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ORIGINAL ARTICLE

Inference of Potential Genetic Risks Associated with Large-Scale Releases of Red Sea Bream in Kanagawa Prefecture, Japan Based on Nuclear and Mitochondrial DNA Analysis

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Abstract Since 1978, millions of hatchery-reared red sea bream (*Pagrus major*) juveniles have been released in Sagami Bay and Tokyo Bay in Kanagawa Prefecture, Japan. The stock enhancement program has contributed to total catch; however, no information regarding the genetic interactions with wild counterparts is available. Here, we combined 15 microsatellite loci and mitochondrial D-loop sequencing to characterize the genetic resources of red sea bream in Sagami Bay and Tokyo Bay and to elucidate the potential harmful genetic effects associated with fish releases. Both types of markers evidenced higher levels of genetic diversity in wild samples (SB and TB) compared with offspring before stocking (H07 and H08) as well as a hatchery-released sample recaptured in Sagami Bay (HR). Microsatellite F_{ST} estimates and Bayesian clustering analysis found significant genetic differences among samples (F_{ST}=0.013–0.054), except for the two wild samples (F_{ST}=0.002) and HR vs. H07 (F_{ST}=0.007). On the other hand, mitochondrial-based Φ_{ST} suggested haplotypic similarity between SB, H07, and HR. The low effective number of females contributing to the offspring over multiple generations may be responsible for the lack of haplotypic differentiation. Moreover, the putative hatchery origin to three fish (8 %) without deformity in the inter-nostril epidermis was inferred for the first time. Our results showed the usefulness of combining nuclear and mitochondrial markers to elucidate genetic interactions between hatchery-released and wild red sea bream and warned about potential harmful genetic effects should interbreeding takes place.

Keywords Genetic risks · Wild hatchery fish interaction · *Pagrus major* · Release · Microsatellite · mtDNA

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Introduction

Genetic and ecological interactions between wild and hatchery fish have been a major focus of discussion over decades (Allendorf and Phelps 1980; Allendorf and Ryman 1987; Laikre et al. 2010). Hatchery specimens are originated from a limited broodstock; then, it is likely that they only carry a small part of the genetic diversity present in the wild (Taniguchi et al. 1983; Ortega-Villaizán Romo et al. 2005). Domestication selection and genetic drift in the hatchery can lead to detrimental differentiation (Perez-Enriquez et al. 1999; Jeong et al. 2007), despite recent improvements in broodstock management practices (Nugroho and Taniguchi 2004; Taniguchi 2004; Blanco Gonzalez et al. 2010). Escapes of fish artificially selected for economic traits comprise additional threats to preserve the

identity of wild stocks (Hansen 2002; Glover et al. 2010, 2011). Nevertheless, many farms and stock enhancement programs worldwide are run without prior baseline genetic characterization of the natural resources, while genetic monitoring is still rarely accomplished (Kitada et al. 2009; Laikre et al. 2010). Hence, efforts directed towards gaining accurate information of wild and hatchery strains as well as their genetic and ecological interactions are required in order to develop sustainable fishery strategies and conserve the genetic resources.

Technological advances in molecular genetics continuously provide new tools to deepen our understanding of fisheries and conservation genetics. In this regard, mitochondrial DNA sequencing and microsatellite loci have been the markers of choice to address wild and hatchery fish interactions (Blanco Gonzalez and Umino 2009; Hamasaki et al. 2010; Glover et al. 2011). In fact, they possess different mechanisms of evolution, complementing each other to provide insight into the processes involved in genetic variation and differentiation (Hansen et al. 2000; Sekino et al. 2002). The smaller effective population size of mitochondrial DNA is expected to be more sensitive to demographic events not detected by microsatellite loci; however, they only represent the maternal inheritance at a single locus. On the other hand, genetic profiles from a considerable number of polymorphic microsatellite loci can add relevant information to investigate biparental contribution and examine levels of differentiation and admixture proportions between wild and hatchery fish based on Bayesian inferences (Falush et al. 2003) and assignment tests (Paetkau et al. 2004). Bayesian approaches appear as an alternative to classical frequentist methods for testing Hardy–Weinberg model-based statistics, estimating probability distributions on the parameters instead of values from available data (Shoemaker et al. 1998; Wakefield 2010). In this manner, it is possible to infer admixture coefficients between wild and hatchery fish when parental information is absent (Hansen et al. 2001).

Red sea bream, *Pagrus major*, is an important species for both commercial and sport fisheries in Japan. In 1963, it was one of the coastal fishery species initially selected for stocking in the Seto Inland Sea (Kitada 1999). Since then, millions of juveniles have been released in the majority of coastal prefectures over the country. Evaluation of the effectiveness of stock enhancement programs for this species has been standardized by fish market surveys (Kitada 1999). The proportion of hatchery-released fish in landings is estimated by their identification based on the presence of a deformity in the internostril epidermis (DIE), a unique morphological character observed in about 70–80 % of farmed fish (Sobajima et al. 1986). By this method, contribution of hatchery-released fish to total production in Kagoshima Bay and Kanagawa Prefecture, two areas where the occurrence of hatchery fish in landings have been extensively monitored, was reported at about 40 % (Kitada and Kishino 2006). Their occurrence in the inner part of Kagoshima Bay was extremely high between 1989 and 1991, representing 64–83 % of all individuals (Kitada 1999). In fact, the large number of juvenile releases was suggested to have exceeded the carrying capacity in Kagoshima Bay (Kitada and Kishino 2006). On the other hand, hatchery-released fish represented 46–74 % of the catches in Kanagawa Prefecture during the period 1990–1994 (Imai 2005); however, no density-dependent problems were observed (Kitada and Kishino 2006). It should be noted that these numbers did not include recreational fisheries, a major problem given that they may exceed commercial catches both in numbers and economically as reported in Kanagawa Prefecture, a common fishing ground for many anglers from the neighboring Tokyo metropolis (Imai 2005). Therefore, the actual contribution of stocked juveniles was likely underestimated, and their ecological and genetic interactions demand a deeper analysis.

Previous genetic studies suggested that red sea bream in Japan comprises a single large panmictic stock (Taniguchi and Sugama 1990; Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001), clearly differentiated from populations of the South-West Pacific (Perez-Enriquez and Taniguchi 1999; Tabata and Taniguchi 2000). At a smaller geographical scale, intensive releases were shown to be responsible for small genetic disturbances observed at certain locations (Tabata and Mizuta 1997; Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001). On the contrary, the stock enhancement program conducted in Kagoshima Bay since 1974 represents one of the best-documented examples reporting harmful genetic effects in relation to excessive number of juvenile releases in marine species (Kitada et al. 2009). Analysis based on three polymorphic microsatellite loci (Shishidou et al. 2008; Kitada et al. 2009) and D-loop mitochondrial DNA sequences (Hamasaki et al. 2010) evidenced significant loss of rare alleles and unique haplotypes in specimens with DIE as well as in the stock inhabiting the inner part of Kagoshima Bay. These studies attributed the erosion of the native gene pool in Kagoshima Bay to the use of a relatively small broodstock over multiple generations and to the high contribution rates of their offspring, limiting the negative impact to within the Bay due to its geomorphology "like a deep pond with a narrow mouth" (Hamasaki et al. 2010).

