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RESEARCH ARTICLE

Identification and quantification of farmed red sea bream escapees from a large aquaculture area in Japan using microsatellite DNA markers

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Abstract – A large number of farmed red sea bream (*Pagrus major*) and their gametes have escaped and dispersed into the wild from net-cage aquaculture since the advent of the technology in the mid-20th century. These escapees have likely been recruited into wild populations, affecting their genetic diversity. In this study, we used assignment methods with microsatellite markers to assess the frequency of escapee farmed red sea bream within the wild populations in a major aquaculture production region (UWJ) and in two regions with relatively lower production (IWG and SIJ) in Ehime Prefecture, Japan. The frequency of escapees in UWJ ranged from 14.1% to 30.2%, as shown by three methods of assignment. Escapees were frequently identified among specimens caught by angling (ranging from 26.7% to 46.7%), suggesting that they stay in the same area even after escape or are less cautious than wild individuals. No escapee was found in IWG and SIJ, areas with less extensive aquaculture. Some wild-caught fish appeared to have hybridized with farmed fish, as indicated by the threshold of membership coefficients obtained by simulation. These results clearly indicate that large numbers of farmed individuals have escaped from UWJ, a major aquaculture area, since the inception of red sea bream farming in Japan. Genetic reshuffle has possibly occurred following hybridization of wild and farmed individuals.

Keywords: Aquaculture / *Pagrus major* / escapee / microsatellite / assignment / hybridization

1 Introduction

Net-cage aquaculture as a cost-effective and easy to implement technology is used worldwide, including in Japan (Miyashita, 2008). However, although these fibers are strong, the cages are easily destroyed by the impact of moving ships and other vessels, natural disasters such as typhoons and tsunamis, and fish handling and harvest, as well allow fish escapements (Jackson et al., 2015). The destroyed or damaged cages allow the farmed fish to escape. Another means of escape into the wild is when fertilized gametes from mature farmed fish drift through the mesh. One way or another, a large number of fish and their gametes have escaped from net-cages into the wild, affecting the genetic diversity of the wild populations (Somarakis et al., 2013).

Escaped farmed fish may pose ecological and genetic impact on wild fish populations (McGinnity et al., 1997; Lynch and O'Hely, 2001; Naylor et al., 2005; Hindar et al., 2006). Farmed fish typically have a faster growth rate as they are usually selected for rapid growth (Murata et al., 1996). Domestication is known to decrease individual fitness such as reproductive capabilities (Araki et al., 2007) and survivability (Tymuchuk et al., 2007). In some case, fish such as gilthead seabream (*Sparus aurata*) are known to adapt quickly to the natural environment (Šegvić-Bubić et al., 2018), posing a risk of hybridization with individuals from the wild. Therefore, the escape of farmed fish, especially in the case of well-domesticated species, is likely to adversely affect natural populations. For example, extensive salmon hybridization in

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the Northwest Atlantic was seen due to lower survival of hybrid and feral offspring over time (Wringe et al., 2018). Therefore, understanding the risks associated with escapes from farms is important for sustainable fisheries management and for fisheries coexistence with net-pen aquaculture.

Red sea bream (*Pagrus major*) is an important aquaculture species in Japan (Makino, 2017). Intensive selective breeding program of red sea bream started in 1960s and resulted with fast-growing strain (Murata et al., 1996). This selected population has been distributed to hatcheries, such that all the red sea bream cultured in Japan is now produced from broodstock originating from the same gene pool (Sawayama and Takagi, 2016).

Ehime Prefecture, located on the west side of Shikoku Island, has the largest cultivated production area for red sea bream in Japan. Approximately 34,000 tons of red sea bream (half of the nation's total production) are annually produced in this area (http://www.pref.ehime.jp/h37100/toukei/documents/5-1-6.pdf). The coastline of the southern part of the prefecture forms fjord-like structures, and the water temperature is amenable for growth even during winter (above 15 °C) because of the adjacent warm-water Kuroshio current. Therefore, the southern part of Ehime Prefecture, such as Uwajima and Ainan, is suitable for red sea bream farming, and the high number of farms located there were the first to be established in Japan. In this study, we identified escapees using microsatellite DNA markers, and evaluated and compared the frequency of escapees at locations that are differentially impacted by red sea bream farming in Ehime Prefecture in Japan. The effect of sampling methods on fish catch and origin was also compared.

