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Studies on genetic management of hatchery broodstock in relation to the stock enhancement of Japanese flounder *Paralichthys olivaceus*[*1]

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Abstract From the viewpoint of genetic conservation, this study presents application of molecular genetic markers to the population genetics studies of Japanese flounder *Paralichthys olivaceus* in order to propose several broodstock management options related to the stock enhancement.

A $(CA)_n$-microsatellite enriched library of Japanese flounder was constructed using an efficient method to isolate microsatellite DNA regions, from which 16 PCR primer pairs to amplify microsatellites with a high amplification effectiveness were developed. Genetic variability of these loci varied depending on the locus, and all but one of the 16 loci conformed to Hardy-Weinberg's equilibrium (HWE). Mendelian inheritability of these loci was verified in five experimental families of Japanese flounder excepting one instance. The locus at which the significant HWE departure was observed had unstable and unreproducible PCR amplification of a particular allele in one family. These results suggest that 15 out of the 16 microsatellites are viable for further applications to the wide range of the Japanese flounder population genetics studies.

Genetic variability and structure maintained in wild Japanese flounder populations (seven populations) were assessed by using 11 microsatellites coupled with the nucleotide sequences of mitochondrial DNA (mtDNA) control region to outline the genetic stock management units. The 11 microsatellites examined showed considerable polymorphisms in all populations, and the section of mtDNA sequences examined was highly variable. Assessment of the genetic structure based on the 11 microsatellites revealed that 11 population pairs in 21 possible pairwise comparisons yielded significant genetic heterogeneities in terms of allele frequency distributions and/or pairwise F_{ST} values, and an overall F_{ST} value provided evidence of the genetic structuring among the seven populations, although these inter-populational genetic heterogeneities were not necessarily supported by the results of mtDNA sequencing analysis. According to the population relationships estimated from the phylogenetic trees based on the genetic distances (D_A and D_{ST}) and the results of several hierarchical AMOVA analyses, which were calculated on the basis of the microsatellite data, the seven populations were possibly assigned into three groups consistent with the oceanogeographic positions of the populations. Although the level of the genetic differentiation between the groups was weak, the genetic management of wild flounder should be applied along with the group definitions so as to preserve the currently detectable genetic stock units.

Level of genetic variability maintained in currently available hatchery strains was compared with that of wild populations. Three Japanese flounder hatchery strains were

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screened by means of microsatellite and mtDNA sequencing analysis, and both the number of microsatellite alleles and mtDNA haplotypes, and mtDNA haplotype diversity showed marked reductions in the hatchery strains relative to the comparable three wild populations.

Pedigree structure of a hatchery strain was detailed in order to illustrate a skewed contribution of broodstock parents to the next generation, which may possibly cause a reduced effective population size, by using microsatellite profiling technique. The pedigrees of offspring including 113 individuals of larvae sampled within 24 hours after hatching, 216 individuals of one-month-old, and 407 individuals of four-month-old were unambiguously identified. The contribution of candidate broodstock parents (18 individuals) to the next generation was highly skewed as one male of the six males used monopolized the contribution to the offspring pool, and a half of the females (6 of 12 females) did not produce any offspring. The contribution of one family to the released fish, which were culled for larger size in total length, was significantly higher, while those of other two families were lower, suggesting that culling operations for particular traits might cause a large family size variance of released fish through selection in favor of a few family lineages. Culling operations for particular traits thus should be avoided as much as possible to minimize the risk of unexpected pedigree structure to be a possible cause of the reduced effective size.

Possible way to retard loss of genetic variations in closed and non-pedigreed captive populations of Japanese flounder was explored by using a microsatellite-based kinship estimator (r_{xy} statistic). The difference of pairwise r_{xy} values among and between full-sib, half-sib, and unrelated categories in a hatchery strain was highly significant, suggesting that the r_{xy} statistic would have a high utility to discriminate kinships. Minimal kinship (*mk*) selection approach based on the r_{xy} statistic, which is a breeding strategy to minimize loss of genetic variability in captive populations, turned out to be effective in retention of both allelic diversity and gene diversity; this strategy however did not necessarily select unrelated fish as parents to create the next generation. It should thus be necessary to preclude the mating between highly related individuals in the selected fish by using the r_{xy} statistic as a kinship indicator so as to minimize the risk of inbreeding.

From the results presented above, the present author discusses here several hatchery management options for further practical hatchery operations to minimize the genetic impact of stocking practice to the wild Japanese flounder populations.

Key words: Japanese flounder, Genetic markers, Population genetics, Conservation genetics, Stock enhancement

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Contents

Chapter 1. General introduction

The Japanese flounder *Paralichthys olivaceus* is an important component of coastal fishery resources in Japan, and is heavily exploited due to its highly commercial significance. Market landings of this species have not been greatly increased over the last decade (Anon, 2001) in spite of extensive fishery management effort such as regulations to counteract over-fishing, and thus a major interest has been directed toward the exploitable resource enhancement by means of stocking of hatchery-reared fish into the natural environment. The hatchery production of Japanese flounder was successfully developed in 1965, and stocking practices of flounder juveniles were embarked some 30 years ago (Furusawa, 1997). With the increase of flounder seedlings production, this species has become the most important target in the stock enhancement programs running in Japan (Anon, 2000); however the extent of stocking effects on the biomass and the ecological, biological, and genetic impacts of stocked fish to the wild populations have not been clearly ascertained. The hatchery fish are usually reared in a protected environment and thus exposed to different constraints from those experienced in the natural environment, which may possibly affect to the survival and growth of hatchery-produced fish. The qualitative disadvantages of hatchery-based fish in several traits have been a growing concern: abnormalities in pigmentation (reviewed in Seikai, 1997) and in ossification (Hosoya and Kawamura, 1996) have been observed frequently in hatchery fish, and the differences of feeding behavior between hatchery and wild fish were reported (Furuta *et al.*, 1997; Furuta, 1998a; Furuta, 1998b). These findings indicate that further massive stocking of hatchery-based fish into the natural environment may have the potential to cause harmful effects upon the wild fish populations, possibly resulting in the reduction of the resource mass.

The potential genetic impact of stocking practices to the wild fish populations also has been a focus of attention. This is because most hatchery strains typically show reduced genetic variations, which may possibly result in the loss of disease resistance, and in the reduction of the population's ability to adapt to a changing environment (Ryman and Stahl, 1980; Allendolf and Phelps, 1980; Allendorf and Ryman, 1987). A large scale and consistent stocking of hatchery-based fish will increase a short-term exploitation of resources; but, at the same time, this may however have potential detrimental genetic impacts on the wild populations (Allendorf and Ryman, 1987), as the reduction of genetic variability in salmonid fishes has been shown to cause harmful effects upon several traits such as mortality and growth rate (*e.g.*, Gjerde *et al.*, 1983; Kincaid, 1983; Su *et al.*, 1996). It is very likely that the loss of genetic variations in hatchery fish would be due to the limited number of effective parents or the occurrence of inbreeding events when the hatchery strains were founded. Therefore, hatchery operations should be made advertently, and a central tenet of a stock enhancement program is thus the notion that the genetic condition of the hatchery strains should be maintained, ideally, the same as that of wild populations (FAO, 1993). A population genetics approach can make fundamental contributions to achieve this purpose. There appears to be two main areas in which population genetics studies should meet the current stock enhancement concept. One is concerned with the assessment of genetic diversity and structure in the wild fish populations that should be conserved, and another is the genetic monitoring of hatchery-based fish in order to further improve the hatchery management, by which any harmful genetic impacts of stocking practice to the wild

populations can be minimized. As regards to population genetics studies of Japanese flounder, initial attempts were made by using allozyme loci as a molecular marker (Fujio *et al.,* 1985; 1989), although allozyme loci failed to detect any significant genetic structure among wild fish populations, and were insufficient to precisely evaluate the extent of the genetic variability maintained in hatchery strains due to the limited number of polymorphic loci available and the low level of polymorphisms. Researchers have therefore explored other classes of molecular markers that are suitable for Japanese flounder population genetics studies.

Microsatellite DNA loci are sequences made up of a single sequence motif such as $(CA)_n$ and $(CT)_n$, which are distributed ubiquitously throughout eukaryotic genomes (Tautz and Renz, 1984; Tautz, 1989). Microsatellites are expected to serve in a highly informative manner as a moleculer marker with the practical use in the wide range of population genetics studies (Goldstein and Schlotterer, 1999 and references therein), and as well in regard to the assessment of genetic structure as reported extensively in several fish species (*e. g.*, Rico *et al.*, 1997; Garcia de Leon *et al.*, 1997; O'Connell *et al.,* 1998; Bagley *et al.*, 1999; Chapman *et al.*, 1999; Perez-Enriquez and Taniguchi, 1999; Ruzzante *et al.*, 2000; Sekino *et al.*, 2001), genetic linkage mapping in tilapia (Kocher *et al.*, 1998), rainbow trout (Sakamoto *et al.*, 2000), Medaka (Naruse *et al.*, 2000), and Zebrafish (Shimoda *et al.*, 1999), and quantitive trait loci identification in rainbow trout (Jackson *et al.*, 1998; Sakamoto *et al.*, 1999; Danzmann *et al.*, 1999; Ozaki *et al.*, 2001). Microsatellites inhere a high amount of allelic variations with several advantages beyond other classes of molecular markers: microsatellites are amplified based on polymerase chain reaction (PCR; Saiki *et al.*, 1985) for which minute quantities and poor qualities of tissue can be used, and microsatellite analysis is comparatively easy to automate with a high throughput possible. While, the mitochondrial DNA genome (mtDNA) is predominantly inherited through a single maternal lineage with no recombination (Avise *et al.*, 1987). Theoretically, the effective population size of mtDNA is approximately one-quarter of nuclear DNA, and thus mtDNA genome seems to be susceptible to stochastic events (Carvalho and Hauser, 1994) such as population bottlenecks. The use of mtDNA as a genetic tool is therefore expected to be highly efficient to understand the genetic condition of a bottlenecked population (Kijima and Matsunami, 1992; Iguchi *et al.*, 1999). Low levels of mtDNA variability have been reported in many marine fish species (Ovenden, 1990), however, several marine fish species maintain a high amount of nucleotide variations in the mtDNA control region (*e. g.*, sword fish, Alvarado Bremer *et al.*, 1995; red sea bream, Tabata and Taniguchi, 2000). Fujii and Nishida (1997) found that the control region of Japanese flounder is also highly variable, documenting the broad applications possible to population genetics studies.

Both microsatellites and mtDNA control region offer several practical advantages in the Japanese flounder population genetics studies related to the stock enhancement. The powerful DNA techniques are expected to throw a new light on the challenging subjects such as the assessment of the genetic structure among wild fish populations or the evaluation of genetic diversity within and between hatchery strains and wild populations. The present author considers that these approaches could provide more insight into further improvement of the currently operated hatchery management.

This thesis aims to propound a view for future hatchery operations for Japanese flounder. This thesis consists of seven chapters including this introductory chapter. Chapter 2 describes the isolation and characterization of Japanese flounder microsatellites with special emphasis on an efficient cloning method of microsatellites, and the variability and inheritability of the isolated microsatellites are also examined. Chapter 3 examines the genetic diversity and structure among wild fish populations. Chapter 4 surveys several hatchery

strains evaluating the genetic diversity. Chapter 5 presents an example of parentage analysis in a hatchery strain that were created through a practical hatchery operation. Chapter 6 explores a possible way to maintain the genetic variations of captive populations in the lack of pedigree information. Chapter 7 proposes several hatchery management options to minimize the loss of genetic variations in hatchery strains, and future prospects of genetic studies in relation to Japanese flounder stock enhancement are also discussed.

Chapter 2. Isolation and characterization of Japanese flounder microsatellite DNA loci

A conventional approach to isolate microsatellite sequences has generally been used (reviewed in O'Reilly and Wright, 1995): a small-insert (300-500 base pairs) partial genomic library is constructed, and the library is screened with a repeat unit probe such as a $(CA)_n$ probe. The frequency of microsatellites in this "conventional" library is however usually low, and is expected to be of 1 per 100-400 colonies (Ostrander *et al.*, 1992), although the frequency of microsatellites varies depending on the species. The conventional method thus requires much labor, and is time consuming since a large number of colony plates need to be screened to isolate a significant number of microsatellites. This is of special concern in species with a low frequency of microsatellites in their entire genome (Takahashi *et al.*, 1996). Therefore, a method to increase the efficiency for cloning species-specific microsatellites has been required. Ostrander *et al.* (1992) proposed a method for the construction of canine genomic libraries in which microsatellites were highly enriched, and Takahashi *et al.* (1996) also developed an efficient method to isolate microsatellites from the chicken genome, of which the expected frequency of microsatellites is tenfold less than those in mammalian genomes (Crooijmans *et al.*, 1993). In this study, the method devised by Takahashi *et al.* (1996) was applied to the Japanese flounder with modifications.

Microsatellites are theoretically inherited according to Mendelian transmission, and thus the mode of microsatellite allele segregation has not been a major issue. At the same time, microsatellite analysis is conducted through the PCR amplification procedures; the misbinding of primers to complementary DNA sequences may result in scoring errors of alleles, commonly known as the presence of null alleles. This causes an apparent incompatibility of genotypes within a family when a simple Mendelian transmission of microsatellite alleles is assumed (Callen *et al.*, 1993; Ede and Crawford, 1995), or inconformity of observed genotype frequencies to Hardy-Weinberg's expectations with significant depression of observed heterozygosity from the expected one in a Mendelian population (Pemberton *et al.*, 1995). The inheritability of microsatellite loci in experimental family lineages should be thus studied prior to its application to population genetics studies.

In this chapter, the present author details the isolation technique of Japanese flounder microsatellites from the genome, and attempts were made to characterize the isolated microsatellites in terms of the variability and inheritability in order to qualify them as a valuable molecular tool.

Materials and methods

Cloning of microsatellites

Samples of muscle tissue taken from Japanese flounder were digested with Proteinase K in 4 M TNES-Urea buffer (Asahida *et al.*, 1996), and genomic DNA was purified by phenol/chloroform extraction with ethanol precipitation (Sambrook *et al.*, 1989). Genomic DNA was fragmented by sonication, and resultant fragmented DNA was blunted by mung bean nuclease (Takara, Shiga, Japan). Fragments were electrophoresed in a 1.2% agarose gel, and the fragments ranging from 300 to 500 bp were recovered. Both ends of the recovered fagments were repaired by T4 DNA polymerase (Toyobo,

Osaka, Japan) and T4 polynucleotide kinase (Toyobo). The fragments were ligated to *Srf* I site of pCR-Script Amp SK(+) phagemid vector (Stratagene, La Jolla, CA, USA), and then the recombinant phagemids were transformed into XL2-Blue MRF' competent cells (Stratagene). An equivalent to 10^{10} pfu of helper phage VCSM13 (Stratagene) was added, and the cultivation was continued overnight. After that, the phagemids were precipitated according to a standard PEG/NaCl precipitation procedure (Sambrook *et al.*, 1989). Contaminating *Escherichia coli* DNA and RNA were digested with DNase I (Takara) and RNaseA (Nacalai Tesque, Kyoto, Japan). The phagemids were recovered with PEG/NaCl precipitation, and single-stranded DNA was purified with phenol/chloroform extraction and ethanol precipitation. Selective second strand DNA synthesis *in vitro* with $(CA)_{12}$ oligonucleotide primer was carried out according to Takahashi *et al.* (1996). Single-stranded DNA remaining in the mixture was digested with mung bean nuclease (Takara). Resultant double-stranded DNA was transformed into XL2-Blue MRF' competent cells again, and the resulting transformants were referred to as a $(CA)_n$-enriched library.

From the $(CA)_n$-enriched library, individual clones were randomly picked up, and phagemid DNAs were purified using GFX *Micro* Plasmid prep kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The DNA sequences were determined in both directions using Thermo Sequenase™ cycle sequencing kit (Amersham Pharmacia Biotech) in combination with the ALFexpress automated DNA sequencer (Amersham Pharmacia Biotech).

Microsatellite variability and inheritability

Primer pairs flanking each repeat array was developed using the Primer Premier software package (Premier Biosoft International, Palo Alto, CA, USA). Microsatellite polymorphisms were screened in a wild Japanese flounder population collected from the Japan Sea off Tottori Prefecture (69 individuals). Polymerase chain reaction (PCR) amplification was carried out in a 20 μL reaction volume, which included 20 pmols of each primer set (one primer in each of pair was 5' end-labelled with Cy5 amidite, Amersham Pharmacia Biotech), 100 μM each dNTP, 10 mM Tris-HCl (pH8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.5 units of DNA polymerase (AmpliTaq Gold™, Applied Biosystems, Foster City, CA, USA), and approximately 50ng of template DNA using PC-960G gradient thermal cycler (Corbett Research, Mortlake, NSW, Australia). PCR amplification cycles were as follows: 12 min at 95℃, 35-40 cycles of 30 sec at 94℃, 1 min at a primer-specific temperature, 1 min at 72℃, and final elongation for 5 min at 72℃. The specific annealing temperature of each primer set is given in Table 2. Following PCR amplification, the reaction mixture was mixed with an equal volume of loading dye (de-ionized formamide containing 0.5% blue dextran), and heated for 5-10 min at 94℃ prior to electrophoresis. Microsatellite polymorphisms were detected in polyacrylamide sequencing gels (ReproGel™ High Resolution, Amersham Pharmacia Biotech) using the ALFexpress DNA sequencer (Amersham Pharmacia Biotech). Alleles were designated according to PCR product size relative to a molecular size marker (ALFexpress Sizer™ 50-500, Amersham Pharmacia Biotech) in combination with an ALLELELINKS version 1.00 software (Amersham Pharmacia Biotech).

