

Low Genetic Variation and Inbreeding Depression in Small Isolated Populations of the Japanese Rosy Bitterling, *Rhodeus ocellatus kurumeus*

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ABSTRACT—The Japanese rosy bitterling, *Rhodeus ocellatus kurumeus*, has been affected not only by the invasion of another subspecies, *R. o. ocellatus*, from China, but also by habitat fragmentation. In this study, the effects of habitat fragmentation on the fitness of *R. o. kurumeus* were investigated. Owing to exclusion by *R. o. ocellatus*, *R. o. kurumeus* in Honshu and Shikoku has disappeared entirely, except for small populations in isolated man-made ponds in Osaka and Kagawa. In Kyushu it still occupies open water systems, into which *R. o. ocellatus* has only recently invaded. Meristic and genetic data show that the diversity of *R. o. kurumeus* is significantly lower in the isolated Osaka and Kagawa populations than the non-isolated Fukuoka population. The Osaka population is inferior to the Fukuoka population in terms of viability and growth. The viability of reciprocal inter-population hybrids between the Osaka and Fukuoka populations was, however, as high as that of the Fukuoka population. In addition to the high scores of band sharing index (BSI) in RAPD-PCR analysis, acceptance of transplanted scales among individuals, irrespective of natal pond, indicates that the Osaka population forms a highly inbred line. These results suggest that low genetic variation is associated with inbreeding depression in the small isolated Osaka populations. Consequently, the management of ponds, including the free movement of individuals, in addition to measures to prevent the invasion of *R. o. ocellatus*, is necessary for the conservation of *R. o. kurumeus*.

Key words: conservation, diversity, inbreeding depression, isolation, *Rhodeus ocellatus kurumeus*

INTRODUCTION

The number of endangered species throughout the world shows increases annually (Myers *et al.*, 2000). At present, biodiversity faces six major threats from human activities; destruction of habitat, habitat fragmentation, environmental deterioration by pollution, overexploitation, introduction of non-native species, and introduction of agents of disease (Primack, 1995). Fragmentation of habitats into smaller patches probably reduces or prevents the dispersal or settlement of species, which thereby increases the risk of local extinction of a species (Wilcove *et al.*, 1986). Small, fragmented populations are particularly susceptible to extinction due to genetic problems, demographic stochasticity, and rapid environmental fluctuations (Primack, 1995). The loss of genetic diversity in small-sized populations can negatively affect individual fitness and thereby the viability of populations, measured in terms of survival, growth and developmental rates, disease resistance and developmental

stability (Allendorf and Leary, 1986).

The Japanese rosy bitterling, *Rhodeus ocellatus kurumeus*, is an endemic cyprinid species of Japan. Before World War II, it was widely distributed in creeks and small rivers in western Japan (Nakamura, 1969; Hosoya, 1982; Uematsu and Aki, 1984; Kawamura, 2003). In the 1940s, the Chinese rosy bitterling *R. o. ocellatus*, which is a subspecies of *R. o. kurumeus*, was accidentally introduced into the Tone River in Japan from the Changjiang River in mainland China along with juvenile grass carp *Ctenopharyngodon idellus* and silver carp *Hypophthalmichthys molitrix* (Nakamura, 1955) which it resembles. In the last six decades since its introduction, *R. o. ocellatus* has expanded its distribution all over Japan, which appears to be a major factor leading to the loss of populations of *R. o. kurumeus* (Nagata, 1997). At present, *R. o. kurumeus* in Honshu and Shikoku occur only in small isolated ponds in the foothills of mountains in Osaka and Kagawa. In Kyushu *R. o. kurumeus* still inhabits open water systems, into which *R. o. ocellatus* has only recently invaded (Fig. 1).

Based on allozyme data, it appears that many populations of *R. ocellatus* in the original range of *R. o. kurumeus*