Considering that the hatchery-released contribution in Kanagawa Prefecture was comparable to that in Kagoshima Bay (Kitada 1999; Imai 2005; Kitada and Kishino 2006) and given the important genetic erosion reported in the latter (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010), it was surprising that no information regarding the genetic interactions between wild and hatchery-released fish was available from Kanagawa Prefecture. Hence, the aim of the present study was to characterize the genetic resources of red sea bream in Tokyo Bay and Sagami Bay, the two bays surrounding Kanagawa Prefecture, and to elucidate any potential harmful genetic effects of the stock enhancement program carried out since 1978. Additionally, we tried to

achieve insight into the presence of hatchery fish without DIE in order to obtain a better approximation of the juvenile contribution. In order to achieve these objectives, we screened 15 microsatellite loci using several multiplex polymerase chain reaction (PCR) panels recently developed for this species (Blanco Gonzalez et al. 2012). These multiplexed assays were expected to be more accurate and provide a better estimation of small genetic differences among stocks (Ruzzante 1998; Koskinen et al. 2004) than the small set of three microsatellite markers used in previous studies. Furthermore, the results were strengthened by combining the analysis with D-loop mitochondrial DNA sequencing.

Materials and Methods

Fish Samples and DNA Extraction

Red sea bream specimens employed in this study comprised a total of 215 fish from five different source populations collected in 2007 and 2008 (see the map for location details, Fig. 1 and Table 1). Two wild samples were collected at Sagami Bay (SB) and Tokyo Bay (TB), two embayments where red sea bream juveniles have been released annually by the Kanagawa Prefectural Fisheries Research Center (KPFRC). H07 and H08 represented hatchery-reared red sea bream juveniles before their release in 2007 and 2008, respectively. The former sample, H07, was originated from eggs bought from Shizuoka Prefecture and grown at KPFRC, whereas eggs of the latter, H08, were produced and bought from Chiba Prefecture. Offspring from H07 were produced by 150 breeders from a commercial hatchery strain while the broodstock of H08 comprised 100 first-generation fish originated from wild fish collected in Chiba Prefecture. Additionally, HR comprised a group of previously released red sea bream that were recaptured at Sagami Bay in 2007 (HR). Their identification as hatchery-reared fish was based on the presence of the characteristic DIE reported in hatchery specimens (Sobajima et al. 1986). Fin tissue from each specimen was preserved in 95 % ethanol. Total genomic DNA was extracted using either the DNeasy kit (Qiagen) or the DNAzol (Invitrogen) protocol. DNA extracted using the latter method was re-suspended in TE buffer. All DNA samples were stored at 4 °C for further analysis.

Microsatellite Genotyping and Analysis

Microsatellite polymorphism was analyzed at 15 polymorphic markers: *Kpm*1, *Kpm*11, *Kpm*7, *Kpm*22, *Kpm*2, *Kpm*23, *Kpm*25, *Pma22-9NCL*, *Kpm*28, *Pma11-45NCL*, *Pma4-32NCL*, *Pma103-59NCL*, *Pma18-41NCL*, *Pma*1, and *Pma*5. Microsatellite loci were amplified using the same fluorescent dye labeling and multiplex PCR protocols previously described by Blanco Gonzalez et al. (2012). Amplified PCR products were run with GeneScan^{Tm}–600 Liz as the size standard on an ABI 3500XL Genetic Analyzer (Applied Biosystems), and individual genotyping was analyzed with GeneMapper v. 4.1 (Applied Biosystems).

Genetic diversity for each sample was estimated with standard descriptive statistics such as number of alleles per locus (A), and expected (H_E) and observed (H_O) heterozygosities using Arlequin ver. 3.5 (Excoffier and Lischer 2010). FSTAT

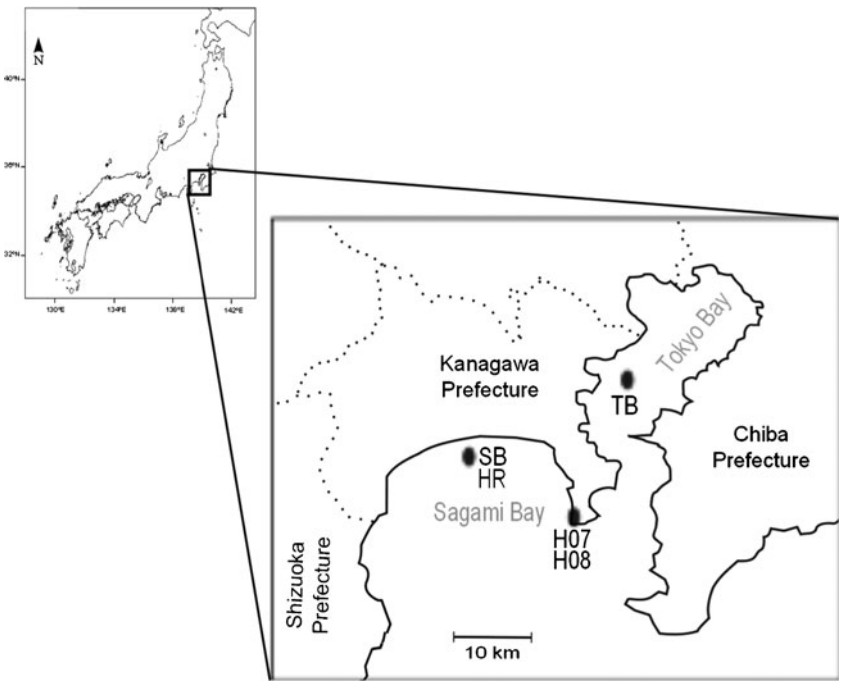

Fig. 1 Map of Kanagawa Prefecture with sampling locations. *SB* Sagami Bay; *TB* Tokyo Bay; *H07* Hatchery 2007; *H08* Hatchery 2008; *HR* Hatchery-released

Table 1 Characteristics of red sea bream samples used in this study

Sample	Sampling location	Fork length range (mm)	Age (years)	Sampling period	Fishing method
SB	Sagami Bay	367–681	3–13	April–September 2007	Long-line, gill net, stationary net
TB	Tokyo Bay	365–640	3–11	May–September 2007	Trawl net, gill net
H07		56–101	0	2007	
H08		42–68	0	2008	
HR	Sagami Bay	152–500	0–6	April–December 2008	Trawl net, gill net, long-line, stationary net

ver. 2.9.3.2 (Goudet 1995) was used to determine allelic richness (A_r), a measure of the mean number of alleles per locus that considers differences in sample sizes with an adjustment to the smallest sample size (HR, n=26). This software was used also to conduct permutation tests to examine deviations from Hardy–Weinberg equilibrium based on significantly higher or lower F_{IS} estimates than expected by chance. Linkage disequilibrium for all pairs of loci in all samples was evaluated in Genepop v.4.0 (Raymond and Rousset 1995) using a Fisher's exact test (10,000 dememorizations; 100 batches; 1,000 iterations per batch). Presence of null alleles was investigated using Micro-Checker v. 2.2.1 (van Oosterhout et al. 2004) and Cervus 3.0 (Kalinowski et al. 2007). The existence of outlier loci was checked with LOSITAN (Antao et al. 2008).

