

## Research Article

## Predicting potential hybridization between native and non-native *Rhodeus ocellatus* subspecies: the implications for conservation of a pure native population in northern Kyushu, Japan

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### Abstract

Hybridization between native and non-native fishes is a serious global problem. Thus, there is a need to develop monitoring methods for predicting potential hybridization to evaluate the risk of genetic introgression and to identify important areas for conservation of pure native populations. Here, we developed a prediction model for intersubspecific hybridization, based on distribution and genetic data. We selected *Rhodeus ocellatus kurumeus* and *R. ocellatus ocellatus* as the native and non-native subspecies, respectively. First, we developed generalized linear models (GLMs) for the species habitat requirements by using presence/absence data and environmental variables. The best-fit models showed river length gave conflicting effects for the two subspecies. Next, we developed a GLM using the ratio of non-native haplotypes in mitochondrial DNA as an objective variable, with the predicted probabilities of the occurrence of each fish and spatial information as explanatory variables. The best-fit model selected the distance from the center of native distribution and the non-native habitat requirement as key factors. Our findings indicate that hybridization occurs highly and/or initially near the margin of native distribution where non-native habitat requirements are available. Our model could identify sites in native habitats with very low potential risk for genetic invasion as important areas for conservation of pure native populations.

**Key words:** Cyprinidae; bitterling; genetic introgression; habitat use; prediction model

### Introduction

The introduction and establishment of non-native species have negative effects on individuals, populations, and communities of native species, and introduced species have contributed to the decline or extinction of native species worldwide (Coblentz 1990; Lodge 1993; Townsend 2003). Hybridization is one of the most detrimental impacts of non-native species on native species (Sato et al. 2010a). Numerous cases of hybridization have been documented in freshwater fishes (e.g., Rhymer and Simberloff 1996; Epifanio and Nielsen 2000; Kawamura et al. 2001; Konishi et al. 2003). Nevertheless, in terms of legislation and regulation little attention has been paid to

the severity of ecological impact from hybridization despite the fact that hybridization can cause extinction of a species, subspecies, or population by genetic extermination with genetic introgression (Rhymer and Simberloff 1996; Allendorf et al. 2001; Sato et al. 2010a).

We can classify the hybridizations of freshwater fishes in Japan into three levels: interspecific, intersubspecific, and intraspecific hybridization. There are reports of interspecific hybridization between the Shinai-motsugo, *Pseudorasbora pumila pumila* Miyadi, 1930, and the topmouth gudgeon, *P. parva* Temminck et Schlegel, 1846 (Konishi et al. 2003), as well as intersubspecific hybridization between the Japanese rosy bitterling, *Rhodeus ocellatus kurumeus* Jordan

and Thompson, 1914 (Rok), and the Chinese rosy bitterling, *R. o. ocellatus* Kner, 1866 (Roo) (Kanoh et al. 2005). The intraspecific hybridizations between different regional populations have been demonstrated in a number of species, including the zezera, *Biwia zezera* Ishikawa, 1895 (Horikawa and Mukai 2007; Horikawa et al. 2007). Rok and *P. pumila* are designated as “critically endangered” in the Japanese Red Data Book (Kawamura 2003), mainly because of hybridization with alien subspecies and closely-related species. By contrast, several alien species, subspecies and populations, including Roo and *P. parva*, are classified as “invasive with high risk” (Onikura et al. 2011), by the Fish Invasiveness Scoring Kit (Copp et al. 2005).

Anthropogenic hybridization between native and non-native fishes in Japan has been confirmed by morphological and genetic studies (Kawamura et al. 2001; Konishi et al. 2003; Konishi and Takata 2004a; Kanoh et al. 2005; Koga and Goto 2005), validation of crossing experiment in aquaria (Konishi and Takata 2004b; Kawamura 2005; Kawamura et al. 2009), analysis of distribution patterns and habitat uses in native and alien subspecies and their hybrids (Miyake et al. 2008; Konishi et al. 2009; Onikura et al. 2012), and evaluation of risk of genetic invasion (Onikura et al. 2011). However, to date no-one has developed monitoring methods for predicting hybridization, or elucidated environmental requirements for genetic introgression from native to non-native subspecies.