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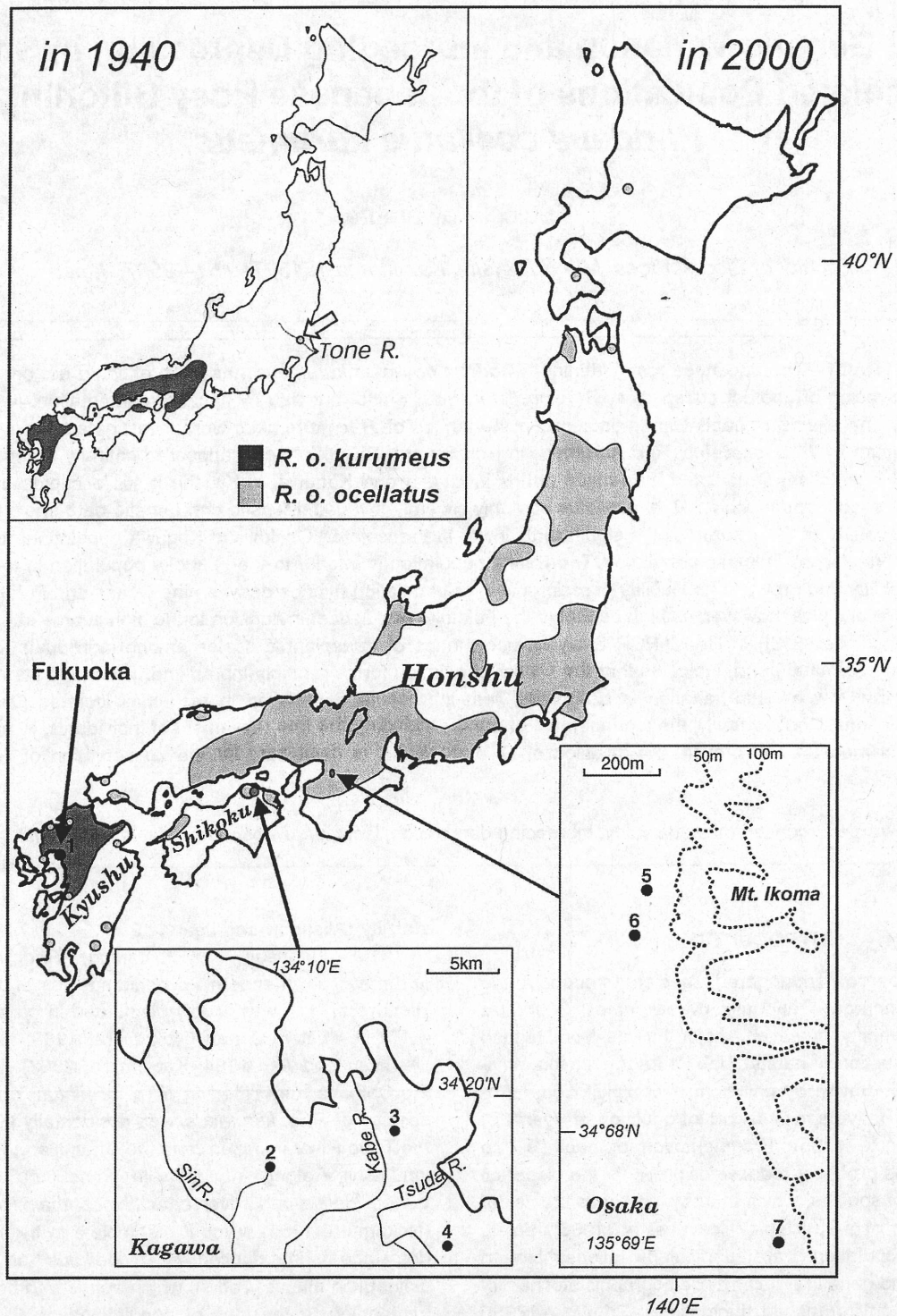


Fig. 1. Changes in the distribution of *R. o. kurumeus* between 1940–2000, showing sampling stations of *R. o. kurumeus* used in this study. (1) Yanagawa, Fukuoka (R. Chikugo system, creeks); (2) Kita, Kagawa (R. Sin system, pond); (3) Ookawa, Kagawa (R. Kabe system, pond); (4) Okawa, Kagawa (R. Tsuda system, pond); (5–7) Yao, Osaka (R. Yamato system, 3 ponds). *Rhodnius o. kurumeus* was widely distributed in the western part of Japan in the 1940's. However, after the introduction of *R. o. ocellatus*, the distribution of *R. o. kurumeus* has markedly declined (Nakamura, 1955; Nakamura, 1969; Hosoya, 1982; Uematsu and Aki, 1984; Japan Wildlife Research Center, 1993).

probably comprise hybrids between *R. o. kurumeus* and *R. o. ocellatus* (Nagata *et al.*, 1996). Kawamura *et al.* (2001a) found that both mitochondrial and genomic DNA of *R. o. kurumeus* was almost completely substituted by that of *R. o. ocellatus* in hybrid populations. Thus, many populations of *R. o. kurumeus* seem to have become hybrids of the two subspecies in the last six decades (Kawamura *et al.*, 2001a).