Pairwise genetic differentiation among samples was calculated based on the variance in allelic identity (F_{ST}) and allelic size (R_{ST}) as implemented in Arlequin ver. 3.5 (Excoffier and Lischer 2010), and the significance levels (P=0.05) were determined following 10,000 random permutations. These tests were supported by a hierarchical analysis of molecular variance (AMOVA) to quantify genetic differentiation in relation to sample origin. Bonferroni adjustments to P values were applied whenever multiple tests were performed (Rice 1989).

The Bayesian clustering method implemented in STRUCTURE 2.3.3. (Pritchard et al. 2000) was performed to determine admixture proportions between wild and hatchery-released fish and to infer the most likely number of populations (K) in the data set without a priori information of population partition. In addition to the five samples from Kanagawa, genotypes of 120 wild fish collected from Iwate, Aichi, and Oita (Blanco Gonzalez et al. 2012) were pooled and included in the analysis. These fish presented a similar genetic composition, and no effect of prior stocking was presumed (Blanco Gonzalez et al. 2012). Red sea bream in Japan has been reported to comprise a large panmictic population (Tabata and Mizuta 1997; Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001); therefore, the above-mentioned specimens could be regarded as representing the original wild stock inhabiting Sagami Bay and Tokyo Bay. We assumed correlated allele frequencies and an admixture model (Falush et al. 2003). Each run consisted of a burn-in of 50,000 MCMC steps, followed by 200,000 steps, for values of K between 1 and 10, and the calculation was done ten times for each K. The most likely number of clusters, K, was estimated as the value which maximized the averaged log-likelihood, log Pr(X|K). Once K was determined, individuals were assigned to the different clusters and plotted with DISTRUCT (Rosenberg 2004). The putative origin of the samples was inferred based on the membership coefficient (q). Individuals were classified as "WILD," "H07," or "H08" when the value of q>0.70 in one cluster. Otherwise, the individual was categorized as "unassigned".

An assignment test was performed with Geneclass2 (Piry et al. 2004) using a partially Bayesian assignment approach (Rannala and Mountain 1997). In contrast to STRUCTURE, Geneclass2 does not assume that all potential source populations have been sampled; it permits the rejection of unknown individuals from baseline populations in case of potentially incomplete baseline information. Self-assignment simulations were performed with 10,000 genotypes and the Monte-Carlo re-sampling method of Paetkau et al. (2004) with a type I error (alpha) of 0.05 to determine the power of microsatellite markers for discrimination between three baseline populations. H07 and H08 were used as two reference hatchery populations. The 120 wild fish collected from Iwate, Aichi, and Oita (Blanco Gonzalez et al. 2012) were pooled together to represent the third baseline population of specimens with no effect of prior stocking, WILD. A direct assignment test was carried out to investigate the putative wild or hatchery origin of the samples from SB and TB as well as the population source of the recaptured fish, HR, setting a threshold probability value at 0.01. Finally, we performed an exclusion test with a type I error (alpha) of 0.01 and 0.05 following the Monte-Carlo re-sampling method of Paetkau et al. (2004) and 10,000 simulated individuals. The exclusion test rejects those individuals of unknown origin from the putative baseline source populations at 99 % and 95 % of significant.

Mitochondrial DNA Control Region Sequencing and Analysis

An approximately 559-bp fragment corresponding to the left domain of the control region flanked by a part of the $tRNA^{PRO}$ was amplified by PCR using the primer pair L-15560 5'-CATATTAAACCCGAATGATATTT-3' and H1067 5'-ATAATAGGGTATCTAATCCTAGTTT-3' (Martin et al.

1992). PCR amplifications were carried out using the IllustraTM puReTaq Ready-To-Go PCR Beads kit (GE Healthcare). For each reaction, 10 pmol of each primer were added to 50 ng of DNA template and ultrapure distilled water to a final volume of 25 μl. PCR conditions consisted of an initial denaturation at 94 °C for 1 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 45 °C and 1 min at 72 °C, with a final extension of 72 °C for 7 min. PCR products were visualized on a 1 % agarose gel and purified with the QIAquick PCR purification kit (Qiagen, Inc.). Cycle sequencing was performed in both forward and reverse directions with the same primers as those used in the PCR using the BigDye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems). The excess dideoxynucleotide terminators were removed with the BigDye® XTerminator™ purification kit (Applied Biosystems) in accordance with the manufacturer's recommendations. Sequences were generated using an ABI 3500xl Genetic Analyzer (Applied Biosystems).

Base calling was performed with KB™ Basecaller v1.41 (Applied Biosystems). Initially, individual sequences were visualized, aligned, and edited manually using BioEdit ver. 7.0.5.5. (Hall 1999). Reverse complements of backward sequences were aligned with the corresponding forward sequences, and the end of each sequence was truncated. A second alignment was created in ClustalW integrated in MEGA ver. 4.0 (Tamura et al. 2007). Additionally, the initial part containing a partial segment of the $tRNA^{PRO}$ gene was truncated for further analysis. The final pruned sequences contained 484-bp including gaps. New sequences were deposited in the DDBJ/EMBL/GenBank database under the accession numbers AB713769–AB713839.

Population descriptive statistics—such as number of haplotypes, H; number of shared and unique haplotypes, haplotype diversity, h; nucleotide diversity, π; mean number of pairwise differences, K; and number polymorphic sites, S—were estimated with MEGA ver. 4.0 (Tamura et al. 2007), DnaSP ver. 5.0 (Librado and Rozas 2009), and Arlequin ver. 3.5 (Excoffier and Lischer 2010). Similarly to the allelic richness estimation for microsatellite DNA analysis, CONTRIB 1.02 (http://www.pierroton.inra.fr/ genetics/labo/software/sontrib/) was used to calculate haplotypic richness following Petit et al. (1998). The rarefaction method was applied to correct for differences in sample sizes, selecting the smallest sample size, HR=25, as rarefaction value.

Genetic differentiation between sample pairs were conducted based on Φ_{ST} using the Kimura 2P genetic distance method (Kimura 1980) implemented in Arlequin ver. 3.5 (Excoffier and Lischer 2010) by 10,000 permutations. The same software and model were employed to perform an AMOVA. Genealogical relationships were inferred constructing a neighbor-joining (NJ) tree based on Kimura 2P genetic distances with MEGA ver. 4.0 (Tamura et al. 2007) and a haplotype network using the median joining method (Bandelt et al. 1999) implemented in Network 4.5.10 (http://www.fluxus-engineering.com/sharenet.htm).

Results

Genetic Diversity

Red sea bream showed a high level of genetic diversity at the majority of microsatellite loci (Table 2). *Kpm*1 showed exceptionally low genetic variability, segregating for only two alleles at H08 and H_E=0.165. On the other hand, SB showed 30 alleles at *Kpm*2 and *Pma*22-9NCL, corresponding to H_E= 0.966 and H_E=0.963, respectively. In fact, red sea bream from SB and TB presented higher allele richness, Ar=15.3±6.7 and 14.1±6.7, respectively, than hatchery offspring, H07 and H08, Ar=11.1±4.6 and 9.4±3.6, respectively, as well as the recaptured sample, HR, Ar=12.8±5.6.