2 Materials and methods

2.1 Fish sampling

Previous findings indicated presence of three sub-groups among farmed populations (called FRM hereafter) (Sawayama and Takagi, 2016), and found that farmed populations could be divided into three sub-groups (FRM-A, −B, and −C). Based on those results, we took broodstock samples from each of the three sub-groups and treated them as a single farmed population (n = 29–40 each). Three different sampling methods (ring-net fishing, basket capture, and angling) were used in Uwajima (UWJ), in area with intensive red sea bream aquaculture, aiming to compare effect of sampling methods on fish catch and origin. A total of 139 individuals {n = 29 by ring-net fishing (UWJ-R), n = 80 by basket capture (UWJ-B), and n = 30 by angling (UWJ-A)} caught around UWJ were used for the genetic analysis. Ring-net fishing was done off the coast of UWJ with no fish farms in the vicinity. Basket capture (see Supplementary Figure 1) was done in the vicinity of cages, because many fish gather around the cages to forage on the feed passing through them. Angling was also done in the farming area, with several farms located nearby. Another wild red sea bream population (n = 103) was sampled using trawls and gill-nets off the Coast of Iwagi Island (IWG), where a red sea bream seed production company is located. Finally, the third wild sample of red sea bream (n = 47) was collected by ring-net fishing off the Coast of Saijo (SIJ), with no farms or hatcheries located near the sample site. The three sampling

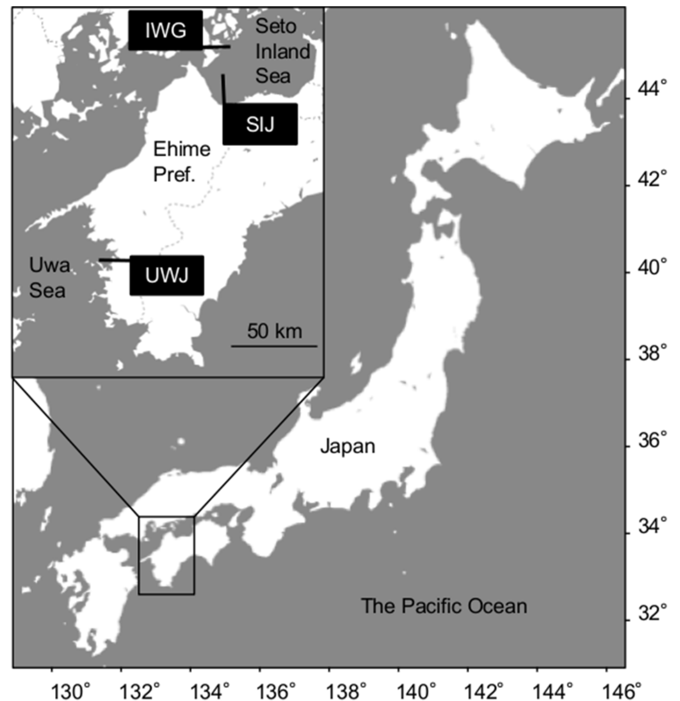

Fig. 1. Sampling locations used in the study. See Table 1 for abbreviations used for the sampling sites.

sites (UWJ, IWG, and SIJ) are shown in Figure 1. In addition, fingerlings produced by artificial breeding at Ehime Prefectural Fishery Research Center (FRC) for the purpose of stock enhancement were also used for genetic analysis (n = 59). Details regarding the samples collected are shown in Table 1.

All wild-caught individuals were visually screened for the phenotype of inter-nostril epidermis (INE). INEs are found in wild red sea bream, but their absence is related to fish reared in aquaculture facilities (Anraku et al., 1999). This provided a physical 'index' to distinguish farmed red sea bream from their phenotype.