The remaining genetically discrete populations of *R. o. kurumeus* are confined to about 20 small ponds, typically with a diameter of less than 20 m, in Osaka and Kagawa (Kawamura *et al.*, 2001b). The ponds were created for irrigation of paddy fields, into which *R. o. kurumeus* were probably accidentally introduced with juvenile crucian carp, *Carrasius cuvieri*, in the 19th century (T Yata, pers. comm.). Renovation of these ponds with concrete walls after World War II isolated them from surrounding water bodies. Consequently, populations of rosy bitterling in each pond are now completely isolated from neighboring impoundments. Kawamura *et al.* (2001b) showed that diversity measured by both meristic characters and mtDNA in *R. o. kurumeus* was markedly lower in the isolated Osaka and Kagawa populations than in non-isolated populations in Fukuoka. It has been reported that low haplotype diversity of mtDNA is related to low fitness in *Poeciliopsis* (Quattro and Vrijenhoek, 1989; Quattro *et al.*, 1996). Therefore, it is possible that the fitness of isolated populations of *R. o. kurumeus* in the isolated Osaka and Kagawa populations with low haplotype diversity of mtDNA may also be reduced.

The objective of the present study was to investigate genetic problems arising from habitat fragmentation in *R. o. kurumeus*. First, the degree of loss of fitness in isolated populations was examined by rearing and hybridization experiments of *R. o. kurumeus* from isolated and non-isolated populations. Second, the magnitude of loss of genetic diversity was estimated by scale transplantation experiments, as well as RAPD-PCR analysis of genomic DNA among populations. Results are discussed in the light of measures to conserve *R. o. kurumeus* within its native range.

MATERIALS AND METHODS

Samples

Samples of *R. o. kurumeus* were collected from open and closed water systems. Fish from open water systems were captured in creeks in the River Chikugo system in Fukuoka (Station [Stn.] 1), while those from closed water systems were collected from three ponds in Kagawa (Stns. 2–4) and three in Osaka (Stns. 5–7) in 1995 (Fig. 1). Samples for rearing experiments and DNA analysis were captured using fish traps in Fukuoka and Osaka, and transported live to the laboratory (n=130 in Stn. 1, 30 in Stn. 5, 30 in Stn. 6 and 100 in Stn. 7). DNA samples from Kagawa populations were collected by non-lethal fin clip from the caudal fin and fixed in 70% ethanol (n=20 in Stn. 2, 20 in Stn. 3 and 20 in Stn. 4). All fish samples were collected from genetically discrete populations of *R. o. kurumeus*, demonstrated by analyses of RAPD-PCR and mtDNA in previous studies (Kawamura *et al.*, 2001a, 2001b).

Rearing and hybridization experiments

Differences in fertility, survival and growth between isolated and non-isolated populations were examined by comparing the Fukuoka (n=120 in Stn. 1) and Osaka (n=97 in Stn. 7) populations of *R. o. kurumeus* by artificial fertilization and rearing experiments, according to the protocol of Kawamura (1998). Artificial hybridizations between populations or subpopulations were carried out for the Fukuoka (n=42 in Stn. 1) and Osaka (n=40 in Stn. 5 and 43 in Stn. 7) populations, to compare the survival of hybrids with that of their parental populations. In addition, the presence of transparent-scaled types was investigated in each hybrid. Transparent-scaled types, which are characterized by low numbers of guanophores in the scales, are a genetic mutant often seen in cyprinid fishes (Kawamura *et al.*, 1998). In *R. o. kurumeus*, it is only observed in the subpopulation from Osaka in an impoundment at Stn. 7. Statistical differences among experimental groups were examined using *t* tests.

Scale transplantation experiment

Scale transplantations were carried out between the Fukuoka (n=30 in Stn. 1) and Osaka (n=30 in Stn. 5, 30 in Stn. 6, and 30 in Stn. 7) populations to examine the level of histocompatibility between individuals in populations. If individuals have high levels of histocompatibility, then it is likely that they are genetically similar (Hyodo-Taguchi, 1980). Following Kurita *et al.* (1995), five scales were exchanged reciprocally between two mature males of about 40 mm TL in each combination. Scales taken from the dorsal region above the operculum of a donor, where the distribution of xantho-

Table 1. Fertility, viability and growth (mean±SD) of two populations of *R. o. kurumeus*.