Allele frequencies and unbiased expected heterozygosity (H_e; Nei, 1987) at each locus were calculated using an ARLEQUIN version 1.1 software package (Schneider *et al.*, 1997). The observed heterozygosity (H_o) was calculated directly from the observed genotypes. Hardy-Weinberg equilibrium (HWE) for each locus was examined by a test analogous to the Fisher's exact test with the Markov chain procedures (Markov-chain steps, 100,000; dememorization, 10,000) by using the ARLEQUIN program.

Inheritability of the microsatellites isolated was examined using five experimental families of Japanese flounder. From five sets of simple 1×1 crosses using three ripe females (FM1,

FM2, and FM3) and four ripe males (M1, M2, M3, and M4), approximately 300 full-sibs were successfully obtained from each cross (family A: FM1×M1, family B: FM2×M1, family C: FM3×M2, family D: FM3×M3, and family E: FM3×M4). Accordingly, the members of family A and B were paternal half-sibs, and family C, D, and E consisted of maternal half-sibs. The inheritance mode of microsatellite alleles was studied in up to 30 F_1 offspring in each family. In order to determine if alleles were inherited according to simple Mendelian fashion, the conformity of the offspring genotypes to the expectations was tested using the chi-square methods (Snedecor and Cochran, 1967).

Results

Of the 80 clones randomly selected from the $(CA)_n$-enriched library, 59 clones contained one or more repeat arrays, of which 25 clones were discarded due to the unreproducible sequencing results flanking the repeat regions. Four of the remaining 34 clones were over-represented with the other clones, and consequently 33 repeat arrays were isolated from the remaining 30 clones (Table 1), of which 27 repeat arrays were available for the development of PCR primers (Table 2) and six repeat regions did not provide sufficient sequence information to develop any primer pairs.

All the 27 primer pairs were available for PCR amplification, out of which 16 primer pairs were used for further assessment of microsatellite polymorphisms in a wild Japanese flounder population. The remaining 11 primer pairs were discarded since they produced unexpected PCR products in an initial sample of Japanese flounder. As shown in Table 3, the genetic variabilities of the 16 microsatellites varied depending on the locus. The number of alleles ranged from 4-40, and the H_o and H_e values ranged from 0.43 to 0.99, and from 0.46 to 0.97, respectively. All but one of the 16 loci conformed to HWE after correction of significance levels for 16 simultaneous tests (Rice, 1989) ($p>0.003$, initial K of sequential Bonferroni correction, $K=16$); at the *Po31* locus, the observed genotype frequency showed a significant departure from HWE ($p<0.003$) with a large discrepancy between the H_o and H_e values (0.34 and 0.91, respectively).

The Mendelian inheritability of each locus was assessed by using five experimental fami-

Table 1. Core repeat sequences isolated from 30 clones randomly selected from the $(CA)_n$-enriched library

Clone No.	Repeat motif (5'-3')	Clone No.	Repeat motif (5'-3')
1	$(TG)_3T_2(TG)_8$	16	$(CACG)_4(CA)_5$
	$(CA)_2GT(AC)_{14}$	17	$(CA)_2CG(CA)_6GA(CA)_5$
2	$(TC)_7TG(TC)_9$	18	$(CAA)_2T(AC)_6(AT)_2$
	$(CA)_5A(AC)_4$	19	$(AC)_{20}$
3	$(CA)_{12}(CGCA)_2(CA)_5CG(CA)_6$	20	$(CA)_{11}(GA)_2GC(GA)_9$
4	$(TG)_3G(AC)_{13}$	21	$(CA)_4$
5	$(GAA)_3(CA)_5$	22	$(TG)_7C(GT)_5\{GC(GT)_5\}_2(CGTG)_2(TG)_4$
6	$(CACG)_4(CA)_4CG(CA)_{18}C(GT)_3$	23	$(CA)_4TA(CA)_2A_6(CA)_3$
7	$(TC)_7T2(TC)_3TA(AC)_{21}$	24	$(CA)_{48}$
8	$(AC)_4C(CA)_2G(AC)_4C(CA)_2G(AC)_5$	25	$(CA)_6T_2(CA)_{16}(CCCA)_2CA_3(CA)_2$
9	$(GATG)_2A_2CA(GATG)_{10}$	26	$(CA)_5AG(CG)_2(TG)_3(CG)_2(CA)_{15}$
	$(CA)_2GA(CA)_4GA(CA)_4$	27	$(CA)_3(AC)_2G(CA)_{26}$
10	$(CA)_6CGCACGGA(CA)_7$	28	$TA_3(CA)_7$
11	$(CA)_4(GA)_2(CA)_{11}$	29	$(CA)_{18}$
12	$(TG)_5T_2(TG)_{10}$	30	$(CACG)_2(CA)_6$
13	$(CA)_7$		
14	$(CA)_9$		
15	$(CA)_5(TA)_{13}(CA)_3$		

Table 2. Repeat motifs, PCR primer sequences, and annealing temperature of 27 Japanese flounder microsatellites

Locus	Repeat motif (5'-3')	Primer sequence (5'-3')	Annealing temperature*1 (℃)	Locus	Repeat motif (5'-3')	Primer sequence (5'-3')	Annealing temperature*1 (℃)
Po 1	$(TG)_3T_2(TG)_8$	F-GCCTTTTGTCAGCCATTAACAGAGC	55	***Po48***	$(CACG)_4(CA)_5$	F-GCCTCCAGAAACATTTATGGGG	55
		R-CTGAGGCCAGACATGACATTACCTT				R-TGTCTTGCCTCTGGTCCTTCTT	
Po7	$(CA)_5A(AC)_4$	F-AACAAACAAACCGGGCTGGCAGCG	56	***Po52***	$(CA)_2CG(CA)_6$	F-TCAGACAGAGGAGCGGGGTTGTTGC	58
		R-AGCCATGTGACGACAGACCCAGCATG			$GA(CA)_5$	R-GCTGTACCCAGGGTTCCGCTGAAGA	
Po9	$(CA)_{12}(CGCA)_2$	F-GCATCAGTGCGTGCAGCATGTTAGTA	58	***Po55***	$(CAA)_2T(AC)_6$	F-TCTTCTGATGCTGCCCAGGCTCCTT	58
	$(CA)_5CG(CA)_6$	R-CTGACAACAGCCGCTTTGAGCATCTA			$(AT)_2$	R-CGACCATGATCACCCCATGAGAACG	
Po13	$(TG)_3GA(CA)_{13}$	F-CGGCCTAAACCTGGACATCCTCTCTA	58	***Po56***	$(AC)_{20}$	F-TCGAGCGTAAACAAACCAGCTAACA	55
		R-CGGGACAACGGAGGTTTGACTGAC				R-GCTGAAAATCGCTTTAGCTTCCCAT	
Po15	$(GAA)_3(CA)_5$	F-TTCAGTACCTGTTCAACCTGATGT	52	***Po58***	$(CA)_{11}(GA)_2$	F-GCCCCTCACTGAGACTGTGACA	52
		R-CACAACTTAAGGTAACAACCTGCT			$GC(GA)_9$	R-CAAGGTATGTGCATGAGCAGGC	
Po20	$(CACG)_4(CA)_4C$	F-TGCTCCTTCACCTGCACGGCCTCAAA	58	***Po69***	$(TG)_7C(GT)_5\{GC(GT)_5\}_2$	F-TGGATTGTACCTTGACTCACCTGCT	53
	$G(CA)_{18}C(GT)_3$	R-TGCACCCTGACCTGTCACTGGGGATT			$(CGTG)_2(TG)_4$	R-GTGTTAATCATATGTGCCACCAGCT	
Po25A	$(GATG)_2A2$	F-TGAGGAGTCAGGTTTCAGGCCACT	55	***Po74***	$(CA)_4TA(CA)_2$	F-ACCTTCAGCAGCATGTCCACATAA	50
	$CA(GATG)_{10}$	R-TCGCAGGAACACCCAGAGTACAGA			$A_6(CA)_3$	R-CACTAGTATGCTTTGGCGGCAAAT	
Po25B	$(CA)_2GA(CA)_4$	F-AAGCTCTGTACTCTGGGTGTTCCTG	55	***Po78***	$(CA)_{48}$	F-GTAGACAACAAATCGGTTGTGCATG	56
	$GA(CA)_4$	R-ACCAACGTCACTATCACTCCTCTGC				R-CGATTGGGACATGAGTTGTTATGAG	
Po26	$(CA)_6CGCA$	F-ACACTGGGCCCTCTGTTAAACAC	55	***Po80***	$(CA)_6T_2(CA)_{16}$	F-TTCTGCATAATGAATGCAGTCCTC	54
	$CGGA(CA)_7$	R-AGAGGAGAAAGGGCACCGAGATA			$(CCCA)_2CA_3(CA)_2$	R-ACACGGCATGCAAATTACTACAGT	
Po31	$(CA)_4(GA)_2(CA)_{11}$	F-AGGGTTAATTATAGAGGACGCAG	57	***Po83***	$(CA)_5AG(CG)_2$	F-TGCGGTCATCATGTCTTTAAAATA	57
		R-CTGAAACAACAACTCAGAAGACG			$(TG)_3(CG)_2(CA)_{15}$	R-AGCAAATGTTTGCTTTTGGATACA	
Po33	$(TG)_5T_2(TG)_{10}$	F-GTTGGTTTAACTGATTCATCTGCAG	55	***Po89***	$TA_3(CA)_7$	F-ATCAGAAGTCATCCATGCACTGGCAC	60
		R-TTACATATCCCACAATGCTTCACTC				R-AGCTACTTATCCACAGGTGTCGACGG	
Po35	$(CA)_7$	F-TGGTTCTAGTGTTTGTCTGGTGA	54	***Po91***	$(CA)_{18}$	F-AGGTTTCAAGGTGTTCATTGCGAGTC	55
		R-CCTACAGCACAGATATGACCTTT				R-TAAAGGAAGTGCCTCACTGTGGAGAA	
Po37	$(CA)_9$	F-ATACGACAACATCTGTCTGCAACACC	53	***Po93***	$(CACG)_2(CA)_6$	F-ACCACATTGAACGAATGACAGCG	56
		R-GATAAACTTGCTGCTCCTTGTGGGTA				R-CTGGGACTTTCTTTGCATATCTCCTG	
Po42	$(CA)_5(TA)_{13}(CA)_3$	F-CGAGCGCTGTTTCAACTACGGTCATT	55				
		R-ATGATGATCTAACCGTCCGGCTCCAT					

lies of Japanese flounder. Of the 16 microsatellites screened, the *Po31* locus had unstable and unreproducible PCR amplification of a particular allele in one family. This locus was thus discarded, and the remaining 15 loci were used for further statistical analysis. Table 4 shows the genotype frequencies estimated for the offspring, and the parental allele segregating patterns among the offspring in the 55 possible combinations of the five families and the 15 loci. Possible scoring errors, as indicated at the *Po31* locus, were not common in the other loci, as all the expected genotypes and no unexpected ones were observed throughout all families. The genotype frequencies of offspring in each family were in accordance with the expectations for almost all of the 15 loci, excepting three instances (the *Po48* locus in family E, the *Po56* locus in family D, and the *Po58* locus in family B, $0.01 < p < 0.05$). These deviations, however, were not significant after the correction of significance levels on the basis of the sequential Bonferroni method (5 simultaneous test, initial $K=5$; significant value at the $\alpha=0.05$ was adjusted at $\alpha=0.01$; For $\alpha=0.01$, the critical χ^2 value is 9.21 for 2*df*, and 13.28 for 3*df*).

Discussion

Efficient method for cloning microsatellites

A schematic of the microsatellite enrichment procedures employed in this study is shown in Fig. 1. This method achieved a high efficiency in the enrichment of Japanese flounder microsatellites: approximately 75% of the clones (59/80 clones) derived from the $(CA)_n$-library contained one or more repeat arrays. Takahashi *et al.* (1996) reported that about a half of clones in the library they constructed were over-represented, and these authors speculated that the results might be caused by a severe bias of DNA fragments due to the use of restriction enzymes at the DNA-fragmentation step. The use of sonicated DNA

Table 3. Variability of the 16 microsatellite loci estimated for a wild Japanese flounder population

Locus	Sample size	No. of alleles	Size range[*1] (bp)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)	p[*2]	GenBank accession no.
Po 1	69	26	162-234	0.87	0.82	0.63	AB046745
Po13	69	27	206-254	0.97	0.92	0.99	AB046746
Po20	69	40	239-379	0.99	0.97	1.00	AB046748
Po25A	69	12	201-253	0.77	0.76	0.31	AB046749
Po26	68	5	141-159	0.74	0.65	0.69	AB046750
Po31	69	25	129-193	0.43	0.91	0.00*	AB046751
Po33	69	10	257-290	0.72	0.68	0.61	AB046752
Po35	69	19	283-333	0.81	0.78	1.00	AB046753
Po42	69	23	164-224	0.88	0.91	0.67	AB046754
Po48	64	7	126-146	0.46	0.46	0.74	AB046755
Po52	69	4	155-163	0.46	0.50	0.61	AB046756
Po56	69	26	139-205	0.94	0.94	0.77	AB046757
Po58	69	27	101-159	0.84	0.90	0.52	AB046758
Po83	68	32	227-313	0.91	0.93	0.18	AB046759
Po89	69	20	252-327	0.86	0.90	0.44	AB046760
Po91	69	34	146-246	0.96	0.94	1.00	AB046761
Mean		21.1		0.79	0.81		

[*1] Size is indicated as the number of the base pairs of PCR products

[*2] p is the exact p-value estimated by a test anologous to Fisher's exact test described by Schneider *et al.* (1997). Significant departure of the observed genotype frequencies from HW-expectations for each locus was determined by adding $*=p<0.003$ with initial K of sequential Bonferroni correction (Rice, 1989), K=16.

Table 4. Inheritance characteristics of microsatellite alleles in 5 experimental families of Japanese flounder

Locus	Family	Parental genotypes	N[*1]	Observed number of offspring in each genotypic class[*2]				χ^2
Po1	**A**	♂162/180		162/162	162/172	162/180	172/180	
		♀162/172	30	11(7.5)	5(7.5)	7(7.5)	7(7.5)	2.53
	B	♂162/180		162/164	162/214	164/180	180/214	
		♀164/214	30	7(7.5)	5(7.5)	10(7.5)	8(7.5)	1.73
	C	♂162/162		162/162	162/214			
		♀162/214	30	16(15.0)	14(15.0)			0.13
	D	♂162/162		162/162	162/214			
		♀162/214	30	16(15.0)	14(15.0)			0.13
	E	♂162/162		162/162	162/214			
		♀162/214	30	15(15.0)	15(15.0)			0.00
Po13	**A**	♂216/230		216/216	216/230			
		♀216/216	30	14(15.0)	16(15.0)			0.13
	B	♂216/230		212/216	212/230	216/268	230/268	
		♀212/268	30	11(7.5)	8(7.5)	5(7.5)	6(7.5)	2.53
	C	♂214/228		214/214	214/216	214/228	216/228	
		♀214/216	30	5(7.5)	8(7.5)	12(7.5)	5(7.5)	4.40
	D	♂208/240		208/214	208/216	214/240	216/240	
		♀214/216	30	12(7.5)	3(7.5)	8(7.5)	7(7.5)	5.47
	E	♂214/246		214/214	214/216	214/246	216/246	
		♀214/216	30	8(7.5)	9(7.5)	8(7.5)	5(7.5)	1.20
Po20	**A**	♂253/255		253/259	255/259			
		♀259/259	30	11(15.0)	19(15.0)			2.13
	B	♂253/255		239/253	239/255	253/263	255/263	
		♀239/263	29	6(7.25)	11(7.25)	3(7.25)	9(7.25)	5.07
	C	♂237/307		237/263	237/293	263/307	293/307	
		♀263/293	30	9(7.5)	6(7.5)	10(7.5)	5(7.5)	2.27
	D	♂265/307		263/265	263/307	265/293	293/307	
		♀263/293	30	7(7.5)	11(7.5)	10(7.5)	5(7.5)	3.33
	E	♂263/271		263/263	263/271	263/293	271/293	
		♀263/293	30	6(7.5)	7(7.5)	9(7.5)	8(7.5)	0.67
Po25A	**A**	♂229/253		221/229	221/253	225/229	225/253	
		♀221/225	30	4(7.5)	5(7.5)	12(7.5)	9(7.5)	5.47
	B	♂229/253		221/229	221/253	229/229	229/253	
		♀221/229	30	7(7.5)	9(7.5)	10(7.5)	4(7.5)	2.80
	C	♂225/229		225/225	225/229			
		♀225/225	30	18(15.0)	12(15.0)			1.20
	D	♂225/229		225/225	225/229			
		♀225/225	30	19(15.0)	11(15.0)			2.13
	E	♂225/225		225/225				
		♀225/225	30	30(30.0)				—

Locus	Family	Parental genotypes	N	Observed number of offspring in each genotypic class				χ^2
Po26	**A**	♂141/159		141/155	155/159			
		♀155/155	30	18(15.0)	12(15.0)			1.20
	B	♂141/159		141/155	141/157	155/159	157/159	
		♀155/157	30	10(7.5)	5(7.5)	3(7.5)	12(7.5)	3.53
	C	♂141/155		141/141	141/155	155/155		
		♀141/155	30	10(7.5)	15(10.0)	5(7.5)		4.17
	D	♂155/155		141/155	155/155			
		♀141/155	30	19(15.0)	11(15.0)			2.13
	E	♂141/155		141/141	141/155	155/155		
		♀141/155	30	5(7.5)	20(15.0)	5(7.5)		3.34
Po33	**A**	♂263/263		263/263	263/286			
		♀263/286	30	14(15.0)	16(15.0)			0.13
	B	♂263/263		263/263	263/288			
		♀263/288	30	13(15.0)	17(15.0)			0.53
	C	♂263/265		263/263	263/265			
		♀263/263	30	12(15.0)	18(15.0)			1.20
	D	♂265/286		263/265	263/286			
		♀263/263	30	13(15.0)	17(15.0)			0.53
	E	♂263/265		263/263	263/265			
		♀263/263	30	17(15.0)	13(15.0)			0.53
Po35	**A**	♂285/317		285/285	285/317			
		♀285/285	30	17(15.0)	13(15.0)			0.53
	B	♂285/317		285/285	285/317	317/317		
		♀285/317	30	9(7.5)	14(15.0)	7(7.5)		0.40
	C	♂285/291		285/285	285/291			
		♀285/285	30	15(15.0)	15(15.0)			0.00
	D	♂285/317		285/285	285/317			
		♀285/285	30	15(15.0)	15(15.0)			0.00
	E	♂285/289		285/285	285/289			
		♀285/285	30	16(15.0)	14(15.0)			0.13
Po42	**A**	♂176/206		176/180	176/200	180/206	200/206	
		♀180/200	30	7(7.5)	10(7.5)	8(7.5)	5(7.5)	1.73
	B	♂176/206		172/176	172/206	176/180	180/206	
		♀172/180	30	4(7.5)	13(7.5)	7(7.5)	6(7.5)	6.00
	C	♂182/200		182/200	200/200			
		♀200/200	30	18(15.0)	12(15.0)			1.20
	D	♂198/198		198/200				
		♀200/200	30	30(30.0)				—
	E	♂176/178		176/200	178/200			
		♀200/200	30	16(15.0)	14(15.0)			0.13

[*1] N is the number of offspring analyzed

[*2] Mendelian expectations in each genotypic class are shown in parentheses. Significant deviation of the observed genotype frequencies from the expectations was determined by adding $*p<0.05$. The critical χ^2 value is: 7.82 for 3*df*, 5.99 for 2df, 3.84 for 1*df*. In 3 stances (the *Po52* locus in family A, the *Po42* locus in family D, and the *Po25A* locus in family E), chi-square analysis was not performed since both parents possessed homozygous genotypes.