Here, we address the hybridization between Rok (native subspecies) and Roo (non-native subspecies) as a serious problem. Rok, which is an endemic cyprinid species of Japan, was widely distributed in creeks, ponds, and small rivers in western Japan prior to the 1940s (Figures 1-2, Kawamura 2005). By contrast, Roo, which belongs to a sister subspecies of Rok, was accidentally introduced into Japan from mainland China in the 1940s (Nakabo 2002; Matsuzawa and Senou 2008). Hybridization between Rok and Roo is a major factor contributing to the loss of Rok populations (Kawamura 2003). Currently, Roo and hybrid populations are expanding throughout Japan, excluding Kyushu Island (Figure 2). The process of genetic invasion of Roo into Rok in Honshu and Shikoku islands is believed to have occurred as follows: occurrence of Rok mainly in man-made ponds; progression of inbreeding depression within each population in each fragmented habitat; inferior fitness level of each

population; invasion of non-native Roo populations; and rapid subspecies hybridization (Kanoh et al. 2005; Kawamura 2005; Kawamura et al. 2009).

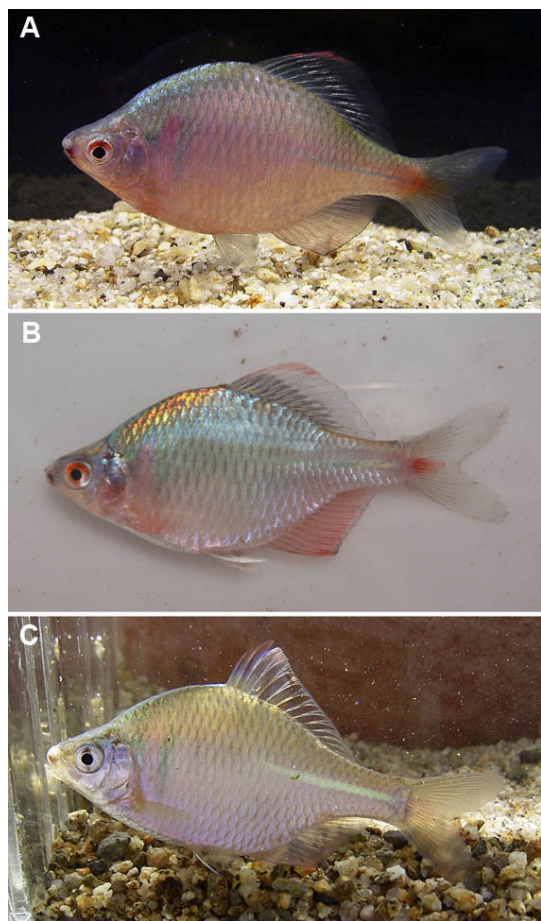
By contrast, Rok populations in northern Kyushu Island have high genetic variation compared with Rok populations in Honshu and Shikoku islands (Kawamura 2005). In Kyushu Island, Rok populations inhabit open-water systems (Kawamura 2005), such as agricultural canal networks and rivers (Miyake et al. 2008; Onikura et al. 2007, 2012) and have a wide range of habitats (Miyake et al. 2008; Onikura et al. 2012). Kawamura (2005) suggested that the high genetic variation of Rok population in this area should be maintained by the superior population size and habitat conditions compared to those in Honshu and Shikoku islands.

On the basis of previous reports on the distributions of Rok, Roo, and hybrid populations in western Japan, we conclude that the prevention of genetic introgression by Roo in northern Kyushu Island is the key to conserve the Rok population of Japan. The aims of this study were to evaluate the risk of genetic introgression by Roo and to identify important areas for Rok conservation in this island. Our previous studies have shown the distributions of Rok, Roo, and their hybrid populations in this island (Figure 3, Miyake et al. 2008; Onikura et al. 2012). Here, we developed a predictive model of genetic introgression by the nonnative subspecies based on genetic and distribution data.

## Methods

### Study design

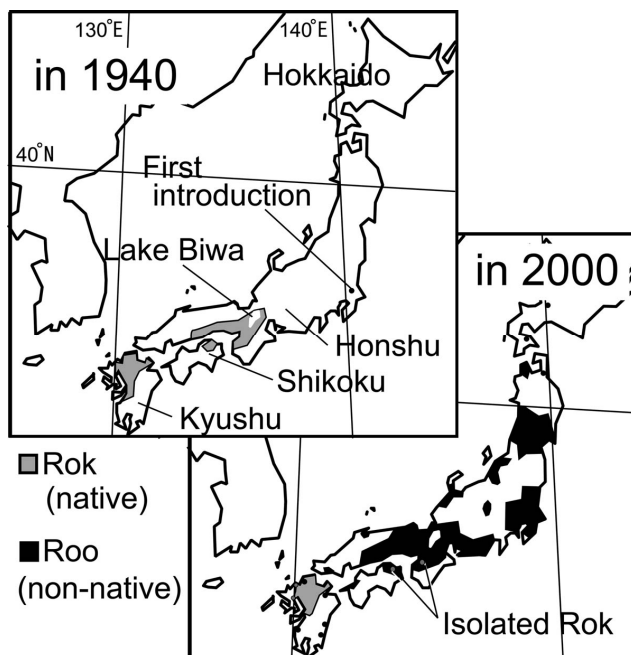
We selected northern Kyushu Island of Japan as our survey area according to Miyake et al. (2008) and Onikura et al. (2012) where Rok, Roo and their hybrids are well distributed. Two estimations were used in developing a genetic invasion model. One estimated habitat requirements of the two subspecies and the other estimated the ease of hybridization in those areas. In the former, the hybrid population was expected to occur in the overlapping area of Rok and Roo distributions based on their habitat requirements. We predicted the distributions of Rok and Roo in northern Kyushu by using models with high accuracy and landscape parameters as explanatory variables in our previous study (Onikura et al. 2012), which indicated that the overlapping areas of distribution of the two subspecies were in limited areas that contained the habitat