Locality	BSP (mm)	CS	HO (%) ^a	SR30 (%) ^b	SR120 (%) ^b	BSO (mm)
Fukuoka (St. 1)	41.3±9.7 (N=120)	9.7±5.1 (B=42)	60.2±37.9 (B=42)	32.6±32.8 (B=42)	6.2±1.5 (B=42)	30.5±3.5 (N=36)
Osaka (St. 7)	42.5±5.9 (N=97)	8.8±6.7 (B=69)	33.5±33.6 (B=69)	14.8±24.0 (B=69)	0.9±0.3 (B=69)	25.4±5.5 (N=6)
Significance (<i>p</i>)	n.s.	n.s.	**	*	**	*

Abbreviations of hierarchical levels: BSP, body size (TL) of parental fish; CS, clutch size; HO, hatchability of offspring; SR30, survival rate of offspring on the 30th day after hatching; SR120, survival rate of offspring on the 120th day after hatching; BSO, body size (TL) of offspring on the 120th day after hatching; B, batch size; N, total number of specimens examined.

* $p < 0.01$; ** $p < 0.001$.

^a Hatchability = (hatched embryos)/(eggs used)

^b Survival rate = (surviving fish)/(hatched embryos)

phores is restricted, were transplanted, using fine forceps, into scale pockets on the ventral unpigmented region of the recipient, from which the original scales had been previously been taken. Fish were reared at 20°C and the characteristics of the grafted scales were checked every three days. The complete breakdown of the

melanophores in all transplanted scales were regarded as rejection, while no change in the appearance of melanophores two weeks after transplantation was regarded as acceptance. Transplanted scales were monitored for two months and the same experiments were performed on ten pairs in each combination (see Table 4) of

Table 2. Viability (mean±SD) of hybrids between populations of *R. o. kurumeus*.

Parental fish		Abbreviation of hybrids	HO (%) ^a	SR30 (%) ^b	SR120 (%) ^b	RT (%) ^c
Female	Male					
Fukuoka (St. 1)	X Fukuoka (St. 1)	FUK1	60.2±37.9 (B=42)	32.6±32.8 (B=42)	6.2±1.5 (B=42)	0 (N=36)
Osaka (St. 5)	X Osaka (St. 5)	OSA5	35.1±28.4 (B=45)	15.1±13.1 (B=45)	1.1±0.2 (B=45)	0 (N=5)
Osaka (St. 7)	X Osaka (St. 7)	OSA7	33.5±33.6 (B=69)	14.8±24.0 (B=69)	0.9±0.3 (B=69)	100 (N=6)
Fukuoka (St. 1)	X Osaka (St. 7)	F1xO7	67.2±29.5 (B=20)	54.9±41.5 (B=20)	15.0±8.4 (B=20)	0 (N=22)
Osaka (St. 7)	X Fukuoka (St. 1)	O7xF1	42.9±17.9 (B=20)	30.3±25.4 (B=20)	13.9±7.4 (B=20)	0 (N=14)
Osaka (St. 5)	X Osaka (St. 7)	O5xO7	32.6±20.5 (B=20)	15.0±19.6 (B=20)	1.2±0.4 (B=20)	0 (N=3)

Abbreviations of hierarchical levels: H, hatchability; SR30, survival rate of offspring on the 30th day after hatching; SR120, survival rate of offspring on the 120th day after hatching; RT, ratio of transparent-scaled type; B, batch size; N, total number of specimens examined.

^a Hatchability = (hatched embryos)/(eggs used)

^b Surviving rate = (surviving fish)/(hatched embryos)

^c Ratio of transparent-scaled type = (transparent-scaled type)/(surviving fish)

Table 3. Pairwise comparison of hybrids between populations of *R. o. kurumeus*. Abbreviation of hybrids are described in Table 2.

		FUK1	F1xO7	O7xF1	OSA7	O5xO7	OSA5
FUK1	HO						
	SR30	—					
	SR120						
F1xO7	HO	n.s.					
	SR30	*	—				
	SR120	***					
O7xF1	HO	*	**				
	SR30	n.s.	*	—			
	SR120	***	n.s.				
OSA7	HO	***	***	n.s.			
	SR30	**	***	*	—		
	SR120	***	***	***			
O5xO7	HO	***	***	n.s.	n.s.		
	SR30	*	***	*	n.s.	—	
	SR120	***	***	***	***		
OSA5	HO	***	***	n.s.	n.s.	n.s.	
	SR30	**	***	*	n.s.	n.s.	—
	SR120	***	***	***	***	n.s.	