Table 4. (continued)

Locus	Family	Parental genotypes	N*1	Observed number of offspring in each genotypic class*2				χ^2
Po48	**A**	♂126/130		126/126	126/130			
		♀126/126	30	16(15.0)	14(15.0)			0.13
	B	♂126/130		126/126	126/130			
		♀126/126	30	15(15.0)	15(15.0)			0.00
	C	♂126/126		126/126	126/130			
		♀126/130	30	15(15.0)	15(15.0)			0.00
	D	♂126/130		126/126	126/130	130/130		
		♀126/130	30	5(7.5)	16(15.0)	9(7.5)		1.20
	E	♂126/126		126/126	126/130			
		♀126/130	29	9(14.5)	20(14.5)			4.17*
Po52	**A**	♂155/155		155/155				
		♀155/155	30	30(30.0)				—
	B	♂155/155		155/155	155/161			
		♀155/161	30	15(15.0)	15(15.0)			0.00
	C	♂155/161		155/161	155/163	161/161	161/163	
		♀161/163	30	6(7.5)	6(7.5)	12(7.5)	6(7.5)	3.60
	D	♂161/161		161/161	161/163			
		♀161/163	30	16(15.0)	14(15.0)			0.13
	E	♂155/155		155/161	155/163			
		♀161/163	30	18(15.0)	12(15.0)			1.20
Po56	**A**	♂159/177		159/175	175/177			
		♀175/175	30	15(15.0)	15(15.0)			0.00
	B	♂159/177		159/163	159/173	163/177	173/177	
		♀163/173	30	4(7.5)	7(7.5)	7(7.5)	12(7.5)	4.40
	C	♂159/165		159/163	159/173	163/165	165/173	
		♀163/173	30	8(7.5)	5(7.5)	5(7.5)	12(7.5)	4.40
	D	♂161/165		161/163	161/173	163/165	165/173	
		♀163/173	30	4(7.5)	14(7.5)	6(7.5)	6(7.5)	7.87*
	E	♂171/177		163/171	163/177	171/173	173/177	
		♀163/173	30	4(7.5)	10(7.5)	8(7.5)	8(7.5)	2.53
Po58	**A**	♂115/117		111/115	111/117	115/153	117/153	
		♀111/153	28	9(7.0)	3(7.0)	7(7.0)	9(7.0)	3.43
	B	♂115/117		115/117	115/139	117/117	117/139	
		♀117/139	30	7(7.5)	15(7.5)	5(7.5)	3(7.5)	11.27*
	C	♂117/123		115/117	115/123	117/117	117/123	
		♀115/117	30	4(7.5)	9(7.5)	4(7.5)	13(7.5)	7.60
	D	♂111/147		111/115	111/117	115/147	117/147	
		♀115/117	30	6(7.5)	9(7.5)	10(7.5)	5(7.5)	2.27
	E	♂125/125		115/125	117/125			
		♀115/117	30	14(15.0)	16(15.0)			0.13

Locus	Family	Parental genotypes	N	Observed number of offspring in each genotypic class				χ^2
Po83	**A**	♂275/287		253/275	253/287	275/301	287/301	
		♀253/301	30	5(7.5)	7(7.5)	7(7.5)	11(7.5)	2.53
	B	♂275/287		271/275	271/287			
		♀271/271	30	15(15.0)	15(15.0)			0.00
	C	♂233/271		233/273	233/275	271/273	271/275	
		♀273/275	30	7(7.5)	5(7.5)	11(7.5)	7(7.5)	2.53
	D	♂261/271		261/273	261/275	271/273	271/275	
		♀273/275	30	7(7.5)	6(7.5)	13(7.5)	4(7.5)	6.00
	E	♂273/273		273/273	273/275			
		♀273/275	20	11(10.0)	9(10.0)			0.20
Po89	**A**	♂283/295		268/283	268/295	270/283	270/295	
		♀268/270	29	10(7.25)	6(7.25)	3(7.25)	10(7.25)	4.79
	B	♂283/295		274/283	274/295	279/283	279/295	
		♀274/279	30	9(7.5)	10(7.5)	3(7.5)	8(7.5)	3.87
	C	♂268/270		268/268	268/270	268/279	270/279	
		♀268/279	30	10(7.5)	8(7.5)	7(7.5)	5(7.5)	1.73
	D	♂268/270		268/268	268/270	268/279	270/279	
		♀268/279	30	8(7.5)	8(7.5)	8(7.5)	6(7.5)	0.40
	E	♂270/279		268/270	268/279	270/279	279/279	
		♀268/279	30	7(7.5)	8(7.5)	8(7.5)	7(7.5)	0.13
Po91	**A**	♂146/162		146/148	146/188	148/162	162/188	
		♀148/188	30	10(7.5)	7(7.5)	7(7.5)	6(7.5)	0.93
	B	♂146/162		146/158	146/160	158/162	160/162	
		♀158/160	29	7(7.25)	7(7.25)	8(7.25)	7(7.25)	0.10
	C	♂192/228		148/192	148/228	160/192	160/228	
		♀148/160	30	5(7.5)	6(7.5)	9(7.5)	10(7.5)	2.27
	D	♂158/192		148/158	148/192	158/160	160/192	
		♀148/160	29	9(7.25)	9(7.25)	6(7.25)	5(7.25)	1.76
	E	♂150/162		148/150	148/162	150/160	160/162	
		♀148/160	30	8(7.5)	7(7.5)	5(7.5)	10(7.5)	1.73

fragments is a possible improvement in this regard as approximately 90% of the clones sequenced were unique (30/34 clones). Likewise, Sekino and Hara (2001) reported that this enrichment procedure was also efficient to clone the Pacific abalone *Haliotis discus* microsatellites: they succeeded in the construction of overall 400-fold enriched library than that constructed based on the conventional method, and all the 39 clones for which they sequenced were unique.

Microsatellite variability and inheritability

The high level of genetic variability detected in the 16 microsatellites suggests that these loci provide greater amounts of genetic information compared with those of the allozymic loci reported (Fujio *et al.*, 1989): four wild populations of Japanese flounder screened for 23 enzyme loci revealed that the average number of alleles

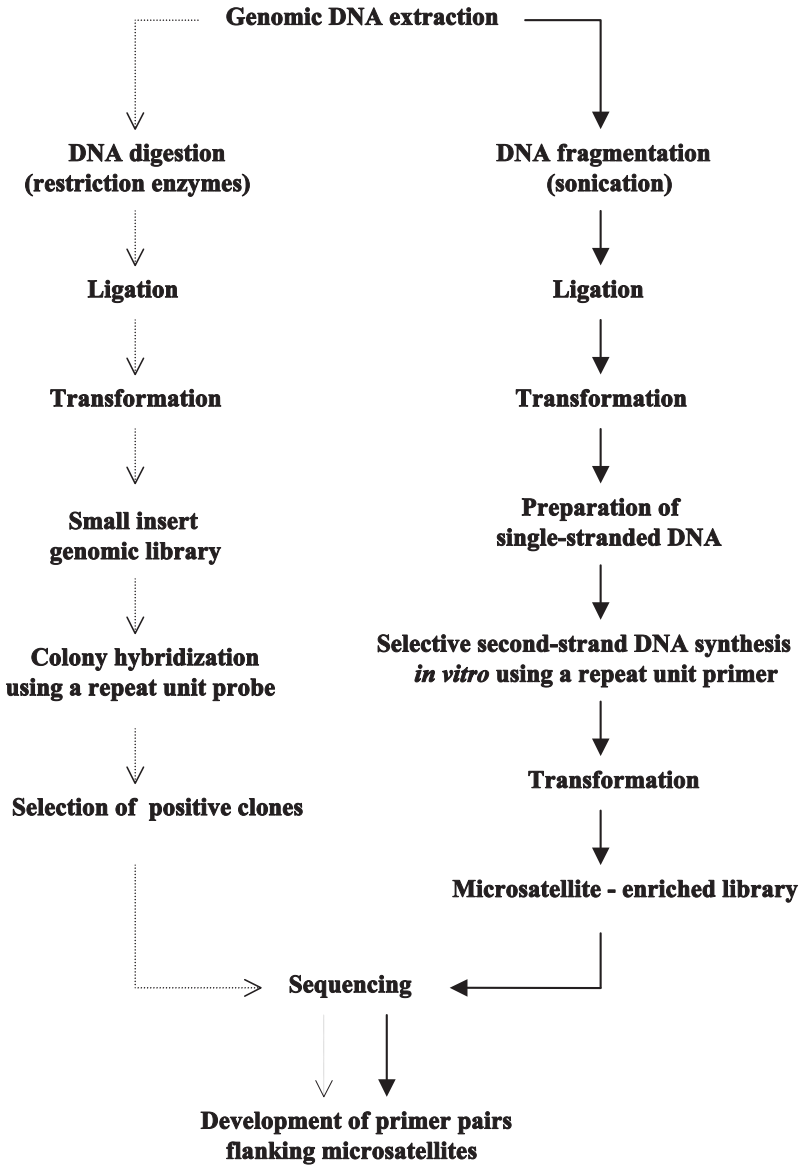

Fig. 1. A schema of the procedures for cloning microsatellites. Dotted lines indicate the conventional method described by Brooker *et al.* (1994), and thick lines indicate the enrichment procedures described by Takahashi *et al.* (1996) and Sekino *et al.* (2000).

per locus (A) and the average of the H_e value were 1.7 and 0.04, respectively. The A and mean H_e values estimated for the Japanese flounder microsatellites (A: 21.1, H_e: 0.81) were roughly similar to those reported in other marine fish microsatellites; as an average of 66 loci in 12 species surveyed, the A and H_e values were 19.9±6.6 and 0.77±0.19 (mean±s. d.), respectively (DeWoody and Avise, 2000). A significant departure from HWE was observed at the *Po31* locus with a large discrepancy between the H_o and H_e value (0.34 and 0.91, respectively). This may be explained by sampling errors due to the limited sample size or substructuring of the samples, however, these seem unlikely since the observed genotype frequencies for all the other loci were consistent with the HWE expectations. Given the fact that the Mendelian inheritability of this locus was not verified in a particular family, the presence of null alleles possibly caused by mutations occurred within the primer binding sites (Callen *et al.*, 1993; Ede and Crawford, 1995; Pemberton *et al.*, 1995) would be most likely to cause these results. Except for the *Po31* locus, it can be concluded that the remaining 15 loci were segregated according to Mendelian transmission.

Summary

This chapter can be summarized by the following two points. First, a highly microsatellite enriched library of Japanese flounder was constructed. Given that the efficient method was applicable to the Pacific abalone (Sekino and Hara, 2001) and the Japanese rockfish *Sebastes thompsoni* (Sekino *et al.*, 2000), it is expected to be generally applicable to other marine organisms. Secondly, the microsatellite markers developed in this study, which possess hyper-variability and Mendelian inheritability, proved to be useful in further applications to various genetic studies of Japanese flounder.

Chapter 3. Genetic diversity and structure of wild Japanese flounder populations

Long distance movements related to feeding or reproduction by adult Japanese flounder are thought to be relatively limited because of their sedentary behavior (reviewed in Minami, 1997), and thus the wide range of distributions seems largely to be related to the extent of egg and larval transport at the pelagic stages that last for as long as 25-50 days (Ochiai and Tanaka, 1986). While geographical variations in morphological and biological traits such as dorsal and anal ray counts of larvae (Kinoshita *et al.*, 2000), growth rate, and fecundity (reviewed in Minami, 1997) have been reported. Although these findings possibly indicate that this species possesses locally adapted populations, little is known about the population genetic structure. Attempts from genetic perspectives have been made to understand as much about the baseline genetic structure by using two classes of molecular markers, that is, allozyme analyses (Fujio *et al.*, 1985; Fujio *et al.*, 1989) and PCR-RFLP mitochondrial DNA (mtDNA) analysis (Asahida *et al.*, 1998): however any significant genetic heterogeneities between populations have not been conclusively demonstrated. These genetic studies indicate that Japanese flounder populations around Japan may have an overall genetic homogeneity. As discussed for other marine fish populations (reviewed in Carvalho and Hauser, 1994), the lack of significant population genetic heterogeneities may be due to an interpopulational homogenization of alleles (or haplotypes) resulting from temporally and/or spatially wide-ranging gene flow among population components, which may be related to the expansive nature of marine environments and the inherent role of the pelagic stages in their life cycles. Indeed, three major oceanic currents flowing around Japan seem to be effective to transport eggs and larvae over long distances. The Tsushima Warm Current flows northeastward from the Japanese coast side of the East China Sea through the Japan Sea, while the Kuroshio Warm Current flows northeastward from the southwestern part of the Japanese coast side of the Pacific Ocean. The Oyashio

Cold Current, which flows southward from the northern part of the Japanese coast side of the Pacific Ocean, encounters the Kuroshio Warm Current at the central part of the Japanese coast side of the Pacific Ocean. In the Japan Sea, it appears that pelagic larvae can be carried approximately 660km by the Tsushima Warm Current (Kinoshita *et al.*, 2000). It is therefore probable to consider that larval passive dispersions by efficient means of oceanic currents may act to homogenize genetic compositions of Japanese flounder around Japan, even if the extent of adult movements is limited. However, other possibilities should be taken into consideration in that the population homogeneities revealed in previous genetic studies may be only due to insufficient information content of the genetic markers employed. A finding of population heterogeneities by means of the sequencing analysis of mtDNA control region (Fujii and Nishida, 1997) seems to indicate that the other classes of highly polymorphic markers may provide a clearer measure of genetic structure in this species.

This chapter assesses whether the wild Japanese flounder populations around Japan should be considered as a single panmictic population or not, and an attempt is made to provide an insight into the genetic relationships among wild populations on a large oceanographic scale basis. Eleven of the 15 microsatellite markers described in the preceding chapter coupled with a section of mtDNA control region were used for these purposes.

Materials and methods

Fish samples

A total of 401 individuals of fish samples were collected at seven sites around Japan including three populations from the Japanese coast side of the Japan Sea in Hokkaido, Niigata, and Tottori Prefectures; three populations from the Japanese coast side of the Pacific Ocean in Iwate, Chiba, and Hyogo Prefectures; and one population from the Japanese coast side of the East China Sea in Nagasaki Prefecture. These fish samples were collected leaving out any individuals who showed hypermelanosis on the blind body side so as to minimize the possible contamination by released hatchery fish: almost all the hatchery-based flounder show blind-side pigmentation, and this provides an efficient indicator to discriminate wild fish from hatchery fish (Seikai, 1997). The localities where each population sample was collected, population abbreviations, dates of sampling, and numbers of individuals sampled are given in Table 5, and the geographical positions of the populations are plotted in Fig. 2.

Microsatellite analysis

PCR primers for the 11 microsatellites described in the preceding chapter (*Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48, Po52, Po56*, and *Po91*) were used to amplify each locus from each flounder DNA sample. PCR amplification for each locus was carried out in the same manner described in the preceding chapter.

Allele frequencies and gene diversity (unbiased expected heterozygosity: H_e) in each population at each locus were calculated using the ARLEQUIN version 1.1 software package (Schneider *et al.*, 1997). The observed heterozygosity (H_o) was calculated directly from observed genotypes. Effective number of alleles (a_e) was estimated based on the formula: $a_e = 1/\Sigma x_i^2$, where x_i is the frequency of the i th allele for each locus (Crow and Kimura, 1965). An estimate of the effective population size under the infinite allele model (IAM; Kimura and Crow, 1964) was calculated according to the formula: $N_e = (H_e/(1\text{-}H_e))/4\mu$, where μ represents the mutation rate per gamete and per generation at microsatellite loci (Crow and Kimura, 1970). In this study, the average microsatellite mutation rate was assumed as of 10^{-4} following Bagley *et al.* (1999) and Garcia de Leon *et al.* (1997) since microsatellite mutation rates are likely to be in the order of between 10^{-5} and 10^{-3} (Dallas, 1992; Weber and Wong, 1993; Ellegren, 1995), and since there have been

limited data available regarding the mutation rate for the 11 microsatellite loci.