2.2 Microsatellite allele detection

Genomic DNA was extracted from fin-clip using a High Pure PCR Template Preparation Kit (Roche Diagnosis, Tokyo, Japan) and stored at −20 °C until further genetic analysis. We used eight di-nucleotide microsatellite markers for genotyping the sampled red sea bream individuals: *Pma-2, -3*, *-4*, and *-5* (Takagi et al., 1997), *Pma22-9* and *Pma4-32* (Hatanaka et al., 2006), and *Kpm2* and *Kpm22* (Gonzalez et al., 2012). Following Sawayama and Takagi (2016) for the PCR protocol, we used 1 μl of extracted DNA, 0.1 μM primer, 0.2 μM deoxynucleotide triphosphates (dNTPs), 0.05 μl 99% formamid, 2.5 units Ex taq polymerase (TAKARA BIO, Shiga, Japan) with 10× buffer in a total volume of 5 μl. We labeled the forward primers with fluorescent dye and tailed the reverse primers to ensure consistency in amplicon size (Applied Biosystems, California, USA). The PCR products were multi-loaded and separated by electrophoresis using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) for the fluorescent-labeled PCR products. Alleles were scored using PeakScanner® Software v1.0 (Applied Biosystems).

Table 1. Location, sampling method used, and other details of the populations used in the study.

Name	Site/Origin	Sampling method	N (w/o INE)[*1]	Collection date	Fork length (cm)
UWJ-R	Uwajima, Ehime Pref.	Ring-net fishing	29 (0)	April 2014–Sep. 2014	19.6 ± 4.3
UWJ-B	Uwajima, Ehime Pref.	Basket capture	80 (6)	Nov. 2014–May 2015	20.2 ± 4.0
UWJ-A	Uwajima, Ehime Pref.	Angling	30 (9)	Oct. 2014–Sep. 2015	31.0 ± 7.4
IWG	Off the coast of Iwagi island, Ehime Pref.	–	103 (0)	Apr. 2014–Aug. 2014	14.6 ± 1.7
SIJ	Off the coast of Saijo, Ehime Pref.	–	47 (0)	Nov. 2014–Nov. 2015	10.9 ± 0.7
FRC	Fingerlings for stock enhancement	–	59	Apr. 2015	–
FRM-A[*2]	Farmed broodstock	–	40	–	–
FRM-B[*2]	Farmed broodstock	–	30	–	–
FRM-C[*2]	Farmed broodstock	–	29	–	–

[*1], number of individuals without inter-nostril epidermis. [*2], see Sawayama and Takagi (2016).

MICROCHECKER software (van Oosterhout et al., 2004) was used to identify possible genotyping errors such as stuttering, large allele dropout, and presence of null alleles.

2.3 Genetic analysis

Basic population genetic parameters (number of alleles, effective number of alleles, allele frequencies, and observed and expected heterozygosities (*Ho* and *He*, respectively)) of analyzed loci were calculated using GenAlEx V.6.3 (Peakall and Smouse, 2006). The genotype distributions of each population for each locus were tested for departure from the Hardy-Weinberg equilibrium (HWE) using GenAlEx V.6.3 (Peakall and Smouse, 2006). The probability of significant deviation from HWE was estimated using the chi-square test ($P < 0.05$). The *P*-value was adjusted by means of the Bonferroni correction (Rice, 1989).

We used the Bayesian clustering method implemented in STRUCTURE, with the admixture method, a prior location (LOCPRIOR) settings, and correlated allele frequency version of the program. To clarify fine genetic structure, the FRM-A, -B, and -C were treated as the different population. We did 10 runs for each value of K with 10^5 iterations following a burn-in period of 100,000, assuming that the data set could be represented by K separate genetic clusters ($K = 1$ to 10). The ΔK-value, based on the rate of change in the log probability of the data between successive Ks, was also estimated (Evanno et al., 2005). ΔK and mean Ln probability of data for each K ($L(K)$) were calculated using STRUCTURE HARVESTER (Earl and von Holdt, 2012). The clustering results from independent runs were averaged using CLUMPP (Jacobsson and Rosenberg, 2007) and visualized using DISTRUCT (Rosenberg, 2004). Discriminant Analysis of Principal Components (DAPC) was also used to examine potential differences between sampling populations using *adegenet* program (Jombart, 2008) in R. The selection of the optimal number of clusters in the lowest Bayesian Information Criterion (BIC) was calculated between cluster 1 to 20. The scatterplot of the DAPC was drawn with population information as a prior.

Analysis of molecular variance (AMOVA) was done on population groupings identified by the STRUCTURE analysis using GenoDive software (Merimans and van Tienderen, 2004). The number of permutations was set at 999. Pairwise *F*st (Weir and Cockerham, 1984) values were calculated using FSTAT version 2.9.3. Statistical significance levels were determined applying a Bonferroni correction (Rice, 1989). Simulations to evaluate statistical power of microsatellite to differentiate populations between wild and farmed were conducted with POWSIM (Ryman and Palm, 2006).