**Figure 1.** A male of *Rhodeus ocellatus kurumeus* from Tsuyazaki creek in northern Kyushu Island, Japan (A); a male of *Rhodeus ocellatus ocellatus* from the Tiaoxi River, Yangtze River basin, China (B); a male of the hybrid between *Rhodeus ocellatus kurumeus* and *Rhodeus ocellatus ocellatus* from Kashima creek in northern Kyushu, Japan (C). A, C – photographs by Dr. J. Nakajima; B - photograph by Dr. Y. Kano, Kyushu University, Japan.

requirements of the two subspecies. Therefore, we calculated the probability of occurrence of each subspecies in each site and used these probabilities as explanatory variables of a genetic invasion model.

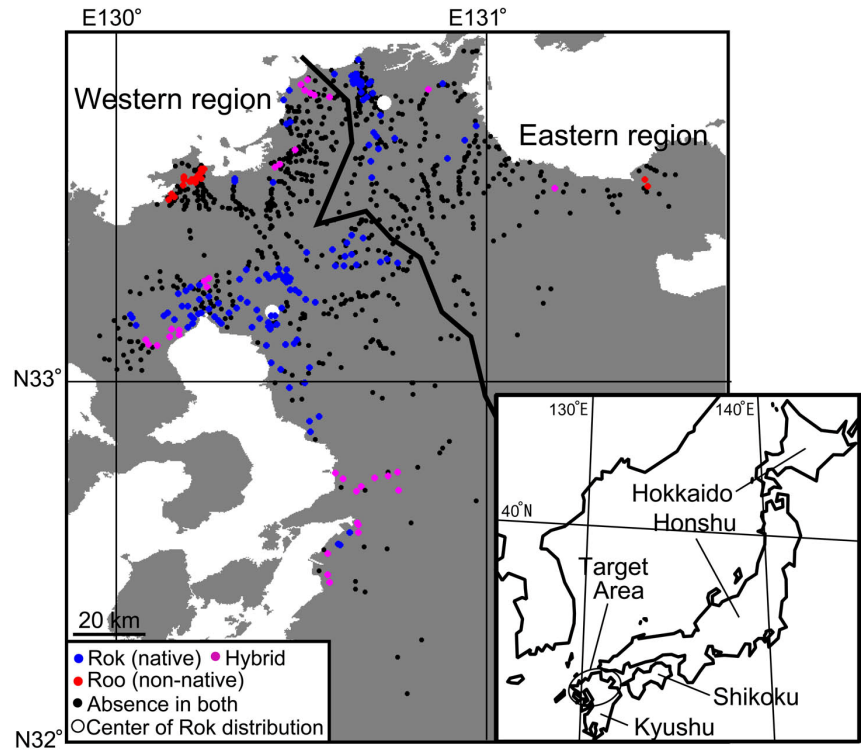
To estimate the ease of hybridization, we first examined previous studies on the mechanism of hybridization. Rok and Roo show gradual pre-mating reproductive isolation in culture experiments (Kawamura et al. 2009). However, Roo and hybrid populations have been expanding throughout Japan since the 1940s (Nakabo 2002; Matsuzawa and Senou 2008). One reason for this expansion



**Figure 2.** The distribution of *Rhodeus ocellatus* subspecies in Japan in 1940 and 2010. The native and non-native subspecies are *Rhodeus ocellatus kurumeus* (Rok) and *R. o. ocellatus* (Roo), respectively. Hybrid populations between these subspecies occur. The distribution of hybrid populations is included in the Roo distribution.

