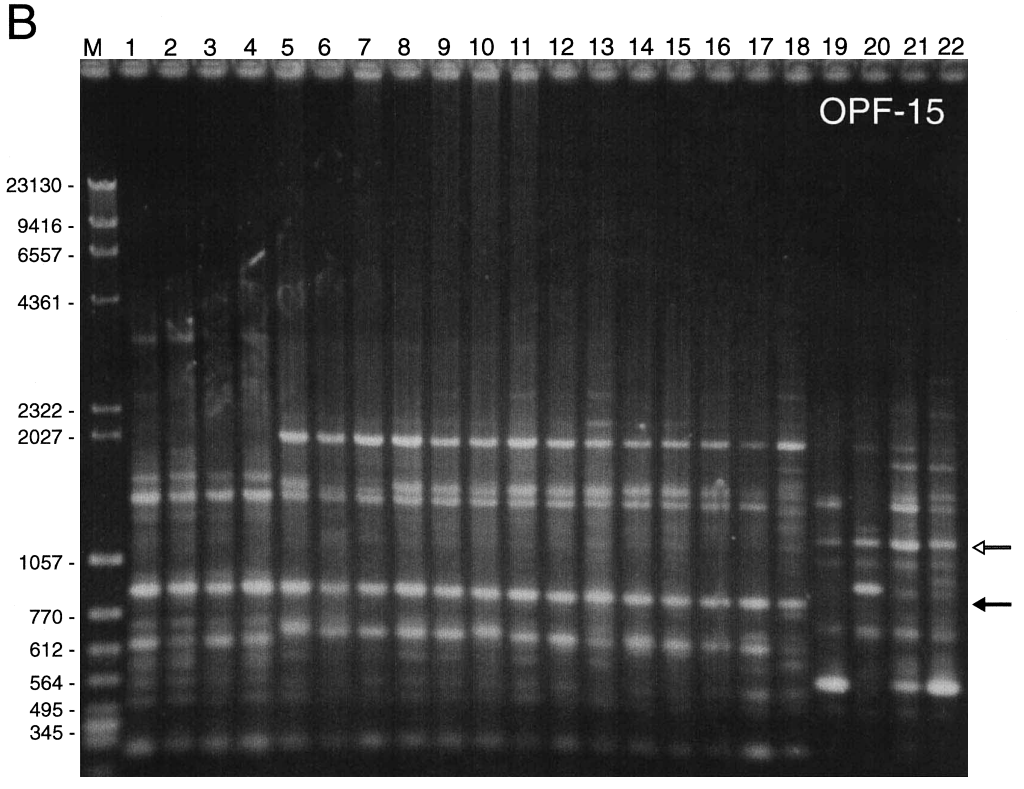
Direction in hybridization

As a general feature, most of the hybrid populations were equipped with more characteristics of *R. o. ocellatus* than those of *R. o. kurumeus* in morph, mtDNA and RAPD markers (Table 5). In particular, the *R. o. ocellatus* mtDNA was predominantly observed in the hybrid populations, except Souja and Okayama. Judging from the morphological and genetic status in hybrids (Table 5), the genomic DNA and mtDNA of *R. o. kurumeus* seemingly tend to be replaced by those of *R. o. ocellatus* in hybridization. Namely, this means a genetic direction from *R. o. kurumeus* to *R. o. ocellatus* in hybridization.

The replacement of genetically pure *R. o. kurumeus* with *R. o. ocellatus* is considered to be caused by the following three factors: (1) difference in body size, clutch size and the duration of spawning period between the two subspecies, (2) ecological competition, especially in mating behavior between the two subspecies and (3) genetic dominance of morphological and physiological characters of *R. o. ocellatus* against *R. o. kurumeus*.