Assignment methods were done using two methods, the frequency-based method of Paetkau et al. (1995) and the Bayesian method of Rannala and Mountain (1997), as implemented in GeneClass2 software (Piry et al., 2004). Assignment threshold was set at 0.05, and missing allele frequency was set at 0.01 for the frequency-based method. The probability was computed using the method of Paetkau et al. (2004) with 1000 MCMC replications.

The optimal threshold values for an individual membership in the STRUCTURE analyses were determined using the approach of Vähä and Primmer (2006). Fifty wild and farmed individuals were selected with q-values > 0.95 obtained from STRUCTURE analysis and used as the parent population of hybrids. Simulated data sets (wild, farmed, F_1 (wild $\times$ farmed), F_2 ($F_1 \times F_1$), back-cross (BC)_wild ($F_1 \times$ wild), and BC_farmed ($F_1 \times$ farmed)) were generated using the R package hybriddetective (Wringe et al., 2017). The simulated data sets were run in STRUCTURE 10 times with $k = 2$ and the above settings. The threshold between hybrids was determined based on the average membership coefficients of hybrids.

3 Results

3.1 Genetic diversity

All microsatellite loci were successfully amplified with no missing genotype within the datasets. Locus *Pma-2* showed several alleles with a 1 bp size difference, and therefore, this locus was omitted from genetic analysis due to possible genotyping errors suggested by MICROCHECKER. The population genetic parameters (allelic number (*Na*), allelic richness (*Ar*), observed (*Ho*) and expected (*He*) heterozygosities, and their ratio (*Ho*/*He*)) are shown in Table 2. The number of alleles ranged from 7.6 (FRC) to 33.7 (UWJ-overall and IWG). Allelic richness showed the same trend as the number of alleles for the loci used. *Ho* ranged from 0.719 (FRC) to 0.906 (SIJ). *He* ranged from 0.714 (FRC) to 0.938 (IWG), and *Ho*/*He* ranged from 0.943 (IWG) to 1.007 (FRC). Among all the wild-caught groups, UWJ-A showed the lowest values of *Na*, *Ar*, *Ho*, and *He*.

Table 2. Population genetic parameters of populations used in this study.

	n	*Na*	*Ar*	*Ho*	*He*	*Ho/He*
UWJ-overall	139	33.7	25.3	0.882	0.926	0.952
UWJ-R	29	29.0	22.3	0.892	0.916	0.974
UWJ-B	80	30.0	21.2	0.882	0.924	0.955
UWJ-A	30	18.3	18.0	0.871	0.887	0.982
IWG	103	33.7	22.6	0.885	0.938	0.943
SIJ	47	26.6	22.2	0.906	0.928	0.976
FRC	59	7.6	7.0	0.719	0.714	1.007
FRM-overall	99	12.1	10.1	0.824	0.835	0.987
FRM-A	40	9.7	7.4	0.789	0.789	1.000
FRM-B	30	7.3	7.1	0.905	0.770	1.175
FRM-C	29	8.3	7.3	0.788	0.779	1.012

n, number of individuals analyzed; *Na*, allelic number; *Ar*; allelic richness; *Ho*, observed heterozygosity; *He*, expected heterozygosity. See Table 1 for abbreviations used for the sampling locations.

Table 3. Pairwise genetic differentiation (*F*st) in three wild caught populations (specimens of UWJ caught by different methods were included in “UWJ-overall”), artificial seedlings for stock enhancement and farmed individuals and its *P*-values.

	UWJ-overall	IWG	SIJ	FRC	FRM-overall
UWJ-overall		**0.0001**	0.0146	**0.0001**	**0.0001**
IWG	0.0063		0.1493	**0.0001**	**0.0001**
SIJ	0.0025	0.0011		**0.0001**	**0.0001**
FRC	0.0837	0.1088	0.1112		**0.0001**
FRM-overall	0.0278	0.0561	0.0458	0.0821	

Lower triangle shows pairwise *F*st values, while Upper triangle shows the corresponding *P*-values; significant differences are shown in bold ($P < 0.005$). See Table 1 for abbreviations used for the sampling locations.