Hardy-Weinberg equilibrium (HWE) for each population at each locus was addressed using a test analogous to the Fisher's exact test in the Markov chain procedures (Markov-chain steps, 100,000; dememorization, 10,000). A fixation index (F_{IS} : Weir and Cockerham, 1984) by which HWE departures within a population can be measured was also estimated evaluating the

Table 5. Sites employed in population analysis of wild Japanese flounder

Locality	Abbreviation	Date sampled	Sample size
Hokkaido	HKD	May, 2000	50
Niigata	NGT	Dec., 1998	50
Tottori	TTR	Nov., 1998	69
Nagasaki	NSK	Jul., 1999	71
Iwate	IWT	Sep., 1997	45
Chiba	CHB	Jan., 1999	72
Hyogo	HYG	Jan., 2000	44

For sampling localities see Fig. 2

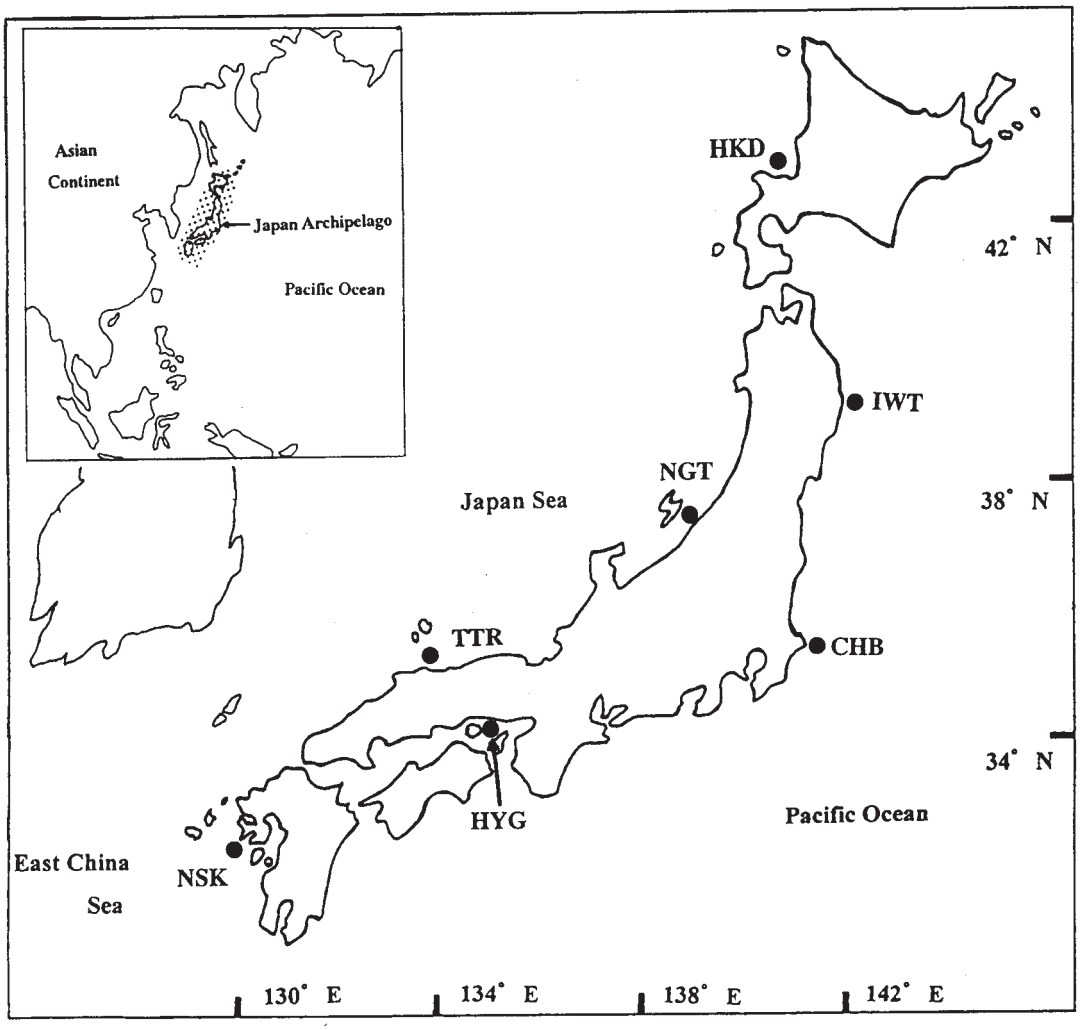

Fig. 2. A map showing sampling locations and abbreviated names for 7 Japanese flounder populations collected from the coastal sea around Japan

significance through random allelic permutation procedures (minimum 10,000 permutations). Genetic structure between populations was assessed by means of comparison of allele frequency distributions together with the tests of the significance of pairwise F_{ST} values (Reynolds *et al.*, 1983; Slatkin, 1995) for all possible population combinations. The probability associated with allele frequency homogeneity was evaluated by a test analogous to the Fisher's exact test using the Markov-chain method. The significance of pairwise F_{ST} values was evaluated through random allelic permutation procedures. These statistical analyses were conducted using the ARLEQUIN program. Significance values for all multiple tests were adjusted following the sequential Bonferroni procedures (Rice, 1989).

Genetic population relationships were estimated constructing neighbor-joining trees (NJ tree) according to the neighbor-joining method (Saitou and Nei, 1987) based on the modified Cavalli-Sforza chord distance (D_A ; Nei *et al.*, 1983) and Nei's standard genetic distance (D_{ST} ; Nei, 1972). The two distance measures were chosen to estimate population relationships since Takezaki and Nei (1996) revealed that D_A is one of the most efficient distance measures in tree topology reconstruction, whereas D_{ST} is more suitable than other distance measures in branch length estimation. The bootstrap values for each tree were calculated by 1,000 bootstrap re-sampling across loci. These procedures were performed on the NJBAFD program (provided by Dr. N. Takezaki, National Institute of Genetics, Mishima, Shizuoka, Japan), and the trees were visualized using the TREEVIEW program (Page, 1996).

A hierarchical Analysis of Molecular Variance (AMOVA: Excoffier *et al.*, 1992) was performed using the ARLEQUIN program to assess whether the groupings of populations estimated from the NJ tree topologies were supported by this approach or not. If the fixation index over all loci (Weir and Cockerham, 1984) among populations within a group (F_{SC}) significantly departed from zero, populations in the group were possibly considered to be subdivided further. Significance associated with the fixation index was evaluated thorough random allelic permutation procedures (minimum 10,000 permutations).

Mitochondrial DNA sequencing analysis

According to the complete nucleotide sequence of Japanese flounder mtDNA genome (Saitoh *et al.*, 2000; GenBank accession AB028664), one set of PCR primer pair was designed to amplify approximately 480 base pairs (bp) segment including the $tRNA^{Pro}$ gene and the left domain of the control region: two primers were placed in the $tRNA^{Thr}$ gene (forward primer: 5'-GTT AGA GCG CCA GTC TTG TA-3') and the middle of the control region (reverse primer: 5'-CCT GAA GTA GGA ACC AAA TGC-3'). PCR amplification was carried out in a 10 μL reaction mixture, which included 10 pmols of each primer, 100 μM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.2 units of DNA polymerase (ExTaq™, Takara, Shiga, Japan), and approximately 50 ng of template DNA. PCR cycles were as follows: 3 min at 95℃, 30 cycles of 15 sec at 95℃, 30 sec at 57℃, 30 sec at 72℃, and final elongation for 5 min at 72℃. Following the PCR amplification, any unconsumed dNTPs and primers were digested with exonuclease I together with shrimp alkaline phosphatase (ExoSAP-IT kit, USB, Cleveland, Ohio). Sequencing analysis for the PCR amplification products was performed using the ABI 373A stretch DNA sequencer (Applied Biosystems) in combination with a Thermo Sequenase™ II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech). Sequences were determined from both directions.

Sequence alignment of mtDNA sequence data was performed by hand using the sequence editor DNASIS software package (Hitach, Tokyo, Japan). The number of variable sites, haplotype frequency distributions, haplotype diversity (h: Nei and Tajima, 1981), and nucleotide diversity (π : Nei, 1987) were calculated using the ARLEQUIN program. This software program

was also used to estimate pairwise Φ_{ST} distance, which is an analogous measure of the F_{ST} distance (Weir and Cockerham, 1984), to measure the level of population differentiation. Probability associated with the Φ_{ST} values was evaluated through random permutation procedures (minimum 10,000 permutations). A hierarchical AMOVA test was performed using the ARLEQUIN program to assess whether the grouping of populations estimated from the microsatellite data was supported by the mtDNA sequence data.

Results

Microsatellite Variability

Table 6 summarizes the genetic variability estimated for each population at each locus (allele frequency distributions for all populations and loci, see Appendix 1). One individual derived from the IWT population showed very weak PCR amplifications for all loci, and thus this individual was excluded from the data. All loci showed polymorphisms in all populations: the allelic diversity (numbers of alleles per locus: A) in the seven populations ranged from 15.2 to 18.2, while the effective number of alleles ranged from 7.2 to 9.2, and the average of the observed heterozygosity (H_o) and gene diversity (H_e) ranged from 0.74 to 0.78, and from 0.74 to 0.76, respectively. There did not appear to be large differences in the genetic variabilities among the seven populations. Four loci, *Po13, Po42, Po56*, and *Po91* harbored a very high level of allelic variation: 20 or more alleles were detected, and the H_e values were more than 0.90 in all populations. There was little discrepancy between the H_o and H_e values in each population at each locus excepting the *Po42* locus in the IWT population (H_o: 0.80 and H_e: 0.91), and consequently the H_o/H_e values on average ranged from 1.00 to 1.03 across the seven populations. Just one instance showed a significant HWE departure (at the *Po42* locus in the IWT population) after correction of significance levels for 11 simultaneous tests ($p<0.005$). The F_{IS} value estimated for the IWT population at the *Po42* locus was also significantly different from zero ($p<0.005$). The estimates of the effective population size (N_e) ranged from 7,100-7,900 with an average of 7,600 among the seven populations (Table 6).

Mitochondrial DNA sequence variability

Approximately 443 bp sequences containing the $tRNA^{Pro}$ gene (71 bp) and the left domain of the control region of mtDNA for a total of 399 individuals were unambiguously determined. It was unable to determine the sequences of one individual from each of the CHB and NSK populations due probably to the poor quality of the template DNA. As shown in Fig. 3 and Table 7, the mtDNA control region of Japanese flounder was highly variable (see also Fujii and Nishida, 1997), and all but three of the variable sites were contained in the control region. There were 150 variable sites comprised of 170 base-substitutions (138 transitions and 32 transversions) with seven indels (single base pair insertion/deletion). At one sequence site (sequence No. 139), almost all the variations detected were transversions. Eleven variable sites (sequence No. 139, 153, 183, 219, 220, 225, 259, 327, 385, 398, and 430) showed especially high levels of polymorphisms. Table 8 summarizes the sequence and haplotype variabilities estimated for each of the seven populations. A total of 287 haplotypes were identified among 399 individuals. The number of observed haplotypes in the seven populations varied from 39 to 65, and more than 50 % of the haplotypes identified in each population were unique. It appears that the number of haplotypes commonly shared between populations and the geographical locations of populations were not correlative (Table 10); for example, the CHB and IWT populations located on the Pacific Ocean commonly shared nine and 10 haplotypes with the TTR population located on the Japan Sea, respectively, while the CHB and IWT population had just three common haplotypes in spite of their geographically proximal locations. The estimates of nucleotide diversity (π) ranged from 0.027 to 0.032, and the difference of the

Table 6. Number of alleles (A), effective number of alleles[*1] (a_e), observed heterozygosity (H_o), expected heterozygosity (H_o), F_{IS} values[*2] and effective population size[*3] (N_e) estimated for the 7 wild Japanese flounder populations on the basis of the 11 microsatellite loci

Locus		Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po1	N	50	50	69	71	44	72	44
	$A(a_e)$	24(7.7)	22(7.7)	26(5.6)	22(5.0)	22(6.3)	28(5.3)	22(5.3)
	H_o	0.86	0.88	0.87	0.76	0.86	0.81	0.82
	H_e	0.87	0.87	0.82	0.80	0.84	0.81	0.81
	p[*4]	0.35	0.24	0.63	0.11	0.48	0.37	0.91
	F_{IS}	0.006(0.35)	-0.009(0.46)	-0.064(0.96)	0.048(0.18)	-0.035(0.65)	0.016(0.42)	-0.014(0.68)
Po13	N	50	50	69	71	44	72	44
	$A(a_e)$	25(16.7)	23(12.5)	27(12.5)	26(16.7)	25(16.7)	26(16.7)	21(11.1)
	H_o	0.90	0.84	0.97	0.92	0.91	0.97	0.93
	H_e	0.94	0.92	0.92	0.94	0.94	0.94	0.91
	p	0.07	0.09	0.99	0.52	0.80	0.09	0.95
	F_{IS}	0.041(0.19)	0.088(0.02)	-0.053(0.98)	0.023(0.28)	0.035(0.15)	-0.038(0.95)	-0.022(0.57)
Po25A	N	50	49	69	71	44	71	44
	$A(a_e)$	11(5.9)	13(5.6)	12(4.2)	13(5.6)	12(5.0)	15(5.6)	13(6.7)
	H_o	0.74	0.88	0.77	0.86	0.77	0.79	0.93
	H_e	0.83	0.82	0.76	0.82	0.80	0.82	0.85
	p	0.05	0.57	0.31	0.86	0.61	0.36	0.75
	F_{IS}	0.113(0.03)	-0.078(0.93)	-0.012(0.63)	-0.031(0.76)	0.038(0.35)	0.037(0.19)	-0.068(0.81)
Po26	N	50	50	68	71	44	72	43
	$A(a_e)$	6(2.6)	4(2.2)	5(2.9)	5(2.8)	5(3.0)	5(3.0)	5(2.6)
	H_o	0.68	0.52	0.74	0.70	0.77	0.67	0.70
	H_e	0.62	0.55	0.65	0.64	0.67	0.67	0.62
	p	0.90	0.64	0.69	0.95	0.16	0.74	0.15
	F_{IS}	-0.092(0.86)	0.057(0.23)	-0.131(0.96)	-0.079(0.87)	-0.151(0.96)	0.009(0.51)	-0.057(0.76)
Po33	N	50	49	69	71	44	72	44
	$A(a_e)$	7(2.5)	7(2.6)	10(3.1)	11(3.1)	9(2.8)	9(2.7)	8(2.3)
	H_o	0.68	0.59	0.72	0.76	0.70	0.58	0.48
	H_e	0.60	0.61	0.68	0.68	0.64	0.63	0.57
	p	0.47	0.20	0.61	0.36	0.97	0.02	0.39
	F_{IS}	-0.141(0.97)	0.068(0.26)	-0.065(0.89)	-0.098(0.95)	-0.109(0.92)	0.076(0.17)	0.165(0.11)
Po35	N	50	50	69	71	44	71	44
	$A(a_e)$	17(5.0)	18(5.0)	19(4.5)	19(5.0)	16(5.3)	18(5.3)	14(4.2)
	H_o	0.84	0.76	0.81	0.80	0.82	0.81	0.70
	H_e	0.80	0.80	0.78	0.80	0.81	0.81	0.76
	p	0.45	0.08	1.00	0.72	0.97	0.14	0.08
	F_{IS}	-0.051(0.73)	0.056(0.21)	-0.046(0.86)	-0.002(0.57)	-0.005(0.61)	-0.007(0.62)	0.071(0.20)

Locus		Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po42	N	50	50	69	71	44	71	44
	$A(a_e)$	23(10.0)	27(11.1)	23(11.1)	24(11.1)	25(16.7)	28(14.3)	22(10.0)
	H_o	0.96	0.94	0.88	0.90	0.80	0.93	0.95
	H_e	0.90	0.91	0.91	0.91	0.94	0.93	0.90
	p	0.87	0.58	0.64	0.50	0.000*	0.33	0.33
	F_{IS}	-0.067(0.98)	-0.031(0.68)	0.025(0.18)	0.010(0.45)	0.159*(0.001)	0.000(0.57)	-0.056(0.95)
Po48	N	50	50	64	71	44	72	44
	$A(a_e)$	6(2.0)	7(1.7)	7(1.9)	8(1.7)	6(1.7)	9(1.9)	6(2.0)
	H_o	0.54	0.42	0.46	0.46	0.39	0.47	0.43
	H_e	0.50	0.42	0.46	0.42	0.41	0.48	0.51
	p	0.49	0.87	0.74	0.22	0.89	0.34	0.16
	F_{IS}	-0.072(0.66)	0.006(0.55)	-0.006(0.58)	-0.110(0.92)	0.063(0.37)	0.023(0.45)	0.156(0.12)
Po52	N	50	50	69	70	44	72	44
	$A(a_e)$	4(1.5)	4(1.7)	4(2.0)	4(2.0)	6(1.6)	4(1.5)	4(1.9)
	H_o	0.34	0.38	0.46	0.51	0.41	0.33	0.41
	H_e	0.34	0.40	0.50	0.51	0.39	0.32	0.46
	p	0.88	0.13	0.61	0.26	0.06	0.66	0.07
	F_{IS}	-0.051(0.54)	0.040(0.26)	0.076(0.25)	-0.018(0.63)	-0.063(0.80)	-0.054(0.81)	0.102(0.23)
Po56	N	50	50	69	71	44	71	44
	$A(a_e)$	22(16.7)	22(16.7)	26(16.7)	24(14.3)	28(25.0)	23(14.3)	22(16.7)
	H_o	0.96	0.94	0.94	0.86	1.00	0.93	0.95
	H_e	0.94	0.94	0.94	0.93	0.96	0.93	0.94
	p	0.49	0.11	0.77	0.29	1.00	0.95	0.16
	F_{IS}	-0.024(0.62)	-0.002(0.38)	0.002(0.54)	0.064(0.03)	-0.048(1.00)	0.000(0.57)	-0.021(0.79)
Po91	N	50	50	69	71	44	72	44
	$A(a_e)$	23(14.3)	26(12.5)	34(16.7)	30(20.0)	28(16.7)	35(20.0)	30(20.0)
	H_o	0.94	0.94	0.96	0.97	0.98	0.96	1.00
	H_e	0.93	0.92	0.94	0.95	0.95	0.95	0.95
	p	0.98	0.67	1.00	0.46	0.98	1.00	0.41
	F_{IS}	-0.011(0.48)	-0.017(0.54)	-0.015(0.77)	-0.028(0.76)	-0.031(0.91)	-0.012(0.74)	-0.050(1.00)
Mean	$A(a_e)$	15.3(7.7)	15.7(7.2)	17.5(7.4)	16.9(7.9)	16.5(9.2)	18.2(8.2)	15.2(7.5)
	H_o	0.77	0.74	0.78	0.77	0.76	0.75	0.75
	H_e	0.75	0.74	0.76	0.76	0.76	0.75	0.75
	H_o/H_e	1.03	1.00	1.03	1.01	1.00	1.00	1.00
	Overall F_{IS}	-0.023(0.88)	0.009(0.29)	-0.028(0.95)	-0.012(0.78)	-0.008(0.62)	-0.001(0.55)	0.002(0.48)
	N_e	7.5×10^3	7.1×10^3	7.9×10^3	7.9×10^3	7.9×10^3	7.5×10^3	7.5×10^3

[*1] Calculated according to the fomula: $a_e=1/\Sigma x_i^2$ (Crow and Kimura, 1965)

[*2] Probability value associated with F_{IS} is shown in parentheshis. F_{IS} values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ with initial K of sequential Bonferroni correction (Rice,1989), $K=11$.