Deviation from HW expectations (F_{IS}) was observed in 12 locus–sample combinations. These departures appeared evenly distributed between samples at the same loci as well as among loci within samples, and only two tests remained significant after Bonferroni correction (Table 2). Both cases were observed in SB and displayed heterozygosity deficit. Nevertheless, the presence of null alleles was not apparent, whereas results of LOSITAN did not suggest the existence of outlier locus. Alternatively, the deficit of heterozygotes may have resulted from the admixture of two distinct stocks, i.e., wild and hatchery. A total of 39 (7.4 %) out of 525 pairwise tests for linkage disequilibrium were statistically significant after Bonferroni correction. Significant outcomes were evenly distributed between pairs of loci across sample, indicating little linkage disequilibrium.

A total of 71 haplotypes were identified by sequencing 484 bp of the control region from wild- and hatchery-reared red sea bream (Table 3). Unique haplotypes counted 58, representing 82 % of the total, divided between SB (40 %), TB (54 %), and HR (6 %), whereas both hatchery-reared offspring samples shared their haplotypes (Table 4). In fact, most haplotypes observed in SB and TB were unique. In the former sample, only two haplotypes were observed in two fish each, and an additional haplotype was shared by seven fish. On the other hand, there were two common haplotypes in the latter sample, shared by two and three specimens, respectively. In contrast, haplotype frequencies were much higher in H07 and H08, evidencing a reduction in haplotypic variability (Table 3). Erosion in haplotypic richness was especially apparent, as values in SB and TB were four- to five-fold higher than in H07 and H08. Haplotype diversity was also considerably lower in the hatchery samples. The group of recaptured fish, HR, presented intermediate values.

Table 2 Summary statistics for genetic diversity at 15 microsatellite loci

		Kpm1	Kpm11	Kpm7	Kpm22	Kpm2	Kpm23	Kpm25	Pma22-9NCL	Kpm28	Pma11-45NCL	Pma4-32NCL	Pma103-59NCL	Pma18-41NCL	Pma1	Pma5
SB																
n= 38	A	3	8	21	24	30	12	15	30	24	15	25	13	15	14	16
	Ar	2.7	7	17.2	20.7	25.8	10.4	12.6	25.6	20.7	13.9	22.1	11.3	12.8	11.3	15.1
	HE	0.411	0.601	0.878	0.947	0.966	0.825	0.792	0.963	0.947	0.915	0.955	0.857	0.837	0.664	0.911
	HO	0.500	0.658	0.868	0.974	0.974	0.737	0.526	0.789	0.974	0.895	0.921	0.789	0.737	0.711	0.947
	FIS	−0.216	−0.095	0.011	−0.028	−0.009	0.107	0.336*	0.180*	−0.029	0.022	0.036	0.079	0.120	−0.071	−0.039
TB																
n= 60	A	4	5	18	23	24	10	12	28	23	17	21	11	13	14	16
	Ar	3.5	4.6	15.4	20.3	21.2	9.4	10.6	23.6	20.6	15.7	18.9	9.7	12.1	11.2	15.2
	HE	0.397	0.469	0.892	0.946	0.954	0.843	0.828	0.948	0.947	0.908	0.936	0.787	0.871	0.693	0.907
	HO	0.350	0.400	0.850	0.900	0.875	0.900	0.750	0.950	0.900	0.950	0.900	0.700	0.775	0.675	0.900
	FIS	0.119	0.148	0.047	0.049	0.083	−0.068	0.094	−0.002	0.050	−0.047	0.038	0.111	0.110	0.026	0.008
H07																
n= 60	A	3	7	10	20	20	11	12	24	23	15	21	7	10	10	17
	Ar	2.8	5.5	8.2	15.5	15	10.2	9.8	17.3	16.2	12.3	17.5	6.8	8.5	7.8	13.4
	HE	0.464	0.712	0.748	0.894	0.908	0.845	0.829	0.899	0.894	0.874	0.930	0.747	0.738	0.591	0.891
	HO	0.433	0.767	0.750	0.817	0.917	0.867	0.667	0.883	0.817	0.850	0.967	0.750	0.783	0.600	0.917
	FIS	0.066	−0.077	−0.003	0.087	−0.010	−0.025	0.195	0.018	0.087	0.027	−0.039	−0.005	−0.062	−0.015	−0.029
H08																
n= 50	A	2	6	8	18	14	9	11	18	15	11	13	7	10	7	14
	Ar	2	5.3	7.2	14.4	12.7	8.4	9.3	15.1	12.7	9.6	11.3	6.5	8.6	6.4	12.1
	HE	0.165	0.642	0.788	0.905	0.901	0.822	0.787	0.905	0.900	0.801	0.869	0.741	0.848	0.608	0.894
	HO	0.180	0.540	0.660	1.000	0.960	0.840	0.860	0.940	1.000	0.820	0.920	0.800	0.800	0.580	0.960
	FIS	−0.089	0.158	0.162	−0.105	−0.066	−0.021	−0.093	−0.039	−0.111	−0.024	−0.059	−0.080	0.056	0.046	−0.073
HR																
n= 26	A	3	5	8	17	21	9	14	21	17	14	18	9	13	8	15
	Ar	3	5	8	17	21	9	14	21	17	14	18	9	13	8	15
	HE	0.51	0.67	0.71	0.87	0.94	0.84	0.87	0.95	0.87	0.90	0.94	0.72	0.81	0.62	0.92
	HO	0.385	0.654	0.654	0.923	0.923	0.731	0.692	0.923	0.923	0.962	0.885	0.769	0.808	0.462	0.808
	FIS	0.244	0.024	0.080	−0.057	0.017	0.126	0.208	0.024	−0.057	−0.070	0.057	−0.065	0.006	0.2565	0.123

n sample size, *A* number of alleles per locus, *Ar* allelic richness, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} Wright's within-population fixation statistic per locus and population

*$P<0.05$ level of significant difference, after Bonferroni correction

Table 3 Summary statistics for mitochondrial DNA diversity

	N	*H*	Haplotypic richness	Shared haplotypes	Unique haplotype	*h*	π	*K*	*S*
SB	38	30	19.5	7	23	0.97	0.027+ −0.014	13.12	62
TB	41	38	22.7	7	31	0.99	0.029+ −0.015	14.01	68
H07	60	8	4.8	8	0	0.76	0.024+ −0.012	11.47	32
H08	50	6	4.1	6	0	0.7	0.017+ −0.009	7.96	26
HR	25	11	10	7	4	0.8	0.023+ −0.012	11.13	42

n sample size, *H* number of haplotypes, *h* haplotype diversity, π nucleotide diversity, *K* number of pairwise differences, *S* number of polymorphic sites

Population Differentiation

Microsatellite pairwise F_{ST} values ranged from 0.002 to 0.054 and evidenced the different genetic composition among samples (Table 5). Except SB vs. TB and H07 vs. HR, all pairwise comparisons were significantly different after Bonferroni correction. On the other hand, only those comparisons involving H08 were statistically significant when tested using R_{ST}. Similarly, pairwise Φ_{ST} based on mitochondrial DNA haplotype data confirmed the differences between H08 and the rest of samples as well as between TB vs. H07 (Table 5). Interestingly, pairwise Φ_{ST} values reflected larger genetic differentiation among wild samples (Φ_{ST}=0.022) than between SB, HR, and H07 (Φ_{ST}=−0.003−0.016), even though all of them were statistically insignificant. AMOVA tests based on F_{ST}, R_{ST}, and Φ_{ST} were performed until differences among samples within groups were not statistically significant at the 5 % level of confidence. The analysis corroborated previous pairwise results (Table 6). In all cases, SB and TB were grouped. On the other hand, AMOVA based on microsatellite allele frequencies, F_{ST}, kept each of the three hatchery samples separate, whereas R_{ST} and Φ_{ST} agreed clustering H07 and HR, leaving H08 as a third group.