3.2 Genetic structure

AMOVA revealed that the global *F*st was 0.044 for the entire group ($P < 0.001$), suggesting that there was a moderate level of genetic differentiation among the populations. Pairwise *F*st was calculated among all the sampled populations (Tab. 3). Significant differences were observed between wild-caught populations (UWJ, IWG, and SIJ) and populations derived from artificial breeding (FRC and FRM). Among wild-caught populations, a significant difference was observed between UWJ and IWG ($P = 0.0001$). An analysis with the computer program POWSIM suggested that our microsatellite DNA dataset had a statistical power of 95% to detect an *F*st as low as 0.005 between wild and farmed populations. The probability of α error calculated by chi-square and Fisher methods was 0.025 and 0.057, respectively.

The genetic structure estimated by STRUCTURE is shown in Figure 2. A Bayesian clustering analysis and application of the ΔK procedure of Evanno et al. (2005) indicated that the most likely value of K was 2, with the mean $L(K)$ similar to ΔK (Supplementary Figure 2a and 2b). However, the mean $L(K)$ showed the secondary peak when $K = 5$, and presence of the five sub-genetic structures was suggested, and FRC and FRM were clearly separated (Supplementary Figure 3). The BIC plot indicated the presence of six genetic populations (Supplementary Figure 4a), and the *adegenet* program also clearly separated FRC and FRM (Supplementary Figure 4b). These results supported the highest pairwise *F*st value between FRC and FRM. The FRM-A, -B, and -C were not largely different even when $K = 5$, and therefore, these three farmed populations were treated as one population for further escapee identification.

3.3 Individual assignment

Simulated datasets generated by the hybriddetective R package yielded 0.90 as the best threshold to use for assigning individuals to wild or farmed categories from the results of STRUCTURE (Supplementary Figure 5 and Supplementary Table 1). Therefore, individuals sharing >90% farmed membership were assigned as escapees. Thresholds of hybrids (F_1 or F_2, BC_wild, and BC_farmed) were also calculated, and the following thresholds of q-values of wild proportion were defined: 0.27 to 0.77 in F_1 or F_2, >0.77 in BC_wild, <0.27 in BC_farmed (Supplementary Table 1). Based on these threshold, possible hybrids were identified (Supplementary Figure 6 and Supplementary Table 2).

No individuals in IWG and SIJ were assigned as escapees by any of the three different methods, but escapees were estimated in UWJ-overall, using three different methods. Among the three methods, the assignment methods of Paetkau et al. (1995) and STRUCTURE detected the higher numbers of

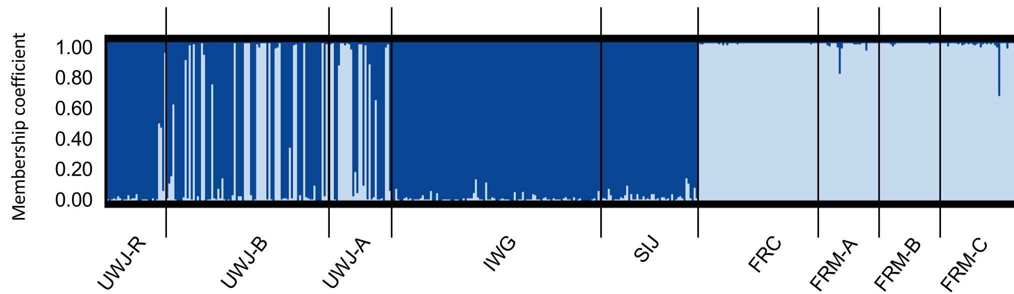

Fig. 2. Results from analysis using STRUCTURE of wild-caught red sea bream (UWJ, IWG, and SIJ), seedlings for stock enhancement (FRC), and broodstock of farmed fish (FRM) used in this study.

Table 4. Percentage of wild and escapees resulted by three methods of assignment.