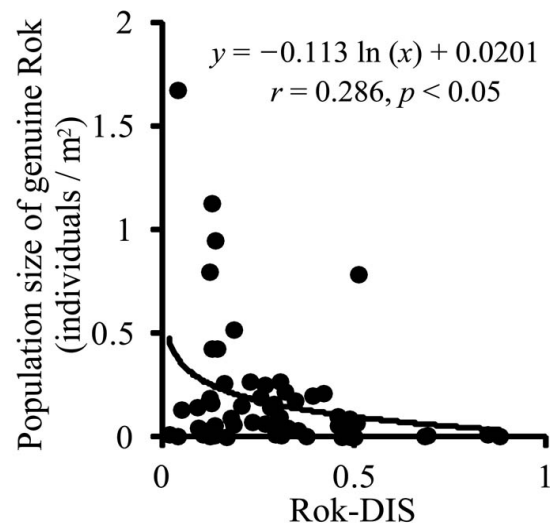
is the progression of inbreeding depression within Rok populations in fragmented habitats (Kanoh et al. 2005; Kawamura 2005; Kawamura et al. 2009). Before the 1940s, Rok inhabited small isolated ponds in western Japan, excluding northern Kyushu Island, and the small populations in each pond showed low genetic variation and inferior fitness levels because of excessive inbreeding. When Roo invaded Rok habitat, introgressive hybridization between the two subspecies progressed rapidly. Therefore, fragmented habitats and small Rok population sizes led to the ease of introgressive hybridization by Roo. In northern

**Figure 3.** The current presence/absence of *Rhodeus ocellatus* subspecies in northern Kyushu, Japan. The distribution data for Rok, Roo, and hybrid populations are summarized in detail in Appendix 1.



Kyushu Island, Rok is widely distributed in open-water systems and there are few fragmented areas near the margin of the distribution (Figure 3). In addition, Rok population sizes near the margins of their distribution are smaller than those near the center (Figure 4), and small Rok populations may lack genetic stability. We, therefore, added the distance from the center of the Rok distribution to each site as an explanatory variable in developing a genetic invasion model.

Roo was accidentally introduced into Japan from mainland China in the 1940s. Since then, this subspecies has expanded its distribution throughout Japan by commingling with other aquatic species in aquaculture and game fishing (Kawanabe et al. 1989; Nakabo 2002; Matsuzawa and Senou 2008). The same phenomenon occurs with alien fishes introduced from domestic regions. When the juveniles of Ayu, *Plecoglossus altivelis* Temminck et Schlegel, 1846, from Lake Biwa located in central Honshu Island were released into rivers throughout Japan, several fish species commingled with them and have been expanding throughout Japan (Matsuzawa and Senou 2008). The Japanese crucian carp, *Carassius cuvieri* Temminck and Schlegel, 1846,



**Figure 4.** The relationship between the distance from each site to the center of Rok distribution (Rok-DIS) and the population size of pure native subspecies (Rok). The logarithmic curve was derived using data from 59 sites with uniform fishing effort.

from Lake Biwa were released throughout Japan for game fishing, several fishes also commingled with it (Kawanabe et al. 1989). More than 10 alien fish species/subspecies that have been intentionally or accidentally introduced from other countries or other domestic regions are currently in northern Kyushu Island (Nakajima et al. 2008). The number of alien fish species may have an effect on the success of Roo introduction. We included the number of other alien species (NAS) as an explanatory variable substitute for the frequency of accidental introduction.

### *Redevelopment of habitat models*

Although our previous study reported predicted Rok and Roo distributions in northern Kyushu Island (Onikura et al. 2012), it was necessary to redevelop the Rok and Roo habitat models to develop a genetic invasion model. We previously analyzed Rok distribution by using native Rok populations as presence data and other populations as absence data (Onikura et al. 2012). However, we assumed that most sites with hybrid populations were originally Rok habitats prior to the invasion by Roo. Thus, in the present study, our presence data included both native Rok populations and hybrid populations. However, some sites with hybrid populations were outside the natural distribution area of Rok. These populations were likely introduced to the sites after hybridization occurred in other regions of Japan and were, therefore, excluded from the Rok presence data. As a result, we used 171/782 sites for presence/absence data (Figure 3). In the analysis of Roo habitat, we used all *Rhodeus ocellatus* populations, excluding native Rok populations, and 56 presence and 897 absence sites (Figure 3).

In our previous study (Onikura et al. 2012), we developed the Rok and Roo habitat models in the western region of northern Kyushu Island and evaluated the performance of each model in the eastern region. In this study, we redeveloped the habitat models by analyzing all the data. In addition, we defined biogeographical information (BGI, 1/0: western/eastern regions) as a dummy variable because we previously demonstrated a small difference in Roo distribution patterns between western and eastern regions (Onikura et al. 2012).