Nagata (1980) commented that *R. o. ocellatus* is superior to *R. o. kurumeus* in body size, clutch size and the duration of spawning period. *Rhodeus o. ocellatus* becomes larger than *R. o. kurumeus* in both males and females (Nakamura, 1969). The clutch size of *R. o. ocellatus* (mean±SD=14.0±3.5, n=69) is approximately twice that of *R. o. kurumeus* (mean±SD =8.8±3.7, n=16), although both subspecies mature within one





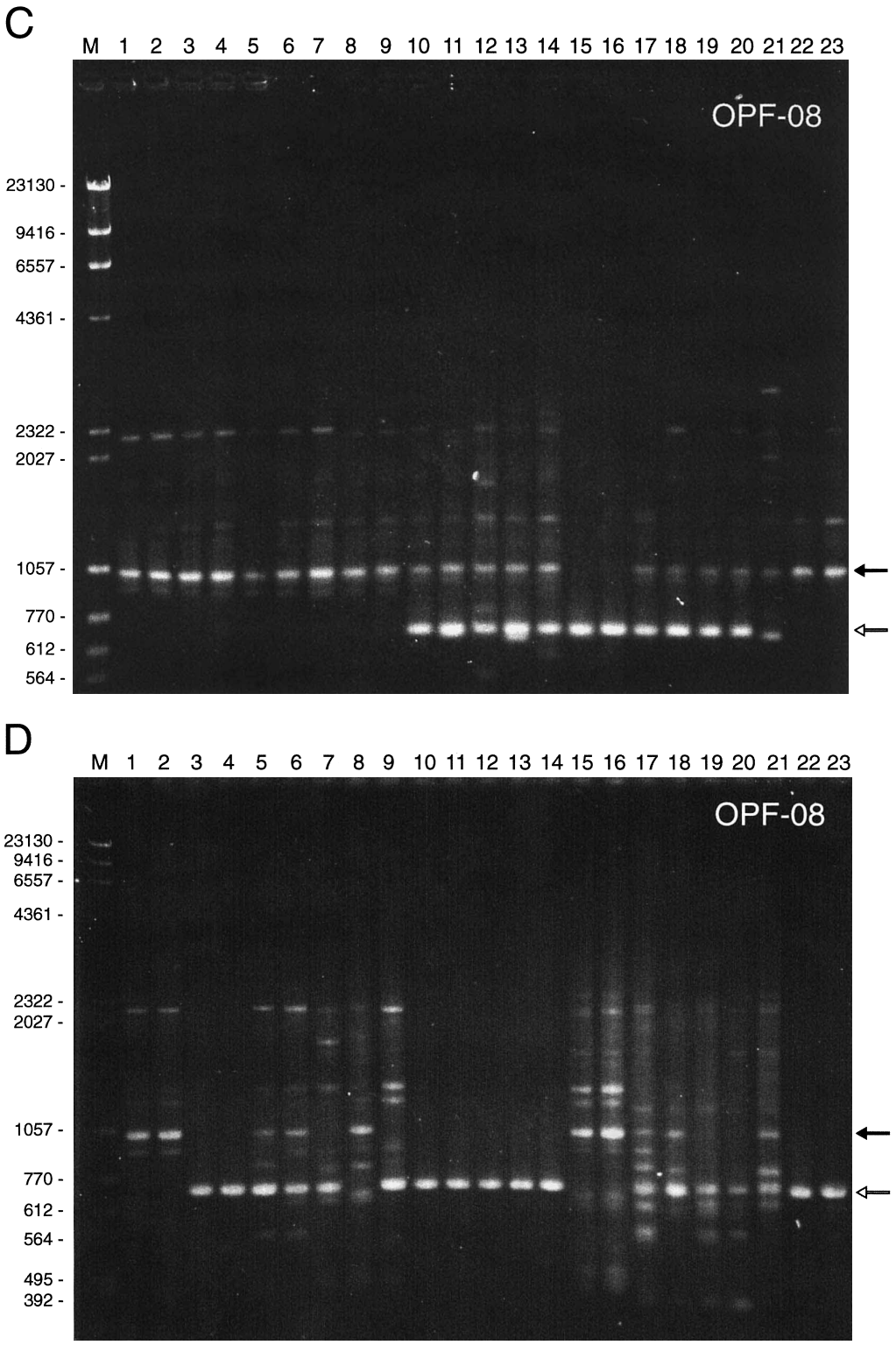


Fig. 4. Examples of RAPD patterns in the rosy bitterling, A, OPF-08 and B, 15, specific to *Rhodeus ocellatus kurumeus* or *R. o. ocellatus*, respectively. *R. o. kurumeus* (lanes 1–18) and *R. o. ocellatus* (lanes 19–22) were characterized by subspecies-specific RAPD markers, respectively. Lanes 1–4, Yanagawa; lanes 5–11, Yao (A); lanes 12–16, Okawa; lanes 17–18, Sanda ; lanes 19–20, Sano; lanes 21–22, Qingpu. C, inheritance of RAPD markers (OPF-08) in F_1 hybrids. Lanes 1–2 and 22–23, *R. o. kurumeus* in Yanagawa (control); lanes 3–7, hybrids between *R. o. kurumeus* in Yanagawa and that in Yao; lanes 8–9, *R. o. kurumeus* in Yao (A) (control); lanes 10–14, hybrids between *R. o. kurumeus* in Yao and *R. o. ocellatus* in Qingpu; lanes 15–16, *R. o. ocellatus* in Qingpu (control); lanes 17–21, hybrids between *R. o. ocellatus* in Qingpu and *R. o. kurumeus* in Yanagawa. D, identification of hybrids of the rose bitterling in wild populations with RAPD markers (OPF-08). Lanes 1–2, Yanagawa (*R. o. kurumeus* as control); lanes 3–5, Souja; lanes 6-9, Okayama; lanes 10–14, Yawata; lanes 15–16, Yao (A) (*R. o. kurumeus* as control); lanes 17-19, Ikoma; lanes 20–21, Ogaki; lanes 22–23, Qingpu (*R. o. ocellatus* as control). Black and white arrows indicate markers specific to *R. o. kurumeus* and *R. o. ocellatus*, respectively. M is a DNA size marker (λ *Hin*d III digest - ϕ X174 *Hin*c II digest).