Abbreviations of hierarchical levels: H, hatchability; SR30, survival rate of offspring on the 30th day after hatching; SR120, survival rate of offspring on the 120th day after hatching.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

donors and recipients.

RAPD-PCR analysis

Genetic variation of genomic DNA in *R. o. kurumeus* was examined by RAPD-PCR analysis in all study populations (Stns. 1–7). Total DNA was extracted from muscle tissue by a standard phenol-chloroform extraction (Lansman *et al.*, 1981). The procedure of RAPD-PCR amplification followed that described in Kawamura *et al.* (2001a) in a 25 μ l reaction volume. 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. To detect molecular polymorphism among populations of *R. o. kurumeus*, three kits (designated A and F), each containing 20 decamer RAPD primers (Operon Technologies, Alameda, CA) were tested, and 17 primers showed amplification of polymorphism. The 17 primers were: OPA-07, OPA-08, OPA-11, OPA-12, OPA-15, OPA-17, OPA-19, OPA-20, OPF-01, OPF-02, OPF-03, OPF-04, OPF-09, OPF-12, OPF-14, OPF-15, OPF-20. Amplifications were repeated with the same samples three times and reproducible banding patterns were obtained with these primers. Twenty samples were examined for each of the seven sampling stations: Fukuoka (Stn. 1), Kagawa (Stns. 2–4) and Osaka (Stns. 5–7).

The presence or absence of each scorable fragment was recorded as binary data. Genetic diversity of each population was measured by using the band sharing index (BSI) of Wetton *et al.* (1987). Resampling of the overall data in each population was performed 1,000 times with the permutation test of Danforth and Freeman-Gallant (1996) for pairwise comparison of genetic diversity in populations.

RESULTS

Fertility, survival and growth

There was no significant difference in body size (BSP) or clutch size (CS) of parental fish between populations of *R. o. kurumeus* from Fukuoka (Stn. 1) and Osaka (Stn. 7) (Table 1). However, survival of offspring after fertilization in the Osaka population was lower than that of the Fukuoka population. Both hatchability (HO) and survival rate on the 30th day after hatching (SR30) of the Osaka population were approximately half those of the Fukuoka population ($P < 0.01$). Survival rates to 120 days after hatching (SR120) in the Osaka population was about one seventh that of the Fukuoka population ($P < 0.001$). Body size of offspring 120

Table 4. Scale transplantation between populations of *R. o. kurumeus*. Each experiment was reciprocally performed with five scales between two individuals in different or same populations ten times. Upper number means no. of recipients accepting grafted scales, while lower means that of recipients rejecting grafted scales. Rejection was considered complete when all melanophores in transplants were destroyed.

Population of Recipients		Population of donors			
		Fukuoka	Osaka		
		St. 1	St. 5	St. 6	St. 7
Fukuoka	St. 1	0/10			
Osaka	St. 5	0/10	10/0		
	St. 6	0/10	10/0	10/0	
	St. 7	0/10	10/0	10/0	10/0

days after hatching (BS120) in the Osaka population was lower than that of the Fukuoka population by about 15% ($P < 0.01$).

In hybridization experiments, inter-population hybrids (F1xO7) between the Fukuoka (Stn. 1, female) and Osaka (Stn. 7, male) populations of *R. o. kurumeus* were always superior to any other hybrids and populations in viability ($P < 0.05$) (Tables 2 and 3). In contrast, reciprocal inter-pop-