We also changed some of the explanatory variables for the habitat models. In our previous study (Onikura et al. 2012), elevation (EL, m)

and river gradient (RG) were highly correlated, and EL was excluded to prevent multicollinearity between predictor variables from affecting the analyses. Information extraction of RG, which was calculated from the distance and the difference of elevation between 2 points, was so complex and difficult that we used EL in this study. The canal network index (CNI, Fukuda et al. 2011) used in our previous study was excluded for the same reason as RG and this study used the number of river to canal connections (RC) instead of CNI. Other explanatory variables used were landscape variables: length of main river (LMR, km), river width (RW, m), area of paddy fields (AP, km<sup>2</sup>), and residential area (RA, km<sup>2</sup>). LMR and EL are needed to develop the habitat models because the distributions of freshwater fishes inhabiting Kyushu Island are influenced by river length and elevation (Nakajima et al. 2006; Onikura et al. 2008). Land use information, including AP and RA, is also needed for model development because Rok mainly inhabit floodplains (Nakajima et al. 2010). Details of the measurements of landscape parameters are described in previous reports (LMR and RW: Onikura et al. 2012; RC, AP, and RA: Fukuda et al. 2011). All data were derived from Digital Map 25,000 (Japan Map Center, Tokyo), edited by the Geographical Survey Institute, Japan. We overlaid digital elevation data on the Map Image and measured environmental characteristics for each site with GIS software KASHIMIR 3D version 8.0.9 (<http://www.kashmir3d.com>). We prepared a correlation matrix, and confirmed the absence of multicollinearity among all variables (Table 1).

We used a GLM (McCullagh and Nelder 1989) to develop the model for Rok and Roo habitats. We conducted a logistic regression for all possible sets of predictor variables, from a null model including no predictors to a full model including all predictors. We used the Akaike Information Criterion (AIC; Akaike 1974) for model selection in which the model with the lowest AIC was defined as the best-fit model. To evaluate the model, we performed a receiver-operating characteristic (ROC) analysis between actual and predicted fish distribution (Manel et al. 2001; Akoberg 2007). An area under the ROC curve (AUC) reflects how well the model distinguishes between the presence and absence of a species: an AUC greater than 0.9 has high accuracy and 0.7–0.9 has moderate accuracy, whereas 0.5–0.7 indicates low accuracy



**Table 1.** Correlation matrix of explanatory variables used during model selection on the habitat models (Pearson's correlation coefficient).

Variables (Acronyms)	1	2	3	4	5	6
1. Length of main river (LMR)	-					
2. Elevation (EL)	0.110	-				
3. River width (RW)	0.047	-0.078	-			
4. Number of river to canal connections (RC)	0.119	-0.275	0.108	-		
5. Area of paddy field (AP)	0.178	-0.463	-0.127	0.229	-	
6. Residential area (RA)	-0.125	-0.428	0.201	0.172	-0.083	-

**Table 2.** Correlation matrix of explanatory variables used during model selection on the genetic invasion model (Pearson's correlation coefficient).

Variables (Acronyms)	1	2	3	4
1. Probability of occurrence of native Rok (Rok-HR)	-			
2. Probability of occurrence of native Roo (Roo-HR)	-0.022	-		
3. Distance from central distribution of Rok (Rok-DIS)	-0.198	0.277	-	
4. Number of alien species (NAS)	0.438	-0.244	-0.205	-

(Akobeng 2007). We used the base package in R v. 2.11.1 (<http://www.R-project.org>) and SPSS v. 15 (SPSS Japan Inc., Tokyo, Japan) for our analyses.

### *Genetic invasion model*

We used previously derived genetic data for Rok and Roo in 68 sites (Miyake et al. 2008), and these specimens have been preserved in the Kyushu University Museum (KYUM-PI-00002235–00002314). These data were based on mitochondrial DNA analysis of 10 to 20 individuals per site, and we indicated the ratio of Roo haplotypes (Roo-GI) in each site in the previous study (Miyake et al. 2008). The frequency of Roo mt-DNA haplotypes in each population increases with increasing genetic introgression of Roo (Kawamura et al. 2001). Therefore, the frequency in each site can be used to estimate genetic invasion by Roo. We used Roo-GI indicated in previous study as an objective variable (i.e., the measure for genetic disturbance).

For the genetic invasion model, we calculated the probability of occurrence of the two subspecies in each site, by using the best-fit habitat models for Rok and Roo (Rok-HR and Roo-HR). In addition, we computed the average latitude and longitude of the sites with Rok

presences in the western and eastern regions. We defined the mean value as the center of Rok distribution in each region, and calculated the distances from the value to each site. We then divided the distance in each site by the maximum distance for the Rok presence in each region and set the obtained value as the indicator for the marginality of Rok distribution (Rok-DIS). We prepared a correlation matrix for Rok-HR, Roo-HR, Rok-DIS, and NAS, and confirmed the absence of multicollinearity among all variables (Table 2).