	Paetkau et al. (1995)		Rannala and Mountain (1997)		STRUCTURE	
	Wild	Escapee	Wild	Escapee	Wild	Escapee
UWJ-overall	71.2	28.8	84.9	14.1	74.1	25.9
UWJ-R	93.1	6.9	100	0.0	96.6	3.4
UWJ-B	70.0	30.0	83.8	16.2	72.5	27.5
UWJ-A	53.3	46.7	73.3	26.7	56.7	43.3
IWG	100	0.0	100	0.0	100	0.0
SIJ	100	0.0	100	0.0	100	0.0

See Table 1 for abbreviation of each sampling location.

escapees ($n=40$ (28.8%) and 36 (25.9%), respectively), while the method of Rannala and Mountain (1997) showed the most conservative result ($n=21$ (14.1%)) among all methods for assignment calculation (Tab. 4). Among the three sampling methods, high frequencies of escapees were detected in UWJ-A (26.7% to 46.7%) and UWJ-B (16.3% to 30.0%), but UWJ-R showed the lowest frequency of possible escapees (0.0% to 6.9%).

We also combined the phenotype data (presence/absence of INE) with the escapee data (Tab. 5). Fifteen individuals caught in UWJ showed unilateral or bilateral INEs (10.8%) and among them, six and nine individuals belonged to UWJ-B and UWJ-A, respectively. Twenty and forty percent of escapees assigned by at least one assignment method lacked INE in UWJ-B and UWJ-A, respectively. Two individuals were genetically assigned as escapees in UWJ-R, but these had the INE. Four individuals (UWJ-B #26, UWJ-A #10 #13 and #16) lacked INE, but these were not identified as escapees by assignment methods.

4 Discussion

To our best knowledge, this study provides the first evidence of genetic introgression into wild populations by farm-raised red sea bream in Ehime Prefecture, where the largest cultivated production area for red sea bream in Japan. Seven microsatellite loci were used to quantify genetic differences between populations of wild and farmed red sea bream and to detect presence of escapees in the specific regions of the above-mentioned area.

Escapees may stay around the farming area even after escape due to forage on excess feed leaving the cages (Gausen and Moen, 1991; Fiske et al., 2006; Šegvić-Bubić et al., 2018). Moreover, farmed red sea bream are acclimated to human presence, unlike wild individuals (Sawayama, personal observation), and therefore, may lack wariness and can be easily captured by angling and basket capture as observed in present study. Domestication is known to change behavior traits such as aggressiveness and schooling (Ruzzante, 1994), and hybridization with wild populations is likely to introduce these traits of domestication into wild populations to their detriment.

Farmed red sea bream have been thoroughly domesticated and are successfully distinguished using the seven microsatellite DNA markers used in this study (Sawayama and Takagi, 2016). Therefore, samples of farmed red sea bream broodstock populations were used as reference for identifying escapees. STRUCTURE or the Paetkau et al. method (1995) are known to be reliable statistical tools to identify escapees and estimate the rate of escape (Glover, 2010). Both yielded similar results in our study. While some wild-captured individuals showed high membership coefficient values for farmed populations, simulation analysis done by hybriddetective software with STRUCTURE indicated that they likely were hybrids. This reflects the long period and decades of fish escapement from aquaculture farms.

In red sea bream, the farmed population has been subjected to high selective pressure for growth since the 1960s (Murata et al., 1996), and several genes related to economic traits have reached fixation (Sawayama and Takagi, 2017; Sawayama et al., 2018a; Sawayama et al., 2018b) as a result of over

Table 5. Individuals identified as escapees by three methods of assignment and inter-nostril epidermis.

Sampling method	No.	Paetkau et al. (1995)	Rannala and Mountain (1997)	STRUCTURE	w/o INE
Ring-net	1	✓			
	2	✓		✓	
Basket	1			✓	
	2	✓			
	3	✓			
	4	✓		✓	
	5	✓	✓	✓	
	6	✓	✓	✓	✓
	7	✓		✓	
	8	✓	✓		
	9	✓	✓	✓	
	10	✓		✓	
	11	✓	✓	✓	
	12	✓	✓	✓	
	13	✓		✓	✓
	14	✓		✓	
	15	✓	✓	✓	
	16	✓		✓	
	17	✓	✓	✓	
	18	✓		✓	✓
	19	✓		✓	
	20	✓	✓	✓	
	21	✓		✓	
	22	✓	✓	✓	
	23	✓	✓	✓	✓
	24	✓	✓	✓	
	25	✓	✓	✓	✓
	26				✓
Angling	1	✓		✓	
	2	✓	✓	✓	
	3	✓			✓
	4	✓	✓	✓	✓
	5	✓	✓	✓	
	6	✓	✓	✓	✓
	7			✓	✓
	8	✓	✓	✓	
	9	✓		✓	✓
	10				✓
	11	✓	✓	✓	
	12	✓	✓	✓	
	13				✓
	14	✓		✓	
	15	✓			
	16				✓
	17	✓		✓	✓
	18	✓	✓	✓	