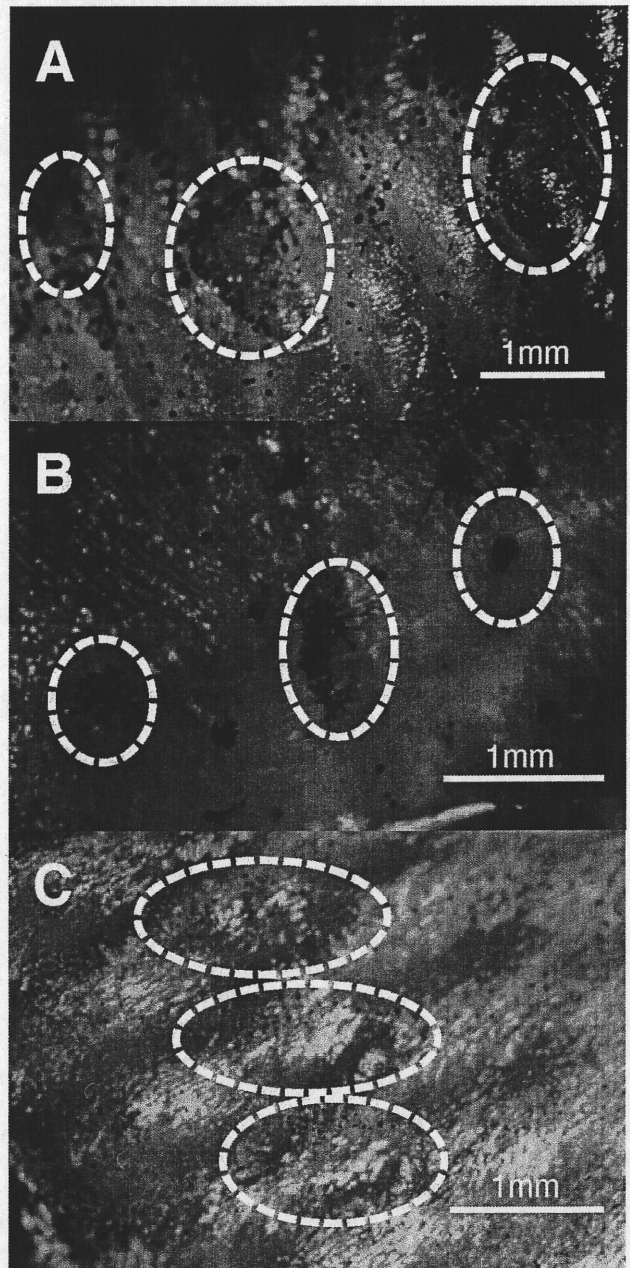


Fig. 2. Successful and unsuccessful transplantations of scales in *R. o. kurumeus*. (A) Transplanted scales immediately after transplantation; (B) Rejected scales one week after transplantation in which melanophores are degrading; (C) Accepted scales two weeks after transplantation. Open circles indicate transplanted scales. All scale bars equal 1 mm.

Table 5. Band sharing index (BSI) of RAPD-PCR analysis among populations of *R. o. kurumeus*. BSIs are presented as mean±S.E. above the diagonal, and significance of BSIs in pairwise comparison is below the diagonal. In each population or sub-population, 20 samples were examined.

		Fukuoka		Kagawa			Osaka		
		Stn. 1	Stn. 2	Stn. 3	Stn. 4	Stn. 5	Stn. 6	Stn. 7	
Fukuoka	Stn. 1	0.798±0.019	0.569±0.026	0.561±0.025	0.535±0.019	0.557±0.022	0.547±0.023	0.573±0.019	
Kagawa	Stn. 2	*	0.937±0.014	0.907±0.021	0.896±0.025	0.744±0.021	0.749±0.027	0.772±0.016	
	Stn. 3	*	n.s.	0.955±0.010	0.954±0.013	0.745±0.012	0.750±0.013	0.774±0.012	
	Stn. 4	*	n.s.	n.s.	0.976±0.014	0.723±0.010	0.733±0.012	0.752±0.014	
Osaka	Stn. 5	*	n.s.	n.s.	n.s.	0.915±0.017	0.923±0.016	0.904±0.010	
	Stn. 6	*	n.s.	n.s.	n.s.	n.s.	0.941±0.013	0.903±0.012	
	Stn. 7	*	n.s.	n.s.	n.s.	n.s.	n.s.	0.951±0.016	

* $P < 0.001$.

ulation hybrids (O7xF1) between the same parental populations were intermediate between the Fukuoka (FUK1) and Osaka (OSA7) populations in both HO and SR30. However, O7xF1 were higher than FUK1 and OSA7 in SR120. Values for SR120 for both F1xO7 and O7xF1 were approximately twice as high as FUK1 ($P < 0.001$). In the Osaka population, inter-subpopulation hybrids (O5xO7) between subpopulations from Stn. 5 and 7 did not statistically differ from OSA5 and OSA7 in HO and SR30, except for the low survival of OSA7 in terms of SR120 ($P < 0.001$). Transparent-scaled types were only observed in individuals from OSA7 ($n=6$), while it was never observed in any other hybrids or populations (Table 2).