We also used a GLM for the model on the genetic invasion by Roo into Rok. We defined BGI (1/0: western/eastern regions) as a dummy variable again, and constructed a GLM as a Gaussian distribution for all possible sets of predictor variables, from a null model including no predictors to a full model including all predictors. We defined the model with the lowest AIC as the best-fit model. For model evaluation, we performed a linear regression analysis between actual and predicted genetic invasion ratios. We used the base package in R v. 2.11.1 and SPSS v. 15 for our analyses. After the model development, we predicted potential hybridization between Rok and Roo by using the best-fit model. We estimated potential risk in each site as very low risk when Roo-GI predicted by using the model was under 1%.

## Results

### *Redevelopment of habitat models for native and non-native subspecies*

The best-fit model for the native subspecies Rok selected four variables. Of these, LMR, RC, and AP were positively correlated, while EL was negatively correlated (Appendix 2). The AUC value of the best model was 0.874. The best-fit model for the non-native subspecies Roo selected five variables. Of these, AP was positively correlated, while LMR, EL, and RW were negatively correlated. The model included BGI as one of explanatory variables. The AUC value of the best model was 0.912.

### *Model for genetic invasion*

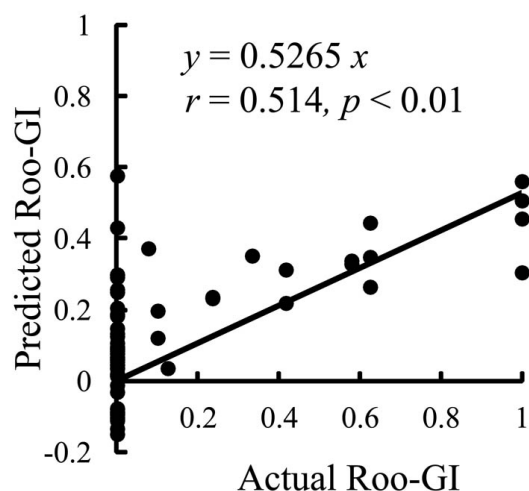
The best-fit model for genetic introgression of non-native Roo into native Rok selected three variables including BGI. Of these, Roo-GI increased with increasing Roo-HR and Rok-DIS (Appendix 3). The linear regression formula between the actual and predicted genetic invasion ratio was as follows:

Predicted =  $0.5265 \times \text{Actual}$  ( $r = 0.514$ ,  $p < 0.01$ , Figure 5).

In the western and eastern regions, the number of actual non-native and hybrid distribution sites was 53 and 4, respectively (Figure 6A), while the number of sites with 1% or more in Roo-GI by predicting hybridization using the best-fit model was 203 and 87, respectively (Figure 6B). Assuming Roo and hybrid populations invade the sites with potential risk (Roo-GI  $\geq 1\%$ ) in the future, our prediction indicated that their distributions will increase from 57 to 290 sites.

In contrast, the number of actual native distribution sites was 95 in the western region and 40 in the eastern region (Figure 6A), while the number of sites with very low risk (Roo-GI  $< 1\%$ ) based on the prediction was 45 and 5, respectively (Figure 6B). Our prediction indicated that the number of sites with only pure Rok populations will decrease from 135 to 50.

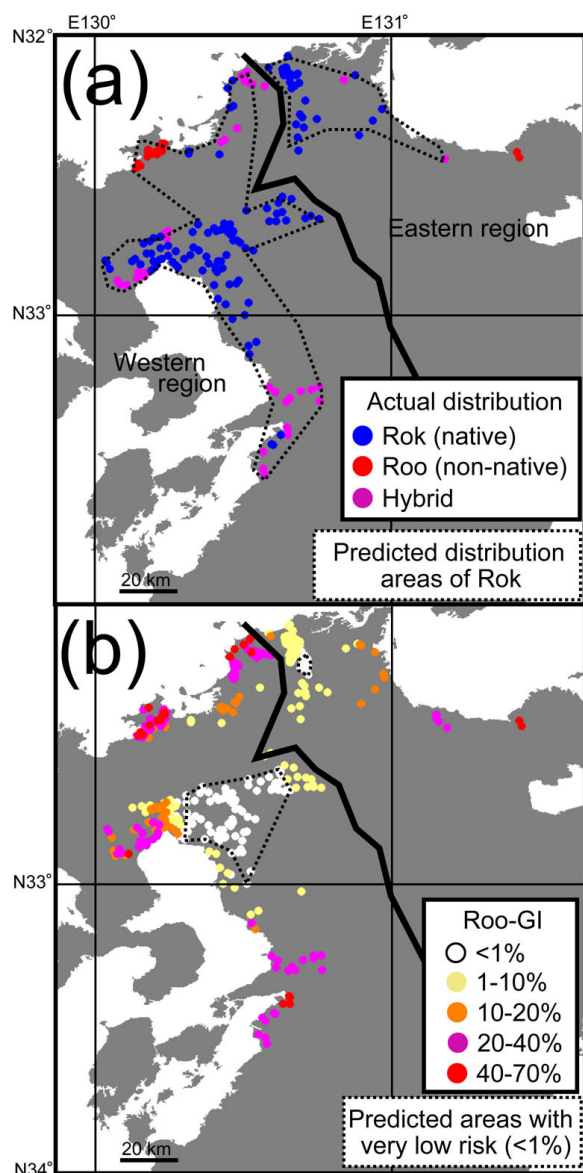
In addition, the enclosure areas of actual native distribution were approximately 1,640 km<sup>2</sup> and 770 km<sup>2</sup> in the western and eastern regions respectively (Figure 6A), while the predicted native distribution areas were approximately 680 and 12 km<sup>2</sup> respectively (Figure 6B). Our prediction indicated a decrease in native Rok distribution areas from 2,410 to 692 km<sup>2</sup>.