The microsatellite-based Bayesian clustering method implemented in STRUCTURE identified three different clusters corresponding to the wild samples (SB and TB) and each of the hatchery strains, H07 and H08 (Fig. 2). While all fish from TB clustered to the wild group, two specimens from SB clustered to H07, suggesting a putative hatchery-released origin even though none of the fish showed the DIE deformity. Membership coefficients of four additional fish were lower than the threshold value of 0.7 and were unassigned to any cluster. It is noteworthy that three of these fish presented higher probability to cluster to H07 than to the wild group. Meanwhile, HR corresponded to a mixture of the three clusters. Considering that all fish in this sample presented DIE, hence evidencing their hatchery origin, clustering to the wild group should not lead to an erroneous interpretation. In this case, the wild cluster should be considered a founder group genetically closer to SB and TB rather than to the two hatchery samples analyzed here, i.e., H07 or H08. The number of fish from HR allocated to the clusters of H07, H08, and wild samples was 12, 6, and 4, respectively, with the remaining four individuals unassigned.

A reconstructed NJ tree based on Kimura 2P model among haplotypes is presented in Fig. 3. Overall, the tree topology confirmed the absence of clustering among fish within samples; instead they appeared scattered throughout the dendrogram. Noteworthy is the close relationship between seven specimens from SB and several hatchery offspring. In fact, three of the fish from SB (SB035, SB039, and SB043) were inferred to have a hatchery origin based on the results of the assignment test (see the next section). The median-joining network (Fig. 4) clearly differentiated two main components; however, clusters did not correspond to the origins of the samples. The central part of the network appeared occupied by unique haplotypes from SB and TB with more frequent haplotypes shared among samples distributed in both extremes of the network. The presence of different terminal hatchery haplotypes may reflect geographically distinct origins.

Assignment Test and Inference of Releases

Correct self-assignment simulations conducted with H07, H08, and a wild sample (see "Materials and Methods" for details) as baseline populations were 64 %, 55 %, and 100 %, respectively, resulting in an overall 80 %. All misclassified fish were assigned to the wild populations while none of the fish was misassigned to a different hatchery baseline source. The difficulty for correct self-assignment and increment of misassignment to the wild population appeared related to relatively modest pairwise F_{ST} values (Table 4), i.e., low genetic differentiation between the source baseline groups. Result of direct assignment and exclusion tests with Geneclass2 were consistent with those of STRUCTURE, as most fish from SB and TB possessed genetic profiles closer to the wild source population, in contrast to the fish from HR that were mainly allocated to

Table 4 Haplotypic composition found for each population sample

	SB n=38	H08 n=50	H07 n=60	TB n=41	HR n=25
H1	1	0	0	0	0
H2	2	0	18	1	1
H3	1	0	0	0	0
H4	1	0	0	0	0
H5	1	0	0	0	0
H6	1	0	0	0	0
H7	7	3	20	1	11
H8	1	0	0	0	0
H9	2	0	0	0	0
H10	1	0	0	0	0
H11	1	0	0	0	0
H12	1	0	0	0	0
H13	1	0	0	0	0
H14	1	0	0	0	1
H15	1	0	0	1	0
H16	1	0	0	0	0
H17	1	0	0	0	0
H18	1	0	0	0	0
H19	1	0	0	0	0
H20	1	23	4	0	0
H21	1	0	0	0	0
H22	1	0	0	0	0
H23	1	0	0	0	0
H24	1	0	0	1	0
H25	1	0	0	0	0
H26	1	0	0	0	0
H27	1	0	0	0	0
H28	1	0	0	0	0
H29	1	0	0	0	0
H30	1	0	0	1	0
H31	0	1	2	1	0
H32	0	2	13	0	0
H33	0	14	1	0	2
H34	0	7	1	0	3
H35	0	0	1	0	1
H36	0	0	0	1	0
H37	0	0	0	1	0
H38	0	0	0	1	0
H39	0	0	0	1	0
H40	0	0	0	1	0
H41	0	0	0	1	0
H42	0	0	0	1	0
H43	0	0	0	1	0
H44	0	0	0	1	0
H45	0	0	0	1	0
H46	0	0	0	1	0
H47	0	0	0	1	0
H48	0	0	0	1	0
H49	0	0	0	1	0
H50	0	0	0	1	0
H51	0	0	0	3	0
H52	0	0	0	1	0
H53	0	0	0	1	0
H54	0	0	0	1	0
H55	0	0	0	1	0
H56	0	0	0	1	0
H57	0	0	0	1	0
H58	0	0	0	1	0
H59	0	0	0	1	0
H60	0	0	0	1	0
H61	0	0	0	2	0
H62	0	0	0	1	0
H63	0	0	0	1	0
H64	0	0	0	1	0
H65	0	0	0	1	0
H66	0	0	0	1	2
H67	0	0	0	1	0
H68	0	0	0	0	1
H69	0	0	0	0	1
H70	0	0	0	0	1
H71	0	0	0	0	1

one of the hatchery samples (Table 7). Interestingly, three fish from SB directly assigned to H07 by STRUCTURE and Geneclass2 could not be excluded from H07 as a potential source population at α=0.05. In fact, the wild reference population was excluded as source of origin for one fish, SB035. On the other hand, the similarity between the reference baseline populations was evident for HR, while an alternative hatchery source population could not be excluded for most fish.