Table 5. Distribution of morphological and genetic characters specific to *Rhodeus ocellatus kurumeus* or *R. o. ocellatus* in local populations of the rose bitterling in Japan

St[1]			10	11	12	13	14	15	16	17	18	19	20	21	22
Locality			Ushizu[2]	Yanagawa[2]	Souja	Okayama	Okawa[2]	Sanda[2]	Yawata	Yao (A)[2]	Yao (B)[3]	Ikoma	Ise	Ogaki	Sano
Sample size			10	10	10	10	14	5	10	26	4	10	10	10	10
WCP (%)[4]			0.0	0.0	100.0	30.0	0.0	0.0	100.0	0.0	100.0	100.0	100.0	100.0	100.0
mtDNA (%)[5]		K	100.0	100.0	20.0	70.0	100.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
		O	0.0	0.0	80.0	30.0	0.0	0.0	100.0	0.0	100.0	100.0	100.0	100.0	100.0
RAPD marker (%)[6]	OPA-02	K	100.0	100.0	20.0	30.0	100.0	100.0	40.0	100.0	50.0	60.0	40.0	0.0	0.0
	OPA-05	K	100.0	100.0	0.0	0.0	100.0	100.0	0.0	100.0	0.0	40.0	0.0	0.0	0.0
	OPA-09	K	100.0	100.0	0.0	40.0	100.0	100.0	10.0	100.0	100.0	40.0	20.0	0.0	0.0
	OPA-16	K	100.0	100.0	20.0	70.0	100.0	100.0	40.0	100.0	100.0	20.0	20.0	10.0	0.0
	OPF-02	K	100.0	100.0	60.0	60.0	100.0	100.0	60.0	100.0	75.0	40.0	0.0	0.0	0.0
	OPF-07	K	100.0	100.0	0.0	70.0	100.0	100.0	10.0	100.0	50.0	0.0	60.0	10.0	0.0
	OPF-08	K	100.0	100.0	0.0	30.0	100.0	100.0	0.0	100.0	50.0	60.0	20.0	40.0	0.0
		O	0.0	0.0	100.0	90.0	0.0	0.0	100.0	0.0	25.0	100.0	100.0	100.0	100.0
	OPF-09	K	100.0	100.0	0.0	40.0	100.0	100.0	0.0	100.0	25.0	40.0	0.0	0.0	0.0
	OPF-15	K	100.0	100.0	20.0	40.0	100.0	100.0	30.0	100.0	75.0	40.0	0.0	0.0	0.0
		O	0.0	0.0	0.0	0.0	0.0	0.0	40.0	0.0	0.0	0.0	10.0	100.0	100.0
	OPF-17	K	100.0	100.0	0.0	40.0	100.0	100.0	30.0	100.0	75.0	40.0	10.0	0.0	0.0
	OPF-18	K	100.0	100.0	0.0	60.0	100.0	100.0	40.0	100.0	50.0	0.0	0.0	0.0	0.0
No. of confirmed RAPD marker (mean±SD)[7]		K	11.0±0.0	11.0±0.0	1.2±1.1	4.6±1.5	11.0±0.0	11.0±0.0	2.7±1.7	11.0±0.0	6.5±1.9	3.8±0.8	1.8±0.4	1.0±1.0	0.0±0.0
		O	0.0±0.0	0.0±0.0	1.0±0.0	0.9±0.4	0.0±0.0	0.0±0.0	1.4±0.5	0.0±0.0	0.3±0.5	1.0±0.0	1.2±0.4	1.8±0.4	2.0±0.0

[1] Sampling stations shown in Fig. 1.