[*3] Calculated according to the formula: $N_e=(H_e/(1\text{-}H_e))/4\mu$, where μ represents the microsatellite mutation rate (Crow and Kimura, 1970). The mutation rate is approximated as $\mu=10^{-4}$.

[*4] p is the exact p-value estimated by a test analogous to the Fisher's exact test in the Markov-chain method. Significant HWE departures for each locus determined by adding *=$p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

π values between populations was not pronounced (Table 8). All populations showed very high levels of haplotype diversity (h) ranging from 0.994 to 0.998, and the regional differences of the h values were also not significant.

Population differentiation and relationships

Table 9 shows the results of the pairwise heterogeneity test based on the microsatellite data. Four of the 11 loci, *Po33, Po52, Po56*, and *Po91*, showed significant differences of allele frequency distributions for at least one population pair (11 simultaneous tests, $p<0.005$). None of the remaining seven loci (*Po1, Po13, Po25A, Po26, Po35, Po42*, and *Po48*) showed significant genetic heterogeneities for all population pairs. Pairwise F_{ST} values varied depending on the locus with a maximum 0.077, which was observed at the *Po52* locus for a population pair of the HKD and NSK population. Significance of the F_{ST} values was observed at five of 11 loci for at least one population pair (11 simultaneous tests, $p<0.005$). Expression of these results in another manner revealed that each population showed significant genetic heterogeneities between one or more populations for at least one locus, and 11 of the 21 pairwise population comparisons yielded significant genetic heterogeneities. The pairwise Φ_{ST} values estimated based on the mtDNA sequence data were however significant at just three population pairs (HKD-NGT, HKD-IWT, and HKD-HYG population pairs) (Table 10).

Genetic distances (D_A and D_{ST}) between populations estimated based on microsatellite allele frequencies are summarized in Table 11, and Fig. 4 shows two types of unrooted NJ-trees reconstructed based on the D_A and D_{ST} distance measures. According to the tree derived from the D_A distance (D_A-NJ tree), it was possible to consider that the CHB, IWT, and HYG populations were clustered as one group and the remaining four populations, HKD, NGT, TTR, and NSK populations were clustered as another group, although it should be emphasized that the tree topology appears not to be highly robust given the short branch length between the groups and low bootstrap values obtained. The tree topology derived from the D_{ST} distance (D_{ST}-NJ tree) was in near agreement with the D_A-NJ tree topology except for the positions of the HKD population: this population was clustered with the TTR population in the D_A-NJ tree, whereas it was placed with the HYG population in the D_{ST}-NJ tree. As Takezaki and Nei (1996) described, this inconsistency might be due to the extent of sampling errors, which is an important factor for efficiency of a distance measure in reconstruction of phylogenetic tree. These authors also proposed that the D_{ST} distance is not so efficient in tree topology construction since it has a relatively large sampling error.

Several hierarchical AMOVA tests were conducted according to the population relationships estimated from the neighbor joining analysis. First, a putative group including all seven populations was defined, assuming that none of the seven populations within the group were structured. In this case, the fixation index was small but significantly differed from zero ($F_{ST}=0.0025$, $p=0.001$), suggesting that there

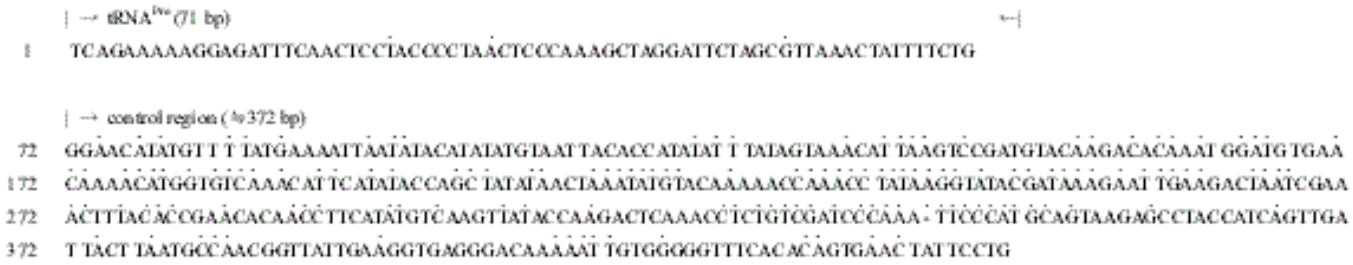

Fig. 3. Sequence including the tRNA^{Pro} gene (71 bp) and the left domain of the control region of Japanese flounder mtDNA. Variable sites found in at least one individual are indicated by adding dots, and the single nucleotide deletion/insertion is represented by a dash (-).

Table 7. Frequency of each nucleotide at each variable site in the Japanese flounder mtDNA sequences including the tRNA^{Pro} gene and the left domain of the control region

Populations	Nucleotides	tRNA^{Pro} ← | → Control region Sequence No.																																							
		25	33	56	74	77	79	84	85	88	95	97	98	122	124	125	134	137	139	140	143	152	153	156	158	159	161	164	165	166	171	172	173	176	177	178	179	181	183
HKD	A	-	50	1	50	48	50	-	-	1	50	-	50	-	-	-	41	-	40	48	-	48	30	-	-	50	48	-	50	-	47	-	50	47	1	49	-	-	14
(*N*=50)	C	-	-	-	-	-	-	2	-	-	-	1	-	4	-	1	-	-	10	-	50	2	-	50	49	-	-	-	-	-	-	50	-	-	48	-	-	-	-	-
	G	-	-	49	-	2	-	-	-	49	-	-	-	-	-	-	9	-	-	2	-	-	20	-	-	-	2	50	-	-	3	-	-	1	-	1	-	50	36
	T	50	-	-	-	-	-	48	50	-	-	49	-	46	50	49	-	50	-	-	-	-	-	-	1	-	-	-	-	50	-	-	-	-	1	-	50	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
NGT	A	-	50	-	50	49	50	-	-	-	50	-	50	-	-	-	36	-	29	49	-	48	19	-	-	50	47	1	49	-	50	-	49	48	-	50	-	-	24
(*N*=50)	C	-	-	-	-	-	-	8	-	-	-	-	-	10	1	-	-	-	21	-	50	-	-	50	50	-	-	-	1	-	-	50	-	-	49	-	-	-	-
	G	-	-	50	-	1	-	-	-	50	-	-	-	-	-	-	14	-	-	1	-	2	31	-	-	-	3	49	-	-	-	-	-	-	-	-	-	50	26
	T	50	-	-	-	-	-	42	50	-	-	50	-	40	49	50	-	50	-	-	-	-	-	-	-	-	-	-	-	50	-	-	1	-	1	-	50	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
TTR	A	-	68	-	69	68	68	-	-	-	68	-	68	-	-	-	51	-	48	67	-	68	39	-	-	69	66	-	69	-	64	-	69	65	-	69	-	-	28
(*N*=69)	C	1	-	-	-	-	-	12	-	-	-	1	-	12	-	-	-	3	21	-	69	-	-	68	69	-	-	-	-	1	-	69	-	-	69	-	-	-	-
	G	-	1	69	-	1	1	-	-	69	-	-	1	-	-	-	18	-	-	2	-	-	30	-	-	-	3	69	-	-	5	-	-	-	-	-	-	69	41
	T	68	-	-	-	-	-	57	69	-	1	68	-	57	69	69	-	66	-	-	-	1	-	1	-	-	-	-	-	68	-	-	-	-	-	-	69	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	-
NSK	A	-	70	1	70	69	69	-	-	-	70	-	70	-	-	-	58	-	50	70	-	67	43	-	-	70	68	-	70	-	66	-	70	69	-	69	-	-	23
(*N*=70)	C	-	-	-	-	-	-	12	-	-	-	-	-	13	-	1	-	1	19	-	68	-	-	70	69	-	1	-	-	1	-	69	-	-	69	-	-	-	-
	G	-	-	69	-	1	1	-	-	70	-	-	-	-	-	-	12	-	-	-	-	3	27	-	-	-	1	70	-	-	4	-	-	-	-	1	-	70	47
	T	70	-	-	-	-	-	58	69	-	-	70	-	57	70	69	-	69	1	-	2	-	-	-	1	-	-	-	-	69	-	1	-	-	1	-	70	-	-
	indels	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
IWT	A	-	45	-	44	44	45	-	-	-	45	-	45	-	-	-	29	-	27	45	-	45	23	-	-	45	40	-	45	-	42	-	45	43	-	44	-	-	18
(*N*=45)	C	-	-	-	-	-	-	13	-	-	-	1	-	11	-	1	1	-	18	-	45	-	-	45	45	-	1	-	-	-	-	45	-	-	45	-	1	-	-
	G	-	-	45	1	1	-	-	-	45	-	-	-	-	-	-	15	-	-	-	-	-	22	-	-	-	4	45	-	-	3	-	-	-	-	1	-	45	27
	T	45	-	-	-	-	-	32	45	-	-	44	-	34	45	44	-	45	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	44	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-
CHB	A	-	71	-	71	71	71	-	-	-	70	-	70	-	-	-	60	-	46	69	-	68	35	-	-	71	66	-	71	-	68	-	69	68	-	69	-	1	28
(*N*=71)	C	-	-	-	-	-	-	10	-	-	-	-	-	11	-	2	-	2	25	-	70	-	-	70	69	-	2	-	-	-	-	71	-	-	71	-	1	-	-
	G	-	-	71	-	-	-	-	-	71	-	-	1	-	-	-	11	-	-	2	-	1	36	-	-	-	3	71	-	-	3	-	-	-	-	2	-	70	43
	T	71	-	-	-	-	-	61	71	-	1	71	-	60	71	69	-	69	-	-	1	2	-	1	2	-	-	-	-	71	-	-	2	-	-	-	70	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-	-	-
HYG	A	-	44	-	44	44	43	-	-	1	44	-	44	-	-	-	31	-	28	43	-	41	20	-	-	43	41	-	44	-	43	-	41	43	-	42	-	-	22
(*N*=44)	C	-	-	-	-	-	-	10	-	-	-	-	-	12	-	-	-	-	15	-	44	-	-	44	42	-	-	-	-	-	-	44	-	-	44	-	-	-	-
	G	-	-	44	-	-	1	-	-	43	-	-	-	-	-	-	13	-	-	1	-	3	24	-	-	1	3	44	-	-	1	-	-	-	-	2	-	44	22
	T	44	-	-	-	-	-	34	43	-	-	44	-	32	44	44	-	44	1	-	-	-	-	-	2	-	-	-	-	44	-	-	3	-	-	-	44	-	-
	indels	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-

Populations	Nucleotides	Sequence No.																																						
		184	185	186	188	191	192	194	195	197	202	203	204	208	209	213	214	215	216	217	218	219	220	221	225	230	231	232	233	234	235	236	237	238	239	240	241	242	244
HKD	A	-	-	50	33	-	-	50	-	-	1	-	-	-	50	49	49	43	-	-	45	-	36	-	39	-	49	50	50	-	-	-	48	-	48	48	-	1	50
	C	-	45	-	-	4	-	-	-	-	2	48	2	-	-	-	-	-	-	-	-	13	-	1	-	50	-	-	-	46	35	-	-	-	-	-	-	-	-
	G	-	-	-	17	-	-	-	-	-	47	-	-	-	-	1	1	7	-	-	5	-	14	-	-	-	-	-	-	-	-	-	1	-	2	2	50	49	-
	T	50	5	-	-	46	50	-	50	50	-	2	48	50	-	-	-	-	-	50	-	37	-	49	11	-	1	-	-	4	15	50	1	50	-	-	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NGT	A	-	-	50	44	-	-	49	-	-	4	-	-	-	50	50	50	48	1	-	50	-	38	-	28	-	49	50	48	-	-	-	50	-	47	46	1	5	50
	C	-	43	-	-	3	-	-	1	-	-	49	-	-	-	-	-	-	-	1	-	22	-	4	-	49	-	-	-	50	42	-	-	1	-	-	-	-	-
	G	-	-	-	6	-	-	1	-	-	46	-	-	-	-	-	-	2	-	-	-	-	12	-	-	-	-	-	2	-	-	-	-	-	3	4	49	45	-
	T	50	7	-	-	47	50	-	49	50	-	1	50	50	-	-	-	-	-	49	-	28	-	46	22	1	1	-	-	-	8	50	-	49	-	-	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TTR	A	-	-	69	58	-	-	69	-	-	9	-	-	-	69	69	68	62	-	-	67	-	41	-	48	-	69	69	67	-	1	-	69	-	66	64	3	3	69
	C	2	54	-	-	6	-	-	-	1	-	66	-	-	-	-	-	-	-	4	-	25	-	8	-	69	-	-	-	63	52	1	-	1	-	-	-	-	-
	G	-	-	-	11	-	-	-	-	-	60	-	-	-	-	-	1	7	-	-	2	-	28	-	-	-	-	-	2	-	-	-	-	-	2	4	66	66	-
	T	67	15	-	-	63	69	-	69	68	-	3	69	69	-	-	-	-	-	65	-	44	-	61	21	-	-	-	-	6	16	68	-	68	-	-	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NSK	A	-	-	70	68	-	-	70	-	-	6	-	-	-	70	69	68	62	-	-	65	-	49	-	50	-	70	70	70	-	-	-	70	-	68	65	2	1	70
	C	-	61	-	-	4	1	-	2	1	-	69	1	-	-	-	-	-	-	1	-	23	-	5	-	70	-	-	-	65	59	-	-	1	-	-	-	-	-
	G	-	-	-	12	-	-	-	-	-	64	-	-	-	-	1	2	8	-	-	5	-	21	-	-	-	-	-	-	-	-	-	-	-	2	5	68	69	-
	T	70	9	-	-	66	69	-	68	69	-	1	69	70	-	-	-	-	-	69	-	47	-	65	20	-	-	-	-	5	11	70	-	69	-	-	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IWT	A	-	-	45	36	-	-	45	-	-	4	-	-	-	44	45	44	44	-	-	44	-	33	-	27	-	45	45	45	-	-	-	45	-	45	40	-	-	45
	C	-	42	-	-	4	-	-	-	1	-	43	-	-	-	-	-	-	-	1	-	21	-	7	-	45	-	-	-	43	39	-	-	1	-	-	-	-	-
	G	-	-	-	9	-	-	-	-	-	41	-	-	-	1	-	1	1	-	-	1	-	12	-	-	-	-	-	-	-	-	-	-	-	-	5	45	45	-
	T	45	3	-	-	41	45	-	45	44	-	2	45	45	-	-	-	-	-	44	-	24	-	48	18	-	-	-	-	2	6	45	-	44	-	-	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHB	A	-	-	70	51	-	-	71	-	-	8	-	-	-	70	71	69	66	-	-	66	-	53	-	46	-	71	69	70	-	-	-	71	-	69	67	1	2	69
	C	1	63	-	-	5	-	-	-	-	1	70	2	1	-	-	-	-	-	1	-	32	-	4	-	71	-	-	-	67	60	1	-	1	-	-	-	-	-
	G	-	-	1	20	-	-	-	-	-	62	-	-	-	1	-	2	5	-	-	5	-	18	-	-	-	-	-	1	-	-	-	-	-	2	4	70	69	1
	T	70	8	-	-	66	71	-	71	71	-	1	69	70	-	-	-	-	-	70	-	39	-	67	25	-	-	2	-	4	11	70	-	70	-	-	-	-	1
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HYG	A	-	-	44	39	-		44	-	-	7	-	-	-	44	44	44	41	-	-	44	-	33	-	27	-	44	43	44	-	-	-	44	-	42	39	2	2	44
	C	1	34		-	1			1	1	-	43	1	-	-	-	-	-	-	-	-	17	-	3	-	44	-	-	-	44	35	-	-	1	-	-	-	-	-
	G	-	-		5	-			-	-	37	-	-	-	-	-	-	3	-	-	-	-	11	-	-	-	-	-	-	-	-	-	-	-	2	5	42	42	-
	T	43	10		-	43	44		43	43	-	1	43	44	-	-	-	-	-	44	-	27	-	41	17	-	-	1	-	-	9	44	-	43	-	-	-	-	-
	indels	-	-		-	-			-	-	-	-	-	-	-	-	-	-	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 7. (continued)