Immunoresponse to grafted scales

When scales were transplanted between individuals from the Fukuoka population (Stn. 1), all scales were rejected in all recipients in ten trials (Table 4). Melanophores in all transplants began to aggregate in the center of scales about three days after transplantation, and the rejection of transplants was clear one week later (Fig. 2A, B). Melanophores in all transplants were completely disrupted after one month. Rejection in the same manner was also observed in the transplantation between individuals of the Fukuoka (Stn. 1) and Osaka (Stns. 5–7) populations in all trials. In contrast, all transplanted scales were accepted in transplantations between individuals from the Osaka populations, irrespective of the ponds from which donors and recipients were derived (Table 4). No signs of aggregation of melanophores were observed in any transplanted scales of recipients in the ten trials. Further, transplants remained unchanged even after one month (Fig. 2C).

Genetic variation in DNA

The BSI of the Fukuoka population (BSI=0.798) was significantly lower than that of the subpopulations in Kagawa and Osaka (BSI=0.937–0.976 in Stns. 2–4 and 0.915–0.951 in Stns. 5–7, $P < 0.001$). There was also no statistical difference between the Kagawa and Osaka populations in BSIs among subpopulations ($P > 0.1$, Table 5). BSIs between sub-

populations in one population (BSI=0.896–0.954 in Kagawa and 0.903–0.923 in Osaka) were not equivalent to those from the subpopulations in the same population. The BSI between the Fukuoka and Kagawa populations (BSI=0.535–0.569) was almost the same as that between the Fukuoka and Osaka populations (BSI=0.547–0.573, $P > 0.1$). BSIs between subpopulations of the Kagawa population and those of the Osaka population were approximately constant, ranging from 0.723 to 0.774.

DISCUSSION

Loss of genetic diversity in isolated populations

Nagata *et al.* (1996) reported that heterozygosity of *R. o. kurumeus* at 16 allozyme loci was zero in the Osaka population, while it was high in the Fukuoka population. Kawamura *et al.* (2001b) found that variation in meristic characters (number of pored lateral line scales, number of vertebrae and unpaired fin rays) were significantly lower in the Osaka and Kagawa populations than in the Fukuoka population. In addition, haplotype diversity of mtDNA was zero in the subpopulations of all 15 ponds in Kagawa and 5 of 11 ponds in Osaka, while it was higher in the Fukuoka population (Kawamura *et al.*, 2001b). In this study, BSI from RAPD-PCR analysis was much higher in the Osaka and Kagawa populations than in the Fukuoka population (Table 5). These results clearly demonstrate that the genetic diversity of *R. o. kurumeus* is markedly lower in the isolated populations in Osaka and Kagawa than in the open Fukuoka population.

The Osaka population of *R. o. kurumeus* was inferior to the Fukuoka population in terms of viability and growth in rearing experiments (Table 1). However, inter-population hybrids showed higher viability after fertilization than both the parental Fukuoka and Osaka populations, while inter-subpopulation hybrids were almost the same as the parental subpopulations in the Osaka population (Tables 2 and 3). One explanation for this result is that the high viability in inter-population hybrids arose from the genetic dominance of the Fukuoka population over the Osaka population in fit-

ness, in addition to heterosis, while the low viability of inter-subpopulation hybrids was not improved by heterosis, because of the low fitness in the subpopulations from Osaka. In term of hatchability, the inter-population hybrid of F1xO7 was significantly different from the reciprocal hybrid O7xF1 ($P < 0.001$). The former approximated the Fukuoka population, while the latter approximated the Osaka population (Tables 2 and 3). Mylonas *et al.* (1992) reported that egg quality is highly correlated with survival from fertilization to hatching. Thus, differences in hatchability between the two inter-population hybrids of *R. o. kurumeus* may be influenced by low egg quality in the Osaka population. Judging from the results of rearing and hybridization experiments (Tables 1–3), the low viability and growth of the Osaka population appears primarily to be due to genetic, not environmental factors.

High histocompatibility among individuals is related to the degree of inbreeding in populations (Hyodo-Taguchi, 1980). In both *Xiphophorus maculatus* (Kallman and Gordon, 1957) and *Oryzias latipes* (Hyodo-Taguchi, 1980), transplanted scales are accepted between individuals when the inbreeding coefficient is more than 0.9. In *R. o. kurumeus*, acceptance of transplanted scales between individuals from multiple impoundments in Osaka indicates that the inbreeding coefficient in the Osaka population is likely to be high in many of ponds (Fig. 6, Table 4). Indeed, it is possible that all the Osaka populations represent a single highly inbred line.