[2] Identified as *R. o. kurumeus* from allozyme analysis in the preceding studies (Nagata *et al.*, 1996; Tabe *et al.*, 1997).

[3] Identified as hybrids between *R. o. kurumeus* and *R. o. ocelaltus* from allozyme analysis in the preceding studies (Nagata *et al.*, 1996; Tabe *et al.*, 1997).

[4] WCP = percentage of specimens with white coloration along the anterior margin of pelvic fins (diagnostic character of *R. o . ocellatus*).

[5] K = *R. o. kurumeus* mtDNA observed; O = *R. o. ocellatus* mtDNA observed.

[6] Eleven RAPD primers used to check the presence of RAPD markers specific to *R. o. kurumeus* or *R. o. ocellatus.* K = *R. o. kurumeus*-specific marker; O = *R. o. ocellatus*-specific marker.

[7] Averaged number and standard deviation of confirmed RAPD markers specific to *R. o. kurumeus* or *R. o. ocellatus* per specimen.

year (K. Kawamura, unpublished data). The duration of spawning period of *R. o. ocellatus* (from April to September; Solomon *et al.*, 1984) is also about two times longer that of *R. o. kurumeus* (from April to June; J. Kitamura, Kyoto University, personal communication). In mating behavior, there is hardly any subspecies-specific difference between the two subspecies, because they spawned together, mixed in an aquarium (Nagata and Nishiyama, 1976). Kanoh (1996) reported that body size of males is significantly important in reproductive success of *R. ocellatus*. Therefore, large *R. o. ocellatus* males are expected to mate with females more successfully than small *R. o. kurumeus* males (Nagata, 1980). Taking these information into consideration, *R. o. ocellatus*, regardless of sex, seems to be completely superior to *R. o. kurumeus* in growth and reproduction.

The growth rate and clutch size of the experimentally produced F_1 hybrids are similar to those of *R. o. ocellatus* (K. Kawamura, unpublished data). F_1 hybrids become almost as large as *R. o. ocellatus* (more than 60 mm TL in both males and females), irrespective of the sex of parental subspecies. Actually, hybrids with a TL of more than 60 mm are commonly observed in the wild. The clutch size of F_1 hybrids (mean±SD =17.4±3.3, n=30) was not inferior to that of *R. o. ocellatus* (mean±SD=14.0±3.5, n=69). In F_2 hybrids and later generations, hybrid breakdown was not observed in growth and reproduction at all. According to Nagata (1985), the duration of spawning period of hybrids is as long as that of *R. o. ocellatus*. Judging from these facts, not only the white coloration in pelvic fins but also other morphological and physiological characters of *R. o. ocellatus* are considered to be inherited dominantly in hybrids. Genetic dominance of these characters of *R. o. ocellatus* in hybrids may suggest ecological dominance of hybrids, as well as *R. o. ocellatus*, against *R. o. kurumeus*.

In conclusion, the expulsion of *R. o. kurumeus* by *R. o. ocellatus* may occur as follows. Once *R. o. ocellatus* is introduced into a *R. o. kurumeus* population, regardless of the number of introduced fish, the genetic identity of the *R. o. kurumeus* population is spoiled by *R. o. ocellatus* through hybridization. In addition, not only *R. o. ocellatus* but also hy-

brids successfully drive away *R. o. kurumeus*, because of ecological dominance against *R. o. kurumeus*. Increased hybrids mate with increased *R. o. ocellatus*, which still more accelerates the replacement of mtDNA and genomic DNA of *R. o. kurumeus* with those of *R. o. ocellatus* in hybrids. Genetically pure *R. o. kurumeus* eventually disappears and hybrids become more and more similar to *R. o. ocellatus*.

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