Populations	Nucleotides	Sequence No. 247	248	250	251	252	253	255	257	258	259	260	261	265	266	267	268	273	274	277	279	280	281	285	290	291	292	298	299	302	307	309	314	324	325	327	328	329	330
HKD	A	-	50	-	50	50	50	49	-	-	43	49	50	-	49	44	-	50	-	-	-	48	-	44	47	-	-	49	2	-	-	-	50	-	-	-	-	-	-
	C	50	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	50	-	46	-	47	-	-	50	46	-	4	50	1	-	-	49	2	12	-	3	50
	G	-	-	-	-	-	-	1	-	-	7	1	-	-	1	6	-	-	-	-	2	2	-	6	3	-	-	1	-	-	-	-	-	-	-	-	50	-	-
	T	-	-	50	-	-	-	-	50	50	-	-	-	49	-	-	50	-	-	50	2	-	3	-	-	-	4	-	44	-	49	50	-	1	48	38	-	47	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NGT	A	-	49	-	47	50	50	50	-	-	29	47	47	-	50	43	-	50	-	-	-	50	-	47	46	-	-	47	1	-	-	-	49	-	-	-	-	-	-
	C	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	50	1	41	-	50	-	-	50	50	-	8	49	2	-	-	49	4	21	-	11	50
	G	-	1	-	3	-	-	-	-	-	21	3	3	-	-	7	-	-	-	-	3	-	-	3	4	-	-	3	-	-	-	-	1	-	-	-	50	-	-
	T	-	-	50	-	-	-	-	50	50	-	-	-	50	-	-	50	-	-	49	6	-	-	-	-	-	-	-	41	1	48	50	-	1	46	29	-	39	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TTR	A	-	69	-	64	69	69	69	1	-	49	64	63	-	68	56	-	69	-	-	1	68	-	68	67	-	-	63	-	-	-	-	69	-	-	3	-	-	-
	C	69	-	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	69	2	62	-	68	-	-	69	66	-	5	69	1	1	-	65	1	22	-	16	69
	G	-	-	-	5	-	-	-	-	-	20	5	6	-	1	13	-	-	-	-	4	1	-	1	2	-	-	6	-	-	-	-	-	-	-	-	69	-	-
	T	-	-	69	-	-	-	-	65	69	-	-	-	69	-	-	69	-	-	67	2	-	1	-	-	-	3	-	64	-	68	68	-	4	68	44	-	53	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NSK	A	-	70	-	64	69	70	70	-	-	54	66	66	-	70	66	-	70	-	-	60	67	66	67	68	-	-	68	-	-	-	-	69	-	-	-	1	-	-
	C	70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	70	-	-	-	-	-	-	69	67	-	3	64	1	1	-	69	1	24	-	12	70
	G	-	-	-	6	1	-	-	-	-	16	4	4	-	-	4	-	-	-	-	4	3	4	3	2	-	1	2	2	-	-	-	1	-	-	-	69	-	-
	T	-	-	70	-	-	-	-	70	70	-	-	-	70	-	-	70	-	-	70	6	-	-	-	-	1	2	-	65	6	69	69	-	1	69	46	-	58	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IWT	A	-	45	-	41	45	44	45	1	-	26	40	39	-	44	41	-	45	-	-	-	40	-	41	45	-	-	42	-	-	-	-	45	-	-	-	1	-	-
	C	43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	2	43	-	42	-	-	45	44	-	2	44	-	1	-	44	3	18	-	13	45
	G	-	-	-	4	-	1	-	-	-	19	5	6	-	1	4	-	-	-	-	2	5	-	4	-	-	-	3	-	-	-	-	-	-	-	-	44	-	-
	T	2	-	45	-	-	-	-	44	45	-	-	-	45	-	-	45	-	-	43	-	-	3	-	-	-	1	-	43	1	45	44	-	1	42	27	-	32	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHB	A	-	71	-	64	68	70	70	-	-	52	69	66	-	71	58	-	70	-	-	67	71	-	68	68	-	-	66	-	-	-	-	71	-	-	1	1	-	-
	C	70	-	1	-	-	-	-	2	1	-	-	-	-	-	-	1	-	70	-	-	-	69	-	-	71	67	-	5	66	2	1	-	70	3	26	-	12	70
	G	-	-	-	7	3	1	1	-	-	19	2	5	-	-	13	-	1	-	-	2	-	-	3	3	-	-	5	-	-	-	-	-	-	-	-	70	-	-
	T	1	-	70	-	-	-	-	69	70	-	-	-	71	-	-	70	-	1	71	2	-	2	-	-	-	4	-	66	5	69	70	-	1	68	44	-	59	1
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HYG	A	-	44	-	41	43	44	44	-	-	30	43	44	-	44	36	-	44	-	-	-	43	-	44	41	-	-	41	-	-	-	-	44	-	-	1	-	-	-
	C	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	43	2	36	-	42	-	-	44	44	-	4	44	1	-	-	43	1	16	-	10	44
	G	-	-	-	3	1	-	-	-	-	14	1	-	-	-	8	-	-	-	-	3	1	-	-	3	-	-	3	-	-	-	-	-	-	-	-	44	-	-
	T	-	-	44	-	-	-	-	44	44	-	-	-	44	-	-	44	-	1	42	5	-	2	-	-	-	-	-	40	-	43	44	-	1	43	27	-	34	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Populations	Nucleotides	Sequence No. 331	333	335	336	337	338	340	341	342	343	344	345	346	347	348	349	350	352	358	368	376	380	384	385	386	398	411	412	413	414	415	419	430	431	435	437
HKD	A	-	-	-	-	50	49	3	1	-	-	-	-	50	-	-	-	-	1	4	50	49	50	-	-	50	37	50	50	50	-	-	1	-	48	50	-
	C	-	14	50	50	-	-	-	-	-	49	48	34	-	-	-	-	49	-	-	-	-	-	50	39	-	-	-	-	-	-	-	-	36	-	-	38
	G	50	-	-	-	-	1	-	-	-	-	-	-	-	-	-	50	-	49	46	-	1	-	-	-	-	13	-	-	-	-	-	49	1	2	-	-
	T	-	36	-	-	-	-	1	49	50	1	2	16	-	50	-	-	1	-	-	-	-	-	-	11	-	-	-	-	-	50	50	-	13	-	-	2
	indels	-	-	-	-	-	-	46	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NGT	A	-	-	-	-	50	50	12	-	-	-	2	-	48	-	-	-	-	1	6	50	50	50	-	-	50	22	49	50	46	-	-	-	-	48	49	-
	C	-	14	48	50	-	-	-	1	1	50	44	40	-	1	-	1	50	-	-	-	-	-	50	28	-	-	-	-	-	1	1	-	29	-	1	48
	G	50	-	-	-	-	-	-	-	-	-	-	1	2	-	-	49	-	49	44	-	-	-	-	-	-	28	1	-	4	-	-	50	-	2	-	-
	T	-	36	2	-	-	-	-	49	49	-	4	9	-	49	-	-	-	-	-	-	-	-	-	22	-	-	-	-	-	49	49	-	21	-	-	2
	indels	-	-	-	-	-	-	38	-	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TTR	A	1	-	-	-	69	69	16	-	-	-	-	1	68	-	-	-	-	-	2	69	69	67	-	-	69	47	69	68	67	-	-	1	-	64	68	-
	C	-	16	62	69	-	-	-	-	-	68	65	56	-	-	-	-	67	-	-	-	-	-	69	48	-	-	-	-	-	-	-	-	44	-	1	63
	G	68	-	-	-	-	-	-	-	-	-	-	-	1	-	-	69	-	69	67	-	-	-	-	-	-	22	-	1	2	-	-	69	-	5	-	-
	T	-	53	7	-	-	-	1	69	69	1	4	11	-	69	-	-	2	-	-	-	-	2	-	21	-	-	-	-	-	69	69	-	25	-	-	6
	indels	-	-	-	-	-	-	52	-	-	-	-	1	-	-	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NSK	A	-	-	-	1	69	70	15	2	-	-	3	1	67	-	1	-	-	2	2	68	70	70	-	-	70	46	70	69	69	-	-	1	-	67	68	-
	C	-	13	70	69	1	-	-	-	2	70	66	58	-	2	-	1	66	-	-	-	-	-	70	50	-	-	-	-	-	-	-	-	45	-	2	70
	G	70	-	-	-	-	-	-	-	-	-	-	-	3	-	-	69	-	68	68	2	-	-	-	-	-	24	-	1	1	-	-	69	1	3	-	-
	T	-	57	-	-	-	-	2	68	68	-	1	11	-	68	-	-	4	-	-	-	-	-	-	20	-	-	-	-	-	70	70	-	24	-	-	-
	indels	-	-	-	-	-	-	53	-	-	-	-	-	-	-	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IWT	A	-	-	-	-	45	45	13	-	-	-	-	-	44	-	-	-	-	1	-	45	45	45	-	-	45	24	45	45	45	-	-	1	-	43	45	-
	C	-	8	44	45	-	-	-	1	1	45	44	36	-	-	-	-	45	-	-	-	-	-	44	27	-	-	-	-	-	1	-	-	25	-	-	45
	G	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	44	45	-	-	-	-	-	-	21	-	-	-	-	-	44	-	2	-	-
	T	-	37	1	-	-	-	32	44	44	-	1	9	1	45	-	-	-	-	-	-	-	-	1	18	-	-	-	-	-	44	45	-	20	-	-	-
	indels	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHB	A	1	-	1	-	71	71	13	-	-	-	-	1	69	-	-	-	-	-	2	71	71	71	-	-	71	46	70	70	69	-	-	-	-	63	70	-
	C	-	13	69	71	-	-	1	-	2	71	71	55	-	1	-	-	67	-	-	-	-	-	71	47	-	-	-	-	-	-	-	-	46	-	1	69
	G	70	-	-	-	-	-	-	-	-	-	-	-	1	-	-	71	-	71	69	-	-	-	-	-	-	25	1	1	2	-	-	71	-	8	-	-
	T	-	58	1	-	-	-	-	71	68	-	-	15	1	70	-	-	4	-	-	-	-	-	-	24	-	-	-	-	-	71	71	-	25	-	-	2
	indels	-	-	-	-	-	-	57	-	1	-	-	-	-	-	71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HYG	A	-	-	-	-	44	43	11	3	-	-	2	-	44	-	-	-	-	-	4	44	43	44	-	-	43	24	44	44	44	-	-	1	-	38	44	-
	C	-	13	43	44	-	-	-	1	-	43	37	39	-	1	-	-	42	-	-	-	-	-	44	37	1	-	-	-	-	-	-	-	26	-	-	40
	G	44	-	-	-	-	1	-	-	-	-	-	-	-	-	-	44	-	44	40	-	1	-	-	-	-	20	-	-	-	-	-	43	1	6	-	-
	T	-	31	1	-	-	-	3	40	44	1	5	5	-	43	-	-	2	-	-	-	-	-	-	7	-	-	-	-	-	44	44	-	17	-	-	4
	indels	-	-	-	-	-	-	30	-	-	-	-	-	-	-	44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 8. Variability of the Japanese flounder mtDNA sequences including the $tRNA^{Pro}$ gene and the left domain of control region

	Populations						
	HKD	NGT	TTR	NSK	IWT	CHB	HYG
Sample size	50	50	69	70	45	71	44
Number of variable sites	76	80	87	95	45	103	76
Number of substitutions	81	81	91	100	74	110	80
Transitions	70	70	80	85	67	95	69
Transversions	11	11	11	15	7	15	11
Number of indels	2	3	3	4	2	3	3
Number of haplotypes	48	45	64	62	44	65	39
Haplotype diversity[*1] (h)	0.998	0.996	0.998	0.996	0.999	0.998	0.994
Nucleotide diversity[*2] (π)	0.027	0.031	0.031	0.028	0.030	0.030	0.032

[*1] Nei and Tajima (1981)
[*2] Nei (1987)

would be some genetic structures within the group. Next, hierarchical AMOVA tests were conducted forming putative groups estimated from each NJ tree topology (Table 12: test panel TD_A, for group definition on the basis of the D_A-NJ tree topology; test panel: TD_{ST}, for group definition on the basis of the D_{ST}-NJ tree topology). The putative groups defined based on the D_A-NJ tree topology were as follows: a group including the HKD, NGT, TTR, and NSK populations (group A1) and another group including the IWT, CHB, and HYG populations (group A2). As shown in Table 12, the fixation index among groups (F_{CT}) was not significant in this case (F_{CT}=0.0009, p=0.197), whereas it was significant among populations within groups (F_{SC}=0.0020, p=0.013). The groups defined based on the D_{ST}-NJ tree topology were as follows: a group including the NGT, TTR, and NSK populations (group ST1) and another group including the HKD, IWT, CHB, and HYG populations (group ST2). In this case, both F_{CT} and F_{SC} were significant (F_{CT}=0.0017, p=0.027; F_{SC}=0.0015, p=0.007). These results suggested that the populations are still structured within at least one group in each test panel since the F_{SC} values estimated in both test panels were significant. Therefore, AMOVA tests were performed for each group (group A1, A2, ST1, and ST2), assuming that none of the populations within each group were structured. In these cases, the F_{ST} values estimated for two groups (A1 and ST2) were significant (group A1: F_{ST}=0.0031, p=0.004; group A2: F_{ST}=0.0000, p=0.461; group ST1: F_{ST}=0.0009, p=0.207; group ST2: F_{ST}=0.0020, p=0.046). Of note, if the HKD population was removed from the group A1 and ST2, the population components of group A1 were identical to those of the group ST1, and the population components of group ST2 were identical to those of the group A2. Therefore, it was possibly considered that the HKD population could be subdivided from other populations as one group, and then the seven populations were putatively assigned into three groups as follows: a group comprised of the HKD population, the ST1 group, and the A1 group. The results of the AMOVA test based on this group definition (test panel: TA) showed that the fixation index among groups was significant (F_{CT}=0.0028, p=0.014), whereas it was not significant among populations within groups (F_{SC}=0.0005, p=0.272). According to the NJ tree topologies coupled with the results of the hierarchical AMOVA tests, the seven populations were possibly assigned into three groups: a group including two populations in the Japan Sea, the NGT and TTR, as well as one population in the East China Sea, the NSK population; a group including three populations in the Pacific Ocean, the IWT, CHB, and HYG populations, and a group comprised of the HKD

Table 9. Pairwise F_{ST} values[*1] between the 7 populations at each locus above the diagonal and results of pairwise comparisons of allele frequency distributions[*2] below the diagonal

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po25A	HKD		0.002	0.017	0.001	0.000	0.005	-0.003
			(0.29)	(0.01)	(0.33)	(0.38)	(0.15)	(0.65)
	NGT	0.17		0.006	-0.007	-0.006	0.000	0.001
				(0.14)	(1.00)	(0.82)	(0.40)	(0.36)
	TTR	0.09	0.18		0.014	-0.001	0.000	0.027*
					(0.02)	(0.45)	(0.35)	(0.0017)
	NSK	0.20	0.99	0.053		-0.001	0.004	-0.003
						(0.47)	(0.14)	(0.64)
	IWT	0.38	0.89	0.34	0.61		-0.003	0.007
							(0.62)	(0.15)
	CHB	0.10	0.71	0.57	0.41	0.84		0.007
								(0.11)
	HYG	0.44	0.54	0.03	0.73	0.56	0.67	

Locus	Pop.	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po56	HKD		0.008	0.001	0.012*	-0.005	0.008	-0.001
			(0.03)	(0.30)	(0.0018)	(0.96)	(0.01)	(0.57)
	NGT	0.01		-0.001	0.009	0.005	0.005	0.008
				(0.52)	(0.01)	(0.07)	(0.05)	(0.03)
	TTR	0.39	0.46		0.000	-0.004	-0.001	-0.002
					(0.40)	(0.90)	(0.61)	(0.67)
	NSK	0.0015*	0.01	0.19		0.004	-0.001	0.002
						(0.13)	(0.54)	(0.25)
	IWT	0.88	0.10	0.89	0.06		0.000	-0.006
							(0.39)	(0.99)
	CHB	0.0029*	0.02	0.35	0.15	0.47		-0.002
								(0.73)
	HYG	0.48	0.02	0.87	0.31	1.00	0.73	

Locus	Pop.	HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po33	HKD		-0.007	0.004	0.004	0.000	-0.001	0.024
			(0.83)	(0.19)	(0.19)	(0.34)	(0.45)	(0.03)
	NGT	0.34		0.001	0.009	0.008	0.004	0.038
				(0.32)	(0.10)	(0.14)	(0.20)	(0.01)
	TTR	0.01	0.26		0.006	0.005	0.004	0.041*
					(0.12)	(0.16)	(0.16)	(0.0021)
	NSK	0.61	0.37	0.07		-0.006	-0.001	0.008
						(0.84)	(0.45)	(0.12)
	IWT	0.21	0.08	0.06	0.93		-0.007	0.004
							(0.93)	(0.24)
	CHB	0.24	0.06	0.0022*	0.50	0.87		0.012
								(0.08)
	HYG	0.01	0.0011*	0.0000*	0.09	0.11	0.02	

Locus	Pop.	HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po91	HKD		0.005	0.000	0.000	0.011	0.007	0.004
			(0.09)	(0.42)	(0.46)	(0.01)	(0.02)	(0.13)
	NGT	0.12		0.001	0.002	0.016*	0.006	0.002
				(0.26)	(0.18)	(0.0013)	(0.03)	(0.30)
	TTR	0.32	0.34		-0.004	0.002	0.003	-0.001
					(0.97)	(0.21)	(0.13)	(0.55)
	NSK	0.47	0.26	0.87		0.001	-0.001	-0.003
						(0.31)	(0.55)	(0.85)
	IWT	0.0004*	0.0037*	0.37	0.22		0.001	0.002
							(0.38)	(0.28)
	CHB	0.02	0.20	0.01	0.15	0.25		-0.003
								(0.90)
	HYG	0.0044*	0.60	0.32	0.33	0.50	0.74	

Locus	Pop.	HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po52								
	HKD		0.010	0.054*	0.077*	0.004	0.004	0.006
			(0.14)	(0.0018)	(0.0000)	(0.20)	(0.23)	(0.24)
	NGT	0.06		0.011	0.023	-0.009	0.002	0.000
				(0.10)	(0.04)	(0.91)	(0.29)	(0.35)
	TTR	0.0004*	0.37		-0.005	0.019	0.048*	0.014
					(0.71)	(0.05)	(0.0015)	(0.09)
	NSK	0.0000*	0.10	0.85		0.033	0.067*	0.030
						(0.02)	(0.0000)	(0.02)
	IWT	0.10	0.89	0.12	0.04		-0.004	0.003
							(0.57)	(0.28)
	CHB	0.14	0.53	0.01	0.0012*	0.56		0.017
								(0.06)
	HYG	0.35	0.31	0.07	0.0040*	0.26	0.09	

Pop.	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD		NS	+	+	+	+	+
NGT			NS	NS	+	NS	+
TTR				NS	NS	+	+
NSK					NS	+	+
IWT						NS	NS
CHB							NS
HYG							

+: population pair showing significant genetic heterogeneities for at least one locus

NS: population pair not showing any significant genetic heterogeneities for all loci

[*1] F_{ST} values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ with initial K of sequential Bonferroni correction (Rice, 1989), $K=11$. The probability for each comparison is also shown in parenthesis.