**Figure 5.** The relationship between actual and predicted values of the ratio of non-native haplotypes of mitochondrial DNA (Roo-GI) in each population. Data are plotted using the best-fit genetic invasion model.

## Discussion

Several studies have shown the potential distributions, habitats, or niches of alien fishes by developing models based on abiotic environmental information (e.g., Iguchi et al. 2004; Céréghino et al. 2005; Spens et al. 2007; Sato et al. 2010b). Such models show proactive analyses, such as predicting occurrences and distribution, are particularly important in preventing further invasion and range expansion of introduced fishes (Sato et al. 2010a). However, there are differences between such models and the model in this study. The former are used to estimate potential presence of each species, while the latter are used to estimate potential hybridization between native and non-native subspecies. As far as we know, this is the first report on a genetic invasion model that predicts potential hybridization from landscape parameters. Hybridization can cause extinction of a species, subspecies, or population by genetic introgression (Rhymer and Simberloff 1996; Allendorf et al. 2001; Sato et al. 2010a). Therefore, the environmental requirement for genetic introgression from native to non-native must be elucidated to preserve native genetic resources. The results of this study can contribute to the management of native fish conservation and the prevention of hybridization with non-native fish.



**Figure 6.** Maps of the actual distribution of *Rhodeus ocellatus* subspecies (A) and the predicted ratio of Roo haplotypes of mitochondrial DNA (Roo-GI) in each population (B). The enclosed Rok distribution areas (A) were predicted using the best-fit Rok habitat model and Roo-GI (B) was predicted using the best-fit genetic invasion model.

The non-native subspecies Roo is expanding its distributions throughout Japan by commingling with other aquatic species in aquaculture and game fishing (Kawanabe et al. 1989; Matsuzawa and Senou 2008). Several alien fishes are becoming established in northern Kyushu Island through the same process (Nakajima et al. 2008). Therefore,

we hypothesized that the number of alien fish species may have an effect on the success of Roo introduction. Nevertheless, the best model of genetic invasion in this study did not select NAS as an explanatory variable. Such accidental introductions occurred mainly through the releases of Ayu and Japanese crucian carp in Japan, and these 2 species occur throughout Kyushu Island. The accidental introductions of Roo may have occurred throughout this island at the same time, resulting in a model that had no effect on Roo introduction.

It is logical that the best model of genetic invasion in this study selected Roo-HR, which represents the habitat requirement of Roo. One of the other selected variables, Rok-DIS, which represents the distance from the center of Rok distribution, indicates a negative effect on Roo-GI. These results suggest that initial and/or high invasion of non-native Roo occur at the margin of the native Rok distribution. In summary, our results demonstrate that genetic invasion by Roo is influenced by the distribution of native subspecies and the habitat requirements of non-native subspecies.