10 generations of selective breeding (Sawayama and Takagi, 2016). Accuracy of escapee identification depends on the history of domestication. Species with a short history of domestication will have weak genetic differences between farmed and wild populations, and, conversely, strong genetic differences will be found in farmed species with a long history of domestication. Glover et al. (2011) identified escapees in Atlantic cod (*Gadus morhua* L.) using neutral microsatellite markers, but escapees and hybrids identification can be challenging in situation of weak population differentiation between farmed and wild fish (Šegvić-Bubić et al., 2014, 2017). For these reasons, we used phenotypic data (inter-nostril epidermis, INE) for escapee identification.

Three individuals with the abnormal INE phenotype were assigned as wild individuals. Fingerling production programs

for stock enhancement generally use broodstock caught from wild populations (Smith, 1994; Perez-Enriquez et al., 1998), and in such cases, it will be difficult to identify the fingerlings using genetic markers geared toward farmed fish. Therefore, we believe these three individuals to be artificially raised fingerlings from wild broodstock for stock enhancement, or simply that they had the abnormal INE spontaneously in the wild. Therefore, INE is not a reliable marker for escape identification, as shown in Table 5.

The FRC population showed the lowest number of alleles and heterozygosity among the analyzed populations. Fingerlings for stock enhancement should be sourced from wild individuals with large populations, as mentioned by Perez-Enriquez et al. (1998). The populations FRC and FRM, both with the high *F*st values among all the populations, were assigned to the different cluster by STRUCTURE when $K=5$ and *adegenet* (Supplementary Figures 3 and 4). FRC population may be bottlenecked because of the limited number of broodstock individuals used for production or encountered in spawning. It is important that approval from the appropriate regulatory agencies be obtained for stock enhancement programs, with proper oversight, in all prefectures conducting stock enhancement programs.

Farmed red sea bream start spawning at the age of two (Kato et al., 1999), which is also when they typically reach market size (around 1.5 kg in body weight) in culture. Some are grown for over three years for larger size (>2 kg) for export to the foreign market, especially South Korea. During these rearing periods, mature farmed red sea bream spawn in the net-cages, and fertilized eggs enter the open sea and become a part of the local wild population. Therefore, fertilized eggs and larvae collected from Uwa Sea area also should be subjected to genotyping and analysis in future studies to reveal possibility of secondary escapees. However, secondary escapees (fish originating from gametes of farmed fish) cannot be easily distinguished with escapees (fish originating from farm), so that additional non-genetic methods, such as otolith shape, scale characteristics, and body shape (Arechavala-Lopez et al., 2012; Talijančić et al., 2019), microchemistry (Adey et al., 2009), and stable isotope analysis (Kaifu et al., 2018) are needed for their identification.

There are several sites well-known as successful farming areas, mainly in western Japan (Yatsushiro Sea and Isahaya Sea in Kumamoto Prefecture, Uranouchi Bay in Kochi Prefecture, and Kagoshima Bay in Kagoshima Prefecture). These areas do not directly front to the open sea, and wave action is minimal, making them suitable for net-cage farming. Escapees may also accumulate in these closed environments. If spawning sites for wild stock are located in these bays, wild fish and escapees could easily hybridize. Moreover, the resulting hybrids will become available for spawning with subsequent generations of wild, escapee, and other hybrids. Since hybridization between wild and farmed fish likely reduces fitness (McGinnity et al., 2003), not only the escapees but hybrids too should be monitored. While DNA markers and specialized statistical software are efficient tools to monitor hybridization between wild and farmed individuals (as shown in this study), genetic/genomic tools should be developed even further for fine-scale identification of hybrids from broader areas.

Supplementary material

Supplementary figures and tables.
The Supplementary Material is available at https://www.alr-journal.org/10.1051/alr/2019024/olm.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical statement

All experiments described in this manuscript were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from Ehime University.

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