[*2] Probability values of homogeneity of allelic frequency distributions (p) estimated by a test analogous to the Fisher's exact test in the Markov-chain method are shown. Significance for each pairwise comparison was determined by adding *=$p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

Six loci, *Po1, Po13, Po26, Po35, Po42*, and *Po48*, did not show any significant genetic heterogeneities of both allele frequency distributions and F_{ST} values for all population comparisons ($p>0.005$)

Table 10. Number of mtDNA haplotypes commonly shared between the 7 populations below the diagonal, and pairwise Φ_{ST} values[*1] estimated based on the mtDNA sequences above the diagonal

	Populations						
Populations	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD	—	0.043**	0.0121	0.0059	0.0399*	0.0099	0.0286*
		(0.007)	(0.076)	(0.168)	(0.010)	(0.099)	(0.023)
NGT	5	—	0.0080	0.0142	-0.0027	0.0048	-0.0097
			(0.135)	(0.071)	(0.450)	(0.188)	(0.878)
TTR	5	4	—	-0.0027	0.0032	-0.0024	-0.00232
				(0.556)	(0.247)	(0.548)	(0.471)
NSK	7	7	7	—	0.0064	-0.0040	0.0044
					(0.169)	(0.697)	(0.213)
IWT	5	5	10	9	—	0.0009	0.0001
						(0.320)	(0.345)
CHB	3	4	9	9	3	—	0.0005
							(0.343)
HYG	3	2	5	5	5	3	—

[*1] Calculated according to Weir and Cockerham (1984). Probability values associated with the Φ_{ST} values are also shown in parenthesis. Significance for each pairwise comparison was determined by adding *=$p<0.05$, and **=$p<0.01$.

Table 11. Modified Cavalli-Sforza chord distances (D_A) between the 7 populations above the diagonal and Nei's standard genetic distance (D_{ST}) below the diagonal

	Populations						
Populations	HKD	NGT	TTR	NSK	IWT	CHB	HYG
HKD	—	0.083	0.072	0.076	0.088	0.083	0.090
NGT	0.011	—	0.061	0.061	0.073	0.066	0.078
TTR	0.017	0.004	—	0.051	0.063	0.061	0.075
NSK	0.025	0.007	0.000	—	0.064	0.061	0.072
IWT	0.008	0.006	0.000	0.000	—	0.056	0.074
CHB	0.014	0.010	0.007	0.009	0.000	—	0.068
HYG	0.014	0.011	0.016	0.003	0.001	0.008	—

population.

The mtDNA sequence data, however, did not support the results derived from the microsatellite data: for the global analysis (including all populations with no subdivision) associated with the Φ_{ST} distance, there was not sufficient evidence to consider that the seven populations were structured ($\Phi_{ST}=0.0062$, $p=0.063$), and also, a hierarchical AMOVA test carried out according to the microsatellite-based group definition did not yield any significant heterogeneities among the groups ($\Phi_{CT}=0.0037$, $p=0.258$).

Discussion

Genetic variability

Genetic variability of the 11 microsatellites in terms of the allelic diversity (A: 15.3-18.2) and the gene diversity (mean H_e value: 0.74-0.76) were roughly similar to those reported in other marine fish microsatellites, and were obviously higher than those observed in allozyme loci (see Chapter 2). Given that the Mendelian inheritability for each of the 11 loci was verified (Chapter 2), and that the H_o values were well in accordance with the H_e values in each

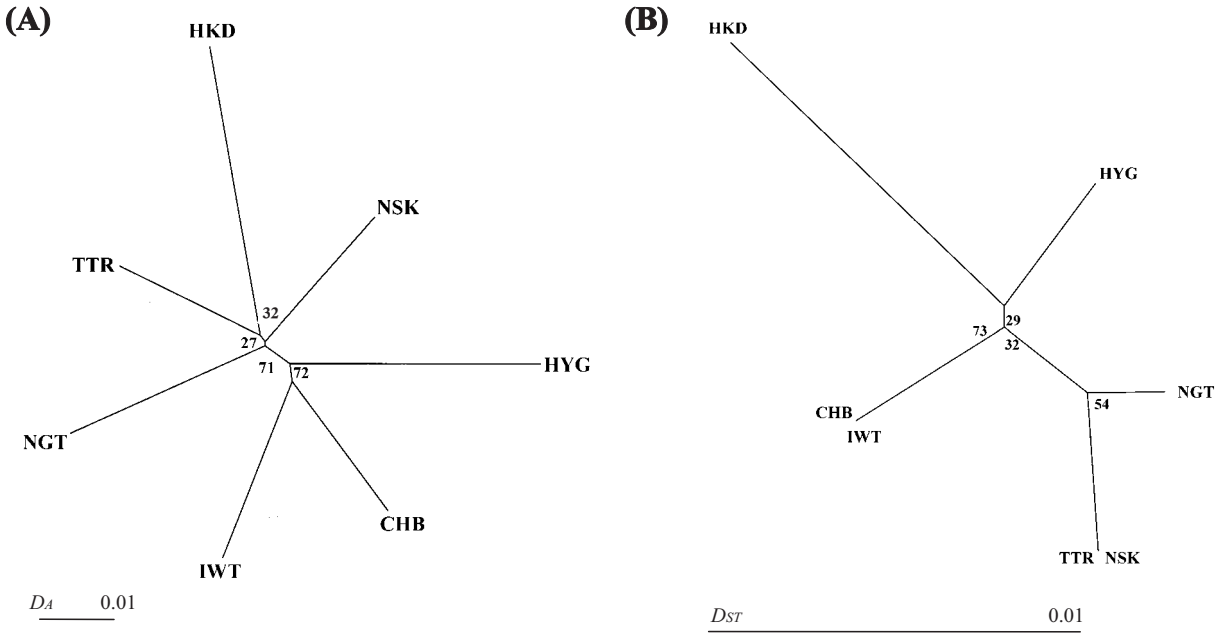

Fig. 4. (A) Unrooted neighbor-joiningtree based on modified Cavalli-Sforza chord distance (D_A; Nei *et al.*, 1983); (B) Unrooted neighbor-joining tree based on Nei's standerd genetic distance (D_{ST}; Nei, 1972). The numbers refer to percentage bootstrap values generated from 1,000 replications of re-sampled loci.

Table 12. Results of hierarchical AMOVAs for groups having several combinations of populations

Test panels[*1]	Number of groups	Group definitions	Among groups (F_{CT})	Among populations within group (F_{SC})	Within populations (F_{ST})
TD_A	2	(HKD-NGT-TTR-NSK)	0.0009	0.0020*	0.0028*
		vs (IWT-CHB-HYG)	(0.197)	(0.013)	(0.001)
TD_{ST}	2	(NGT-TTR-NSK)	0.0017*	0.0015*	0.0032*
		vs (HKD-IWT-CHB-HYG)	(0.027)	(0.007)	(0.001)
TA	3	HKD *vs* (NGT-TTR-NSK)	0.0028*	0.0005	0.0033*
		vs (IWT-CHB-HYG)	(0.014)	(0.272)	(0.001)

[*1] See text for details

Probability values associated with *F*-statistics are shown in parenthesis. *F*-statistics significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.05$.

population at each locus, a possible presence of null alleles seems unlikely to be a major concern for the 11 loci. It is also unlikely that the significant HWE departure observed at the *Po42* locus in the IWT population was caused by several possible factors such as inbreeding, assortative mating or Wahlund effects since all loci but the *Po42* locus did not show any significant HWE departures in this population. Alternatively, it would be plausible to consider that the HWE departure was caused by random variations.

An average of the effective population size (N_e) among the seven populations was estimated to be of 7,600. This value was comparably lower than that reported in other marine fish species such as the vermilion snapper (N_e =24,500; Bagley *et al.*, 1999) and European sea bass (N_e =11,000-15,000; Garcia de León *et al.*, 1997), although the estimate of N_e was calculated based on several assumptions (*e. g.*, microsatellite mutation rates and mutation models), and thus the N_e value may entail a wide range of errors.

The mtDNA control region of Japanese

flounder has been shown to be highly variable (Fujii and Nishida, 1997), and as well this study also bore out the large amounts of nucleotide variations in the control region, in which 147 variable sites among 399 individuals were detected. A high level of nucleotide variations maintained in the control region was also found in other marine fish species such as the swordfish (Alvarado Bremer *et al.*, 1995) and red sea bream (Tabata and Taniguchi, 2000). A selective constraint operates on mutations in coding regions, possibly having an effect on the mutation rates (Carvalho and Hauser, 1994). The mtDNA control region is however generally known to be a non-transcribed region, and hence it is likely that the neutrality of the mtDNA control region may allow itself to be highly variable. The high levels of mtDNA and microsatellite variabilities detected in the Japanese flounder indicate that a severe stock depression resulting in population bottlenecks has not occurred for the long term evolutionary history of wild Japanese flounder populations.

Population differentiation and relationships

The first point that should be discussed about the Japanese flounder genetic structure is that the results derived from the microsatellite and mtDNA data were inconsistent with each other. The overall F_{ST} value estimated from the microsatellite data showed a significant genetic structure among the seven populations (overall $F_{ST}=0.0025$, $p=0.001$), however, the mtDNA data did not reject the null hypothesis of overall genetic homogeneity among the populations (overall $\Phi_{ST}=0.0062$, $p=0.063$). A previous allozymic analysis (Fujio *et al.*, 1989) and PCR-RFLP of mtDNA (Asahida *et al.*, 1998) also failed to detect any significant genetic heterogeneities between several regional populations, although it seems likely that the population homogeneity revealed by the previous studies are largely accounted for by the limited number of available polymorphic loci of allozymes (2 of the 23 loci examined were available as polymorphic markers), and the small a number of individuals examined in the PCR-RFLP analysis (up to 10 individuals in each population). It is widely recognized that different parts of the genomes have passed different evolutionary processes (Avise, 1994; Huelsenbeck *et al.*, 1996), possibly leading to the existence of different mutation rates between the nuclear and mitochondrial genomes. Nielsen *et al.* (1997) assessed the population structure of Pacific trout in California and Mexico using the nucleotide sequences of mtDNA control region coupled with microsatellites, and they also found an incongruity of genetic structure between the two molecular markers. These authors inferred that the incongruity would be caused by the different mutation rates between the microsatellites and mtDNA. Although the results presented here are not enough to determine the causes of the conflicting results derived from the two molecular markers, microsatellites distribute ubiquitously throughout the nuclear genome, and it is thus conceivable that the microsatellite data would better represent the entire genomic information than the mtDNA data, which are derived from a small portion (approximately 440 bp) of the control region. The present author therefore considers that the microsatellites serve as a more appropriate marker to assess the genetic structure, rather than the sequences of mtDNA control region. Here, it is useful to look more closely at the significant genetic structure found in the microsatellite data.

None of the 11 microsatellites showed significant genetic heterogeneities between the three populations located in areas affected by the Tsushima Warm Current (the NGT, TTR, and NSK populations), and between the three populations in the Pacific Ocean (the IWT, CHB, and HYG populations) in terms of both allele frequency distributions and pairwise F_{ST} values. Japanese flounder larvae are possibly transported approximately 600km or more by the Tsushima Warm Current (Kinoshita *et al.*, 2000), and this prevailing oceanic current may homogenize the allele frequencies among populations from the Japanese coast side of the East

China Sea through the Japanese coast side of the Japan Sea. Little is known about the mode and extent of larvae dispersal in the Pacific Ocean, it is also probable that the Oyashio Cold Current and the Kuroshio Warm Current, which mixes with the Oyashio Cold Current, may effectively mix the genetic components among populations throughout the Japanese coast side of the Pacific Ocean. Therefore, these genetic similarities among populations are due most likely to the frequent occurrence of inter-populational gene flow by means of eggs and larvae transport as provided by the prevailing oceanic currents. While, Kinoshita *et al.* (2000) proposed population subdivisions in the Japan Sea from morphological points of view; these authors found geographic variation in dorsal and anal ray counts of juvenile Japanese flounder with significant differences between northern and southern groups in the Japan Sea. According to the geographical areas surveyed together with group definitions proposed by these authors, the NGT and TTR populations are assigned to the northern and southern groups, respectively. The present study did not support the findings inferred from the morphological study: neither the microsatellite data nor the mtDNA sequence data yielded significant genetic heterogeneities among populations within the areas for which they surveyed.

The estimate of F_{ST} among the seven populations was indeed significantly different from zero but it was very small ($F_{ST}=0.0025$, $p=0.001$), indicating that Japanese flounder populations around Japan are not well-structured. Even if the NJ tree topologies coupled with the results of AMOVA tests could illustrate the outline of population relationships, that is, it might be accounted for by oceanographical separation, the small F_{ST} value estimated among the populations and the low robustness of the NJ tree topologies indicate a very weak genetic integrity among populations. The mtDNA sequencing analysis employed in this study failed to detect this subtle genetic population differentiation. A geographical barrier such as the Japanese Archipelago separating the two oceanographic areas (the Japan Sea and the Pacific Ocean) seems not to be effective to maintain genetic population integrity between the two areas. This is indicated by the facts that several population pairwise comparisons such as IWT-TTR, IWT-NSK, and CHB-NGT population pairs did not yield any genetic heterogeneities for all microsatellites, despite that the two populations in each population pair were separated from one another by the Japanese Archipelago. The mtDNA sequence data also indicate the weak genetic integrity between the areas since population pairs between the two populations in the Pacific Ocean (the IWT and CHB populations) and the two populations in the Japan Sea (the TTR and NSK populations) shared a comparably large number of common mtDNA haplotypes (9-10 haplotypes). Bagley *et al.* (1999) reported the absence of genetic heterogeneities among vermilion snapper populations off the southeastern USA using several microsatellite markers, and these authors described that level of gene exchange in terms of N_em (where N_e is the effective population size, and m is the inter-populational migration rate per generation under the infinite island model) estimated among the populations between the South Atlantic Bight and the Gulf of Mexico was sufficient to prevent genetic population differentiation between the two oceanographic areas ($N_em>100$). According to the formula $N_em\fallingdotseq(1/F_{ST}-1)/4$ (Slatkin, 1985) together with the $F_{ST}=0.0025$ estimated for Japanese flounder, the number of migrants can be roughly estimated to be of $N_em\fallingdotseq100$, which is a similar order of magnitude as estimated for the vermilion snapper. Given the average of N_e values estimated for Japanese flounder ($N_e=7{,}600$), the migration rate (m) among populations is estimated to be of 0.013 (1.3%) per generation. This value is sufficiently high to counteract a significant genetic drift: according to a simulation analysis studied by Lacy (1987), the effect of genetic drift in an isolated population, in which 120 individuals are included, will be diminished if one immigrant arrives every generation in

the population (m=0.008).

The genetic structure of wild Japanese flounder could be summarized as follows: overall, gene flows would occur frequently from one area to adjacent areas resulting in the subtle inter-populational genetic differentiation, but not occur with an equal probability between areas as this study provided evidence of the significant genetic heterogeneity among the three groups. It is possible to consider that the genetic heterogeneity among the groups has been kept in the evolutionary history of Japanese flounder populations; however, further analyses for other populations with extended period of time should be conducted to depict the genetic structure of Japanese flounder in finer scale.