Population size and genetic variation of native subspecies contribute to the correlation between Roo-GI and Rok-DIS in the genetic invasion model. A lack of heterosis (or hybrid vigor) (Kanoh et al. 2005; Kawamura et al. 2009) and gradual pre-mating reproductive isolation (Kawamura et al. 2009) have been confirmed as ecological and genetic characteristics among native Rok, non-native Roo, and hybrid populations. In Honshu, Roo and hybrid populations invade almost all Rok distributions, and Roo and hybrid populations predominate ecologically (Kawamura et al. 2001) because of their superior fitness in fragmented and isolated habitats (Kanoh et al. 2005; Kawamura et al. 2009). Thus, the extinction of Rok populations results not only from hybridization, but also from inferior fitness (Kawamura et al. 2009). However, in our study area in northern Kyushu Island, Rok populations have high genetic variation compared with Rok populations in Honshu and Shikoku islands (Kawamura 2005). In addition, Rok populations inhabit open-water systems (Kawamura 2005), such as agricultural canal networks and rivers (Miyake et al. 2008; Onikura et al. 2007, 2012), and have a wide range of habitats (Miyake et al. 2008; Onikura et al. 2012). These results suggest that the high genetic variation of Rok populations in this area are likely to be maintained by superior



population sizes and habitat conditions compared to those in Honshu and Shikoku islands (Kawamura 2005). We believe that initial and/or high genetic invasion by Roo occurs near the margin of Rok distribution because population sizes and genetic variation are smaller and lower than near the center. Using Rok population size data from 59 sites, we derived a logarithmic curve showing significant regression between Rok-DIS and Rok population sizes.

While anthropogenic hybridizations are detrimental to many species through direct and indirect means (Rhymer and Simberloff 1996), natural hybridizations play important roles in the evolution of many animal taxa (Allendorf et al. 2001). Allendorf et al. (2001) provide a categorization of hybridization to help guide management decisions: Types 1–3, shown as natural hybridization, are a natural part of the evolutionary legacy of taxa, and Types 4–6, shown as anthropogenic hybridization, have different consequences from a conservation perspective. Hybridizations are without backcrosses in Type 4, with backcrosses and widespread introgression in Type 5, and with backcrosses and complete admixture in Type 6. Type 4 is detrimental because there is wasted reproductive effort rather than genetic mixing. Type 6 results in the extinction of pure populations. Hybridization between Rok and Roo falls under Type 6. Allendorf et al. (2001) mention that few, if any, pure populations remain and there does not appear to be any selection against the hybrids in this case. Therefore, we must make effective use of the prediction of our current model.

In Japan, the Invasive Alien Species Act (Ministry of the Environment, Japan 2004) went into effect in June 2005. Several fish species, such as mosquitofish (*Gambusia affinis* Baird et Girard, 1853), bluegill (*Lepomis macrochirus* Rafinesque, 1819), and largemouth black bass (*Micropterus salmoides* Lacépède, 1802), are designated as invasive alien species in the act, and several actions, such as cultivation and transportation, are regulated for these species. However, Roo is designated as an adventive species without regulation in the act. This study predicts a drastic increase in the number of sites with genetic invasion (from 57 to 290), and large decreases in the number of sites (from 135 to 50) and area (2,410 to 692 km<sup>2</sup>) with pure native populations. These predictions suggest very high risk of Roo invasion. Another problem is genetic homogenization (Olden et al. 2004). Genetic homogenizations occur throughout Japan, and

the hybridization between Rok and Roo contributes to this homogenization (Watanabe 2012). We emphasize the need for legal and management actions to prevent the expansion of Roo and hybrid populations in this area.

In this study, Rok-DIS shows significant correlation with Rok population sizes, implying higher potential risk than our predictions for genetic invasion. Even if a site is near the center of Rok distribution, high genetic invasion may occur there via negative environmental change with decreasing Rok population size. Recently, in northern Kyushu, the habitat conditions of Rok, including the center of Rok distribution, have become depleted because earthen agricultural canals are now being replaced with concrete revetments, and canal networks with complicated intersections are being reconstructed to create simple, uncrossed networks (Onikura et al. 2007). The Rok populations in canals with concrete revetments are smaller than those in canals with earthen revetments (Onikura et al. 2007). Rok populations affected by such negative impacts may be more susceptible to the genetic invasion by Roo. We believe that large Rok populations depend not only on the locations within the Rok distribution area but also on habitat conditions. The genetic invasion model could identify sites with very low potential risk for genetic invasion. Therefore, we recommend selecting actual Rok habitats with very low Roo invasion risk as target sites, and take actions to restore their habitats in order to maintain Rok populations in the selected sites.ecosystems.

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## Supplementary material

The following supplementary material is available for this article:

**Appendix 1.** Presence data of *Rhodeus ocellatus kurumeus* (Rok), *R. o. ocellatus* (Roo) and the hybrid populations between these species in this study.

**Appendix 2.** Results of statistical analyses and selected explanatory variables of top 5 and null habitat models for native *Rhodeus ocellatus kurumeus* (Rok) and non-native *R. ocellatus ocellatus* (Roo).

**Appendix 3.** Results of statistical analyses and selected explanatory variables of top 5 and null genetic invasion models for native *Rhodeus ocellatus kurumeus* (Rok) by non-native *Rhodeus ocellatus ocellatus* (Roo).

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