Discussion

Genetic Diversity

In this study, a large set of 15 microsatellite markers and mitochondrial DNA sequencing were combined to characterize the genetic resources of red sea bream in Kanagawa Prefecture for the first time. Red sea bream from SB and TB showed high levels of genetic variability at both microsatellite and mitochondrial DNA markers, in contrast to the offspring as well as the hatchery-released group who evidenced an important reduction in genetic variability. Microsatellite genotyping detected the loss of many rare alleles and reduction in heterozygosity, whereas mitochondrial D-loop sequences highlighted

Table 5 Pairwise F_{ST}, R_{ST} (microsatellite), and Φ_{ST} (mtDNA) values based on Kimura 2P

	Microsatellites		mtDNA
	F_{ST}	R_{ST}	Φ_{ST}
SB vs. TB	0.002 (P=0.150)	−0.008 (P=0.960)	0.022 (P=0.067)
SB vs. H07	0.021* (P=0)	0.006 (P=0.145)	0.016 (P=0.104)
SB vs. H08	0.029* (P=0)	0.038* (P=0.001)	0.227* (P=0)
SB vs. HR	0.013* (P=0.001)	0.008 (P=0.146)	−0.003 (P=0.380)
TB vs. H07	0.028* (P=0)	0.010 (P=0.063)	0.083* (P=0.002)
TB vs. H08	0.027* (P=0)	0.029* (P=0)	0.127* (P=0)
TB vs. HR	0.019* (P=0)	0.012 (P=0.058)	0.067 (P=0.013)
H07 vs. H08	0.054* (P=0)	0.044* (P=0)	0.282* (P=0)
H07 vs. HR	0.007 (P=0.012)	−0.003 (P=0.560)	0.011 (P=0.25)
H08 vs. HR	0.037* (P=0)	0.031 (P=0.006)	0.301* (P=0)

*P<0.05 level of significant difference, after Bonferroni correction k=10

a drastic decline in the number of haplotypes. The reduction of genetic diversity in hatchery-released offspring has commonly stem from the small number of breeders reared in hatchery facilities and to the resultant stochastic genetic drift and inbreeding that produce further detrimental effects (Allendorf and Ryman 1987; Taniguchi 2004; Blanco Gonzalez and Umino 2012). In this regard, Ryman and Laikre (1991) warned about the potential reduction in the effective size of the wild stock in case a large proportion of offspring are originated from a small proportion of captive breeders, suggesting a potential trade-off between the genetic erosion and the success of the stock enhancement program. The loss on genetic diversity is a key issue not only in stock enhancement programs but also in conservation genetics, as it involves the adaptive and evolutionary response of the organisms to environmental changes. Recently, Araki and Schmidt (2010) reviewed the existing literature, questioning the benefits of stock enhancement and restocking initiatives based on the negative effects reported in most of case studies. In contrast, Kitada el al. (2009) found no decline in fitness in marine fish species arguing that the magnitude of the genetic impacts can be small, local, and temporary.

In Japan, red sea bream juveniles released as part of stock enhancement programs are usually produced from relatively large broodstocks, 100–250 fish (Perez-Enriquez et al. 1999; Nugroho and Taniguchi 2004; Hamasaki et al. 2010). Nevertheless, pedigree analysis revealed that only about 40 % of them contributed to the offspring, while sex ratio was highly male-skewed (Perez-Enriquez et al. 1999; Nugroho and Taniguchi 2004). In fact, even though holding an equal proportion of parental male and female fish, Perez-Enriquez et al. (1999) found 34 female contributors among 250 breeders genotyping three microsatellites, while the mtDNA analysis conducted by Nugroho and Taniguchi (2004) identified 8–15 dams among 152 parental fish. The loss of rare alleles and low number of mtDNA haplotypes found in our hatchery samples is in line with results of those

Table 6 AMOVA results for microsatellite (F_{ST} and R_{ST}) and mtDNA (Φ_{ST}) data

Microsatellite		
F_{ST}		SB-TB vs. HR vs. H07 vs. H08
	F_{CT}	0.026 (P=0.1)
	% Variation	2.63
	F_{SC}	0.003 (P=0.15)
	% Variation	0.26
	F_{ST}	0.029 (P=0)
	% Variation	97.11
R_{ST}		SB-TB vs. H07-HR vs. H08
	R_{CT}	0.030 (P=0.059)
	% Variation	2.98
	R_{SC}	−0.005 (P=0.88)
	% Variation	−0.53
	R_{ST}	0.024 (P=0)
	% Variation	97.56
mtDNA		
Φ_{ST}		SB-TB vs. H07-HR vs. H08
	Φ_{CT}	0.125 (P=0.058)
	% Variation	12.50
	Φ_{SC}	0.022 (P=0.087)
	% Variation	1.94
	Φ_{ST}	0.144 (P=0)
	% Variation	85.56

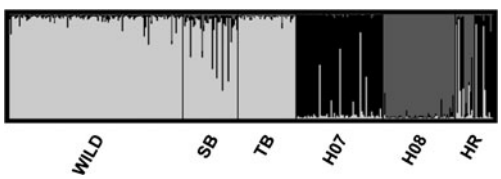

Fig. 2 Results of Bayesian clustering with STRUCTURE (K=3) based on 15 microsatellite loci

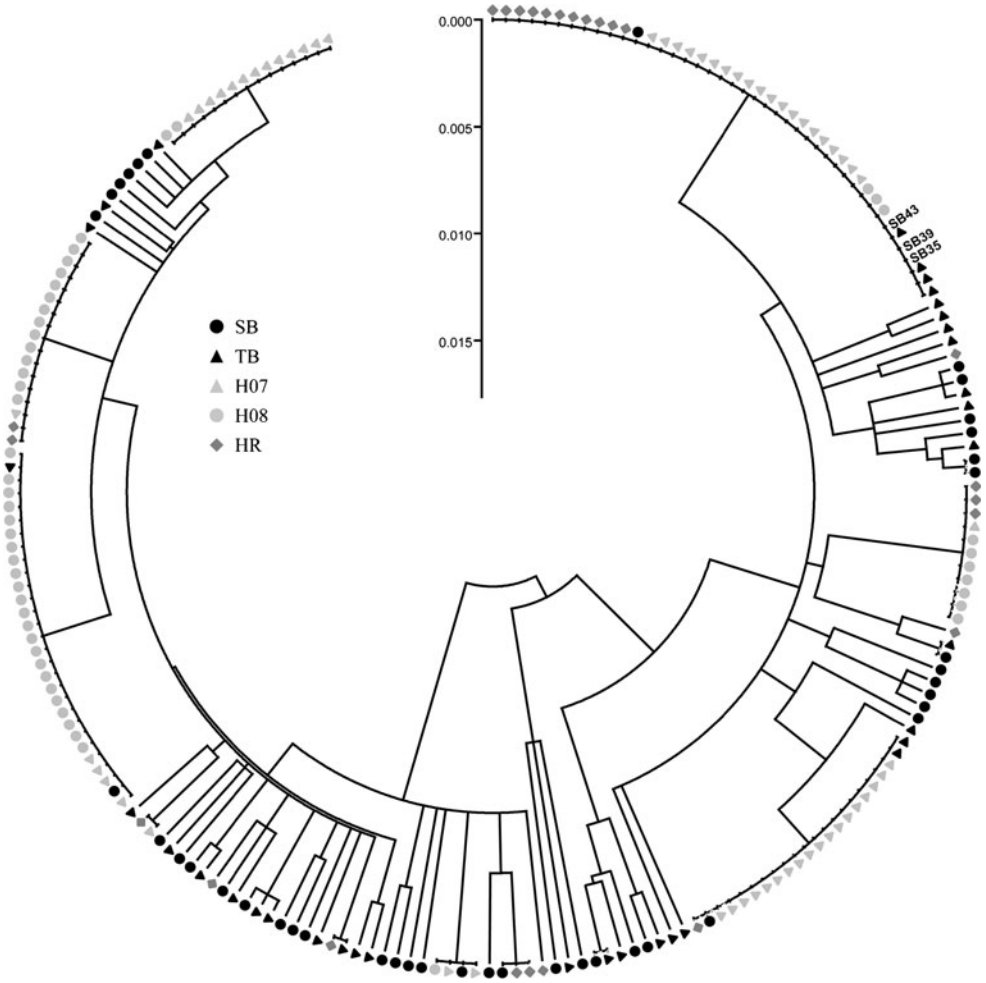

Fig. 3 Neighbor-joining tree of mtDNA D-loop haplotypes based on Kimura 2P distances

studies, as offspring from H07 were produced from 150 breeders while the broodstock of H08 comprised 100 fish. Genetically cognizant broodstock management procedures have been proposed to preserve the genetic diversity of stock enhancement programs (Miller and Kapuscinski 2003; Taniguchi 2004). Recent advances in broodstock