Vrijenhoek (1996) observed that small isolated populations of *Poeciliopsis* that showed rapid loss of genetic diversity also suffered from a decline in fitness; manifested as poor competitive ability, low growth rate, developmental instability, and reduced resistance to parasites. The same phenomena can be seen in Osaka populations of *R. o. kurumeus*. Osaka populations, which have a low genetic diversity (Kawamura *et al.*, 2001b; Table 5), also showed significantly lower viability and growth than the Fukuoka population, which has higher genetic diversity (Tables 1–3). The tentative conclusion to be drawn from this result is that the Osaka populations of *R. o. kurumeus* display lower fitness as a direct consequence of a loss of genetic diversity.

Risk of low genetic diversity

Due to their isolation in small impoundments away from human habitation, populations of *R. o. kurumeus* in Osaka and Kagawa seems to have escaped displacement by *R. o. ocellatus*. However, they face another threat in the form of inbreeding and low genetic diversity. Populations in small isolated water bodies are particularly susceptible to environmental change, such as water pollution, exposure to pathogens, and drought. Drastic changes in the environment can lead to substantial reductions in population size, which leads to a concomitant loss of genetic diversity through inbreeding and genetic drift. For example, in one impoundment (144 m² in area) in Osaka, the population density of *R. o. kurumeus* had fluctuated between 2 and 40 individuals/m² from 1999

to 2003, mainly owing to a drought in 2002 (Y Kanoh, pers. comm.). Since the origin of populations of *R. o. kurumeus* in these man-made ponds is probably through immigration, their original genetic diversity was probably low owing to founder effects. The instability and low carrying capacity of the environment in these small ponds also seems likely to have contributed to the erosion of the genetic diversity of *R. o. kurumeus* populations in them.

The loss of genetic diversity from populations can reduce the fitness of the individuals in them (Harada, 1999). A loss of overall genetic diversity is often accompanied by the disappearance of rare alleles and an increased frequency of deleterious recessive alleles. The lower fitness of *R. o. kurumeus* evident in Tables 1–3 and the presence of transparent-scaled types in one pond (Strn. 7) in the Osaka population are probably symptoms of inbreeding depression, resulting from the loss of genetic diversity. Small populations are more susceptible to demographic stochasticity in birth and mortality rates, and the expression of deleterious genes, both of which can increase the risk of population extinction. Gilpin and Soulé (1986) termed such a situation an extinction vortex. It is possible that just such an extinction vortex exists in the isolated study populations of *R. o. kurumeus* in Osaka and Kagawa.

In conclusion, the risk of extinction of populations of *R. o. kurumeus* may occur through two different mechanisms. Invaded *R. o. ocellatus* displaces genetically distinct *R. o. kurumeus*, through ecological competition and hybridization (Kawamura *et al.*, 2001a). In addition, isolated populations of *R. o. kurumeus* in ponds, which have hitherto escaped invasion by *R. o. ocellatus*, are at risk of inbreeding depression caused by a loss of genetic diversity. Reduced genetic diversity arises through a combination of founder effects in small populations and repeated genetic bottlenecks as a consequence of high demographic stochasticity in isolated and environmentally unpredictable habitats. The effects of inbreeding depression not only reduce overall population size but also increase the probability of local extinction (Eilstrand and Elam, 1993).

Biological conservation

Before the recent renovation of impoundments with concrete walls, and the invasion of *R. o. ocellatus*, *R. o. kurumeus* inhabited many ponds in Osaka and Kagawa. Formerly, aquatic organisms, including *R. o. kurumeus*, could freely move from one pond to another (Y Kanoh and H Ohtaka, pers. comm.). Individual populations probably contributed to one large metapopulation in each region (Nicholson and Bailey, 1935), which thereby prevented an overall loss of genetic diversity, since populations were constantly recolonised from the metapopulation. However, at present low genetic diversity seems to be prevalent in populations of *R. o. kurumeus* in many impoundments in Osaka and Kagawa, judging from the results of this study (Table 5) and mtDNA analyses (Kawamura *et al.*, 2001b).

For the conservation of *R. o. kurumeus* in the study

ponds it is necessary to prevent the further decline of abundance in each impoundment, which often occurs through environmental deterioration, such as water pollution and drought (Kawamura, 2003). In addition, to achieve satisfactory conservation of this species, not only must the quality and quantity of water in ponds be maintained, but also the free movement of individuals among ponds to conserve genetic variability is needed. This goal could be achieved by the removal of concrete walls from ponds and their neighboring drainage channels as soon as possible.

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