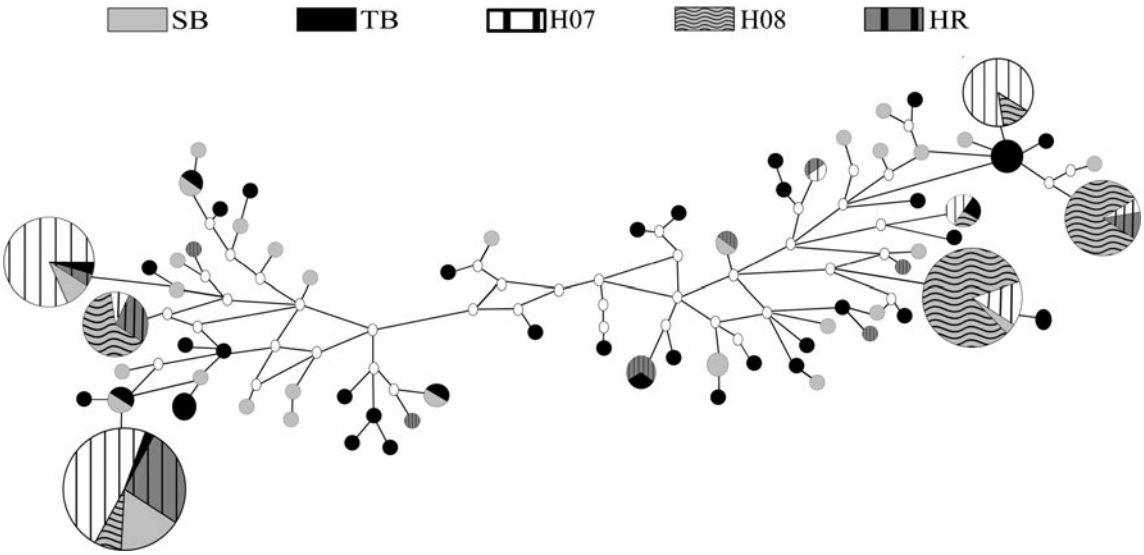

Fig. 4 Haplotype median joining network based on the mitochondrial D-loop analysis. Each *circle* represents a haplotype, and its size is proportional to the number of fish sharing that haplotype. *Empty circles* are median vectors indicating not sampled or missing haplotypes

Table 7 Results of assignment tests performed on red sea bream from SB, TB, and HR using Geneclass2

	SB (n =38)			TB (n=40)			HR (n=26)		
	WILD	H07	H08	WILD	H07	H08	WILD	H07	H08
Direct	33	5		39	1		7	14	5
Excluded (α=0.01)	2	24	36	1	29	40	1	6	20
Excluded (α=0.05)	8	33	37	4	37	40	1	9	21

management practices, e.g., mating designs and egg collection strategies, have shown promising results to increase the proportion of breeders and number of families presented in the offspring for release, without an increment in the rate of inbreeding (Doyle et al. 2001; Nugroho and Taniguchi 2004; Blanco Gonzalez et al. 2010). Hence, the suitability of such means to maximize the genetic variability of the offspring and minimize potential detrimental effects of large-scale red sea bream juvenile releases should be investigated in the program carried out in Kanagawa Prefecture.

The lower levels of genetic variability detected in hatchery-released fish, HR, compared with SB and TB resembled the situation reported for red sea bream in Kagoshima Bay (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010), where numbers of juveniles stocked and their contribution to total production was similar to that in Kanagawa Prefecture (Imai 2005; Kitada and Kishino 2006). However, in contrast to the program carried out in Kagoshima Bay, none of the samples inhabiting Sagami Bay or Tokyo Bay evidenced considerable deterioration in the genetic variability. It is noteworthy, though, that the only haplotype shared by more than two fish in SB was identified in as many as seven fish (Table 4). This finding is surprising given the large number of haplotypes at very low frequencies observed in the wild samples here as well as previously reported by Hamasaki et al. (2010). Bayesian clustering as well as assignment and exclusion tests reinforced the suspicion that some of the specimens were likely hatchery-released fish without DIE or hybrid offspring from a hatchery-origin female (see assignment results for further discussion). Similar to black sea bream (*Acanthopagrus schlegelii*) in Hiroshima Bay (Blanco Gonzalez et al. 2008, 2009; Blanco Gonzalez and Umino 2009), Kitada et al. (2009) attributed the loss of genetic diversity in red sea bream from Kagoshima Bay to density-dependent effects associated to large-scale releases and limitation on the carrying capacity of the Bay, and suggested that the negative impact remained within the Bay because it is "like a deep pond with a narrow mouth". In contrast, Sagami Bay opens to the Pacific Ocean, facilitating red sea bream migration, i.e., gene flow, a fact that may attenuate the putative genetic deterioration related to fish releases. Hatchery-released fish may have exhibited also lower reproductive fitness than wild counterparts (Araki et al. 2007), with a small effective number of females interbreeding with wild males. However, low reproductive fitness was rejected for red sea bream in Kagoshima Bay (Kitada et al. 2009); then, the same conclusion may be applicable to the program in Kanagawa Prefecture. Finally, we should not exclude the possibility that stocked fish increased commercial and sport fishery catches, but only a small portion of hatchery-released fish remained and contributed to the next generation. Nevertheless, we also considered this hypothesis unlikely given the important reduction in gene pool variability reported in the natural stock inhabiting Kagoshima Bay (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010).

Population Differentiation

All three genetic differentiation metrics ($F_{ST,}$ R_{ST}, Φ_{ST}) agreed in terms of high similarity between the wild samples (SB and TB) and differentiation of H08 from the other groups. However, they also showed some incongruent results (Table 5) due to the intrinsic characteristics of the markers and metrics (Sefc et al. 2007). Microsatellite-based F_{ST} reflected the results of using different founder stocks and the reduction in allelic diversity in relation to genetic drift observed in hatchery samples. In contrast to the differences in allele frequencies, the populations showed similar allele sizes that likely led to insignificant R_{ST} values. Meanwhile, Φ_{ST}-based inferences complemented previous information elucidating the maternal contribution, in particular the high frequency of haplotype H7 in SB, which may explain the lack of significant genetic differentiation between this sample, H07, and HR. Values of the pairwise estimates in this study, based on both microsatellite ($F_{ST,}$ R_{ST}) and mitochondrial DNA (Φ_{ST}) markers, were higher than previously reported among wild stocks (Perez-Enriquez et al. 2001), as well as for the stock enhancement program carried out in Kagoshima Bay (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010). Of special concern is the fact that the highest pairwise values, independent of the estimator, were observed between the two groups of offspring intended for release (Table 5). This differentiation contradicted the general objective of stock enhancement programs to produce offspring carrying similar genetic material to the recipient wild stock (Taniguchi 2004) and warns about current broodstock management practices and potential hatchery-wild fish interactions in Kanagawa Prefecture.

The differential genetic composition of the offspring may compromise the original gene pool and result in problems of local adaptation and individual fitness (Araki and Schmidt 2010).

Results of hierarchical AMOVA (Table 6) and Bayesian clustering (Fig. 2) supported those of pairwise comparisons, clustering SB and TB and discriminating them from both offspring samples. Nevertheless, Bayesian clustering with STRUCTURE assigned most of the fish from HR to one of the hatchery groups, in disagreement with AMOVA tests performed based on F_{ST} that differentiated the hatchery-released group from both H07 and H08. DIE confirmed the hatchery origin of all fish; however, results of AMOVA and exclusion tests with Geneclass2 suggested that a large proportion of fish from HR were descendants of a founder stock not sampled here. Hence, the contradictory results are probably due to limitations of STRUCTURE to determine the correct number of genetic clusters when all putative source populations are not present (Pritchard et al. 2000).

On the other hand, the haplotype network and NJ dendrogram based on mitochondrial DNA sequences found no clustering corresponding to sample location. Despite the complexity of the results at the population level, they provided relevant complementary information to microsatellite analysis to understand maternal inheritance and hatchery-release effects at individual level.

Inference of Hatchery-released Fish Without DIE and Interaction with Wild Conspecifics

The combination of Bayesian clustering, assignment, and exclusion-probability tests based on 15 polymorphic microsatellite markers suggested the presence of three (8 %) hatchery-released fish with normal inter-nostril epidermis in the sample collected at Sagami Bay, SB. The hatchery origin of these fish was further supported by mitochondrial D-loop sequencing analysis. These specimens may also represent hybrid offspring of a wild-male and a hatchery-female. However, we presume that, given the moderate pairwise F_{ST} values among samples (0.021–0.054), it would be too challenging to identify hybrid fish using assignment methods, as the accuracy of these tests greatly depends on the level of genetic differentiation among samples (Hansen et al. 2000; Paetkau et al. 2004; Glover et al. 2010, 2011).

Our findings may represent a reference point in the ecological–genetic evaluation of large-scale marine stock enhancement programs carried out on red sea bream in Japan, as this is the first time that hatchery-released juveniles without DIE were inferred. To date, assessment of the contribution and genetic implications of the releases relied on the preliminary identification of previously released fish presenting DIE (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010), even though experimental research found no DIE in about 20–30 % of hatchery-reared fish (Sobajima et al. 1986). In other instances where fish had normal epidermis, the analysis was based on RFLP (Tabata and Mizuta 1997) or a handful of microsatellite loci (Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001); hence, the authors were limited to suggest some genetic disturbance at population level. Combined analysis using the set of microsatellites and mitochondrial DNA sequencing may overcome those limitations, in order to infer the proportion of released fish without DIE. In conjunction with DIE evaluation, the genetic approach implemented in this study would contribute to provide a more accurate estimation of the current contribution of red sea bream releases conducted throughout the country.

Levels of genetic differentiation among baseline source populations used here were much larger than previously reported in red sea bream using three microsatellite loci (Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001; Shishidou et al. 2008; Kitada et al. 2009); nevertheless, the ability of our markers to infer the hatchery origin of the fish lacking the DIE was surprising to some extent. In Norway, ten microsatellite loci were genotyped to identify the source population of Atlantic cod (*Gadhus morhua*) escaped from aquaculture facilities (Glover et al. 2010, 2011). Although pairwise F_{ST} values here were in the same range as those in studies on cod and in both cases wild-hatchery fish interactions were investigated, it is important to distinguish the achievements given the different nature and history of the samples. Escaped cod were likely subjected to intentional selection for desirable traits in the hatchery (Glover et al. 2010, 2011), in contrast to red sea bream releases that aimed at producing viable fish of similar characteristics as their wild counterparts in order to interbreed with them and increase the natural resources in future generations (Kitada and Kishino 2006; Blanco Gonzalez et al. 2008). Therefore, while differential genetic composition between cods produced at different farms should be not surprising, founder red sea bream stocks were expected to possess similar genetic material to the wild recipient population, and genetic assignment tests conducted here should have failed to elucidate their putative hatchery source of origin (Paetkau et al. 2004; Glover et al. 2011). Our results highlighted the differential genetic composition between wild and hatchery samples and emphasized the need to identify the appropriate source population to be released (Miller and Kapuscinski 2003; Taniguchi 2004; Blanco Gonzalez et al. 2008; Laikre et al. 2010).

It is important to interpret our results in the overall management and conservation context for the species in the country. In Japan, red sea bream is considered to comprise a large panmictic stock (Taniguchi and Sugama 1990; Perez-Enriquez and Taniguchi 1999; Perez-Enriquez et al. 2001), suggesting that the genetic erosion observed in

Kagoshima Bay remained within the bay due to its peculiar geomorphology (Shishidou et al. 2008; Kitada et al. 2009; Hamasaki et al. 2010). In contrast, Sagami Bay is open to the sea, facilitating wild and hatchery-released fish interactions. Then, assuming that hatchery-released fish do not present lower fitness than wild counterparts (Kitada et al. 2009) and given the large-scale and contribution of the releases (Imai 2005; Kitada and Kishino 2006), there are fundamental questions still to be clarified regarding the effects of the releases: Do levels of genetic diversity in Kanagawa Prefecture correspond to those in other locations where juvenile releases have not been carried out? Have there been impacts on the fitness of the receiving population? What is the level of genetic differentiation between wild and hatchery offspring in the programs conducted by other prefectures? Or, how can broodstock management practices in large-scale programs be improved? In sum, we inferred the potential genetic risks associated to large-scale releases of red sea bream in Kanagawa Prefecture; nevertheless, there is further research needed to conserve the natural resources of this species, as well as many other marine fishes commonly released in the world.

Conclusion

The combination of 15 microsatellites and mitochondrial DNA sequencing unveiled important implications concerning the genetic interactions between wild and hatchery-released red sea bream in Sagami Bay and Tokyo Bay, a region where millions of juveniles have been released since 1978, and their contribution to total production estimated to be about 40 % (Kitada and Kishino 2006) even reaching 74 % in some years (Imai 2005). Wild red sea bream samples evidenced significant higher genetic variability than any of the offspring produced for release as well as the recaptured hatchery-released group presenting the DIE. In addition, the distinct genetic composition among baseline source populations facilitated the insight into the presumptive hatchery origin of fish without DIE for the first time. Our results warn about the potential genetic erosion in wild stocks in case interbreeding takes place, demanding effective monitoring of hatchery-wild fish interactions and urging revision of the current management strategy of the stock enhancement program in Kanagawa Prefecture. Moreover, combining nuclear and mitochondrial DNA techniques have proven useful for inferring hatchery-released fish without DIE and elucidating a more realistic estimation of the contribution of red sea bream releases conducted throughout the country.

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