Summary

As discussed above, genetic integrity among wild Japanese flounder populations around Japan is not pronounced; nevertheless the present author suggests that the Japanese flounder should not be treated as an overall panmictic population. This is an important finding in this study since almost all of the previous genetic studies have failed to detect any significant genetic population heterogeneities around Japan (but see Fujii and Nishida, 1997). Even if the extent of the genetic population differentiation is subtle, wild Japanese flounder populations should not be managed as a single stock unit; Brown *et al.* (1987) studied the exchange rate between yellowtail flounder stocks, and these authors reported that two stocks with an exchange rate of 10% (m=0.10), which is approximately ten-fold higher than that estimated for among Japanese flounder populations (m=0.013), still reacted independently to the exploitation. The stocking practices of Japanese flounder should be conducted along with the concept of stock-conservation taking into consideration the genetic stock units. The present author recommends that the genetic management of wild Japanese flounder populations, if possible, be executed at every local area. The extent of genetic stock units that should be managed, however, can be extended by within each geographical area in which each of the three groups defined in this study is comprised.

Chapter 4. Assessment of genetic diversity within and between hatchery strains of Japanese flounder

The preceding chapter revealed that natural Japanese flounder populations possess a high amount of genetic variations. On the other hand, loss of genetic variation in hatchery strains is typical, and it is very likely that the reduced genetic variabilities are caused by the small number of founders and/or inbreeding events that occurred when the strains were founded, as clearly shown by recent direct DNA examinations (*e. g.*, Atlantic salmon, Norris *et al.*, 1999; ayu fish, Iguchi *et al.*, 1999). Regarding the Japanese flounder hatchery strains, genetic assessment has been employed using several classes of molecular markers. An allozymic analysis (Liu *et al.*, 1997) found significant genetic differentiation between several hatchery strains, and also between hatchery strains and wild populations; however, allozyme loci did not clearly show the reductions of genetic variability in the hatchery strains, this seemed to be due to the limited number of available polymorphic loci and the low levels of allelic diversity. More powerful techniques such as mitochondrial DNA (mtDNA)-RFLP (Sugaya *et al.*, 1999) and microsatellite analyses (Yoshida *et al.*, 2000) detected significant reductions of genetic variability in several hatchery strains. The molecular markers employed in these studies were found to be appropriate to detect reduced genetic variability in hatchery strains; nevertheless the present author considers that a simultaneous use of mtDNA and nuclear DNA-based markers such as microsatellite markers can clearly demonstrate a loss of genetic variations in hatchery strains.

This chapter presents an application of two

classes of molecular markers, that is, microsatellites and nucleotide sequences of mtDNA control region, to the hatchery strains of Japanese flounder derived from three hatchery stations. Of the hatchery strains examined, two strains were first-generation offspring of wild caught fish, while the other strain was founded using both wild and broodstock, which were hatchery-reared potentially over several generations. It should be noted that a loss of genetic variability might possibly occur even in the first hatchery generation (Verspoor, 1988). This chapter aims to deduce the genetic diversity within and between the hatchery strains to be stocked into the natural environment, documenting the potential uses of the two molecular markers for further monitoring of genetic conditions in hatchery strains.

Materials and methods

Fish samples

Hatchery fish were provided from a hatchery station in Hokkaido Prefecture (abbr. HY; 100 individuals), in Tottori Prefecture (abbr. HF; 100 individuals), and in Miyagi Prefecture (abbr. HC; 100 individuals). The HY strain was founded using approximately 110 wild caught flounder including 50 females and 60 males which were sampled from off Hokkaido Prefecture, and all fish in this strain were F_1 offspring of the wild captives. The HF strain was founded using approximately 300 individuals: 100 individuals including 50 females and 50 males were mated in each of three aquarium tanks, and the offspring sampled from each tank were communally reared in a single tank. The candidate broodstock were both wild caught flounder sampled from off Tottori Prefecture and fish maintained potentially over several generations in this hatchery. Unfortunately, there are no available records regarding how many of the sibs were used for the founding of this strain. The HC strain originated from approximately 60 wild flounder including 30 females and 30 males caught in off Miyagi Prefecture, and this strain comprised of F_1 offspring of the wild captives. Genetic information of the candidate broodstock for all hatchery strains was not available.

The genetic variability in each of the three hatchery strains was measured against that of the geographically proximal wild population examined in Chapter 3, that is, the HY strain was compared with the HKD population, the HF strain with the TTR population, and the HC strain with the CHB population.

Microsatellite and mitochondrial DNA sequencing analysis

Eleven microsatellites described in Chapter 2, *Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48, Po52, Po56*, and *Po91*, and a section of the mtDNA control region (see the preceding chapter), were screened for all fish samples. Microsatellite genotyping and mtDNA sequencing procedures are described in the preceding chapter.

Microsatellite allele frequencies and gene diversity (unbiased expected heterozygosity: H_e) of each strain at each locus were estimated using the ARLEQUIN version 1.1 software package (Schneider *et al.* 1997). Differences of mean H_e values between the hatchery strains and wild populations were tested using the Kruskal-Wallis rank sum analysis (Sokal and Rohlf 1997). The ARLEQUIN program was used for an assessment of the Hardy-Weinberg equilibrium (HWE) at each locus using a test analogous to the Fisher's exact test using the Markov-chain method (the Markov-chain parameters used were: steps, 100,000; dememorization, 10,000). An overall inbreeding coefficient (F_{IS}; Weir and Cockerham 1984) was also estimated to measure the HWE departures evaluating the probabilities through random permutation procedures (minimum 10,000 permutations). The value of significance associated with the HWE analysis was adjusted following sequential Bonferroni procedures (Rice 1989).

As regards the mtDNA sequence data, the number of variable sites, haplotype frequency distributions, and haplotype diversity (h) were calculated using the ARLEQUIN program. The

h values were compared between the hatchery strains and wild populations by the Kruskal-Wallis analysis.

Overall F-statistics (Weir and Cockerham, 1984) were estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}) using the ARLEQUIN program to assess the strain/population differentiation. Probability associated with the F_{ST} (Φ_{ST}) values was evaluated through random permutation procedures (minimum 10,000 permutations).

Results

Microsatellite variability

Allele frequency distributions for the three hatchery strains at each locus are listed in Appendix 2, and Table 13 summarizes the variabilities with the comparable wild population data. In the three hatchery strains, the allelic diversity (number of alleles per locus: A) ranged from 5.9 to 10.7, and the mean H_o and H_e values ranged from 0.57 to 0.72, and from 0.59 to 0.71, respectively. The A values estimated for the three hatchery strains were substantially reduced compared with those estimated for the three wild populations (15.3-18.2), despite the fact that the sample size of each hatchery strain (100 individuals) was larger than that of each wild population (50-72 individuals). Comparison of the mean H_e values revealed that the values estimated for the hatchery strains (H_e=0.59-0.71) were significantly lower than those estimated for the wild populations (H_e=0.75-0.76) (Kruskal-Wallis test, 1df, H=29.6, $p<0.01$). The HF strain showed a marked reduction of H_e value compared to all other strains/populations. There appeared not to be large discrepancies between the H_o and H_e values for almost all of the 11 loci, the *Po42* locus, however, showed a significant depression of heterozygous individuals compared with the expected ones in the HF strain (H_o=0.56 and H_e=0.72). Significant HWE departures were observed in all hatchery strains depending on the locus (11 simultaneous tests, $p<0.005$). This is in contrast to the results of the wild populations analyzed: the 11 loci did not show any significant HWE departures in all the three wild populations (Table 13). Negative values of overall inbreeding coefficients (F_{IS}) were estimated excepting one instance: a comparably higher F_{IS} value was estimated for the HF strain (F_{IS}=0.018), although the value was not significantly different from zero (p=0.20).

Mitochondrial DNA sequence variability

Within approximately 443 bp sequences containing the $tRNA^{Pro}$ gene (71 bp) and the left domain of the control region of mtDNA, a total of 48 variable sites were detected among the 300 individuals in the three hatchery strains. Haplotypic variabilities of the three hatchery strains are summarized in Table 14 with the wild population data. A total of 25 haplotypes were identified across the three hatchery strains, whereas 160 haplotypes were identified among 190 individuals in the three wild populations. The small number of haplotypes identified in hatchery strains (4-14 haplotypes; see also Fig. 5) was in contrast to the large a number of haplotypes observed in the wild populations (48-66 haplotypes). The three hatchery strains did not share any common haplotypes with each other, while several haplotypes were commonly shared between the hatchery strains and the wild populations (Table 15). The hatchery strains had a significantly lower haplotype diversity (h=0.692-0.798) than that estimated for the wild populations (h=0.998 for all the wild populations) (Kruskal-Wallis test, 1df, H=33.5, $p<0.01$). The HF strain, in which the microsatellite variability was substantially reduced, also showed an extremely low haplotype variability.

Strain/population differentiation

Overall F-statistics were estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}), and Table 16 shows the values estimated for the various sample combinations. A high level of genetic differentiation with statistically significant F_{ST} (Φ_{ST}) was estimated among all samples (no subdivisions), and among the

Table 13. Microsatellite variabilities of the 11 loci in the 3 hatchery strains and 3 wild populations of Japanese flounder

Locus		Hatchery strains			Wild populations			Locus		Hatchery strains			Wild populations		
		HY	HF	HC	HKD	TTR	CHB			HY	HF	HC	HKD	TTR	CHB
Po1	Sample size	100	100	100	50	69	72	***Po42***	Sample size	100	100	100	50	69	71
	$A(a_e$[*1])	12(3.0)	5(1.5)	12(4.8)	24(7.7)	26(5.6)	28(5.3)		$A(a_e)$	15(6.3)	7(3.6)	15(7.7)	23(10.0)	23(11.1)	28(14.3)
	H_o	0.67	0.30	0.84	0.86	0.87	0.81		H_o	0.83	0.56	0.85	0.96	0.88	0.93
	H_e	0.67	0.33	0.79	0.87	0.82	0.81		H_e	0.84	0.72	0.87	0.90	0.91	0.93
	p[*2]	0.000*	0.021	0.385	0.35	0.63	0.37		p	0.000*	0.000*	0.000*	0.87	0.64	0.33
Po13	Sample size	100	100	100	50	69	72	***Po48***	Sample size	100	100	100	50	64	72
	$A(a_e)$	15(6.7)	8(2.4)	15(9.1)	25(16.7)	27(12.5)	26(16.7)		$A(a_e)$	5(2.2)	2(1.7)	6(2.4)	6(2.0)	7(1.9)	9(1.9)
	H_o	0.85	0.56	0.96	0.90	0.97	0.97		H_o	0.53	0.36	0.65	0.54	0.46	0.47
	H_e	0.85	0.58	0.89	0.94	0.92	0.94		H_e	0.55	0.39	0.59	0.50	0.46	0.48
	p	0.000*	0.001*	0.004*	0.07	0.99	0.09		p	0.000*	0.60	0.67	0.49	0.74	0.34
Po25A	Sample size	100	100	100	50	69	71	***Po52***	Sample size	100	100	100	50	69	72
	$A(a_e)$	13(6.7)	6(4.5)	7(4.3)	11(5.9)	12(4.2)	15(5.6)		$A(a_e)$	3(1.1)	2(1.1)	3(1.2)	4(1.5)	4(2.0)	4(1.5)
	H_o	0.91	0.81	0.79	0.74	0.77	0.79		H_o	0.06	0.05	0.20	0.34	0.46	0.33
	H_e	0.85	0.78	0.77	0.83	0.76	0.82		H_e	0.06	0.05	0.19	0.34	0.50	0.32
	p	0.000*	0.01	0.31	0.05	0.31	0.36		p	1.00	1.00	0.69	0.88	0.60	0.66
Po26	Sample size	100	100	100	50	68	72	***Po56***	Sample size	100	100	100	50	69	71
	$A(a_e)$	3(2.9)	3(2.8)	3(2.1)	6(2.6)	5(2.9)	5(3.0)		$A(a_e)$	16(8.3)	7(3.7)	18(12.5)	22(16.7)	26(16.7)	23(14.3)
	H_o	0.71	0.72	0.58	0.68	0.74	0.67		H_o	0.86	0.72	0.94	0.96	0.94	0.93
	H_e	0.65	0.64	0.52	0.62	0.65	0.67		H_e	0.88	0.73	0.92	0.94	0.94	0.93
	p	0.04	0.04	0.06	0.90	0.69	0.74		p	0.000*	0.001*	0.05	0.49	0.77	0.95
Po33	Sample size	100	100	100	50	69	72	***Po91***	Sample size	100	100	100	50	69	72
	$A(a_e)$	7(3.6)	7(3.3)	5(2.3)	7(2.5)	10(3.1)	9(2.7)		$A(a_e)$	17(7.7)	10(4.3)	15(8.3)	23(14.3)	34(16.7)	35(20)
	H_o	0.75	0.67	0.60	0.68	0.72	0.58		H_o	0.91	0.80	0.92	0.94	0.96	0.96
	H_e	0.72	0.70	0.57	0.60	0.68	0.63		H_e	0.87	0.77	0.88	0.93	0.94	0.95
	p	0.14	0.34	0.50	0.47	0.61	0.02		p	0.000*	0.004*	0.003*	0.98	1.00	1.00
Po35	Sample size	100	100	100	50	69	71	***Mean***	$A(a_e)$	10.7(3.4)	5.9(2.4)	10(3.3)	15.3(4.0)	17.5(4.2)	18.2(4.0)
	$A(a_e)$	12(5.9)	8(4.0)	11(2.9)	17(5.0)	19(4.5)	18(5.3)		H_o	0.72	0.57	0.72	0.77	0.78	0.75
	H_o	0.87	0.77	0.61	0.84	0.81	0.81		H_e	0.71	0.59	0.70	0.75	0.76	0.75
	H_e	0.83	0.75	0.66	0.80	0.78	0.81		Overall $F_{IS}(p)$[*3]	−0.021(0.86)	0.018(0.20)	−0.037(0.98)	−0.023(0.88)	−0.028(0.95)	−0.001(0.55)
	p	0.000*	0.46	0.01	0.45	1.00	0.14								

[*1] a_e: effective number of alleles was calculated according to the formula: $a_e = 1/\Sigma x_i^2$ (Crow and Kimura, 1965)

[*2] p is the exact p-value estimated by a test analogous to Fisher's exact test in the Markov-chain method. Significant HWE departure for each locus was determined by adding $*=p<0.005$ with initial K of sequential Bonferroni correction, $K=11$.

[*3] Probability value associated with the F_{IS} is shown in parenthesis. The F_{IS} values significantly greater than zero, based on random allelic permutation testing, are noted by adding $*=p<0.005$ with initial K of sequential Bonferroni correction (Rice, 1989), $K=11$.

hatchery strains ($p<0.001$). These values were higher than those estimated for among the wild populations. Each hatchery strain was compared with the geographically proximal wild population (*i. e.*, the HY strain with the HKD population; the HF strain with the TTR population; the HC strain with the CHB population). These sample combinations were expected to minimize differences between the hatchery strains and wild populations caused by any regional differences in frequencies of microsatellite alleles and mtDNA haplotypes. The F_{ST} and Φ_{ST} values estimated for all sample combinations were significantly different from zero in these cases ($p<0.001$). The *F*-statistics were also estimated for between the hatchery group into which the three hatchery strains were pooled and the wild group into which the three wild populations were pooled. The estimate of F_{ST} and Φ_{ST} was also significant in this case ($p<0.001$). As was reported in brown trout populations (reviewed in Ferguson *et al.*, 1995), it appears that the Φ_{ST} values estimated from the mtDNA data were approximately double the F_{ST} values estimated from nuclear DNA (microsatellites) data with the exception of one instance (for a comparison among wild populations).

Discussion

Genetic variability

Microsatellite variability of the hatchery strains was characterized as substantial reductions of allelic diversity in terms of the number of alleles per locus. This would be caused by the loss of many low frequency alleles due most likely to the small number of effective parents when each strain was founded, suggesting that each strain was bottlenecked. Allelic diversity has been shown to be variable depending on the sample size, although the sample size of all the three hatchery strains screened in this study was larger than those for the wild populations. While, the mtDNA genome is theoretically transmitted thorough a single maternal line with no recombination, and mtDNA variabilities are thus expected to be highly susceptible to stochastic events such as population bottlenecks (Carvalho and Hauser, 1994). In practical terms, there were marked reductions as regards both the number of mtDNA haplotypes and haplotype diversity in the hatchery strains, consistent with population bottlenecks. With the facts that the large number of haplotypes were observed in the wild populations (160 haplotypes among 190 individuals), and the HY and HC strains were the first generation of wild caught flounder, it is reasonable to assume that the number of haplotypes detected in the HY strain (14 haplotypes) and the HC strain (7 haplotypes) represents the actual number of female parents by which each strain was founded. Given that the HY strain was founded using approximately 50 females and the HC strain using 30

Table 14. MtDNA sequence variabilities including the $tRNA^{Pro}$ gene and the left domain of the control region detected in the hatchery strains and wild populations of Japanese flounder

	Hatchery strains			Wild populations		
	HY	HF	HC	HKD	TTR	CHB
Sample size	100	100	100	50	69	71
Number of variable sites	43	29	37	76	87	103
Number of substitutions	42	28	35	81	91	110
Transitions	39	24	33	70	80	95
Transversions	3	4	2	11	11	15
Number of haplotypes	14	4	7	48	65	66
Haplotype diversity (h)[*1]	0.798	0.692	0.793	0.998	0.998	0.998

[*1] Haplotype diversity is given by $h=(1-\Sigma x_i^2)\ n/(n-1)$, where x_i is the frequency of a haplotype and n is the sample size (Nei and Tajima, 1981)

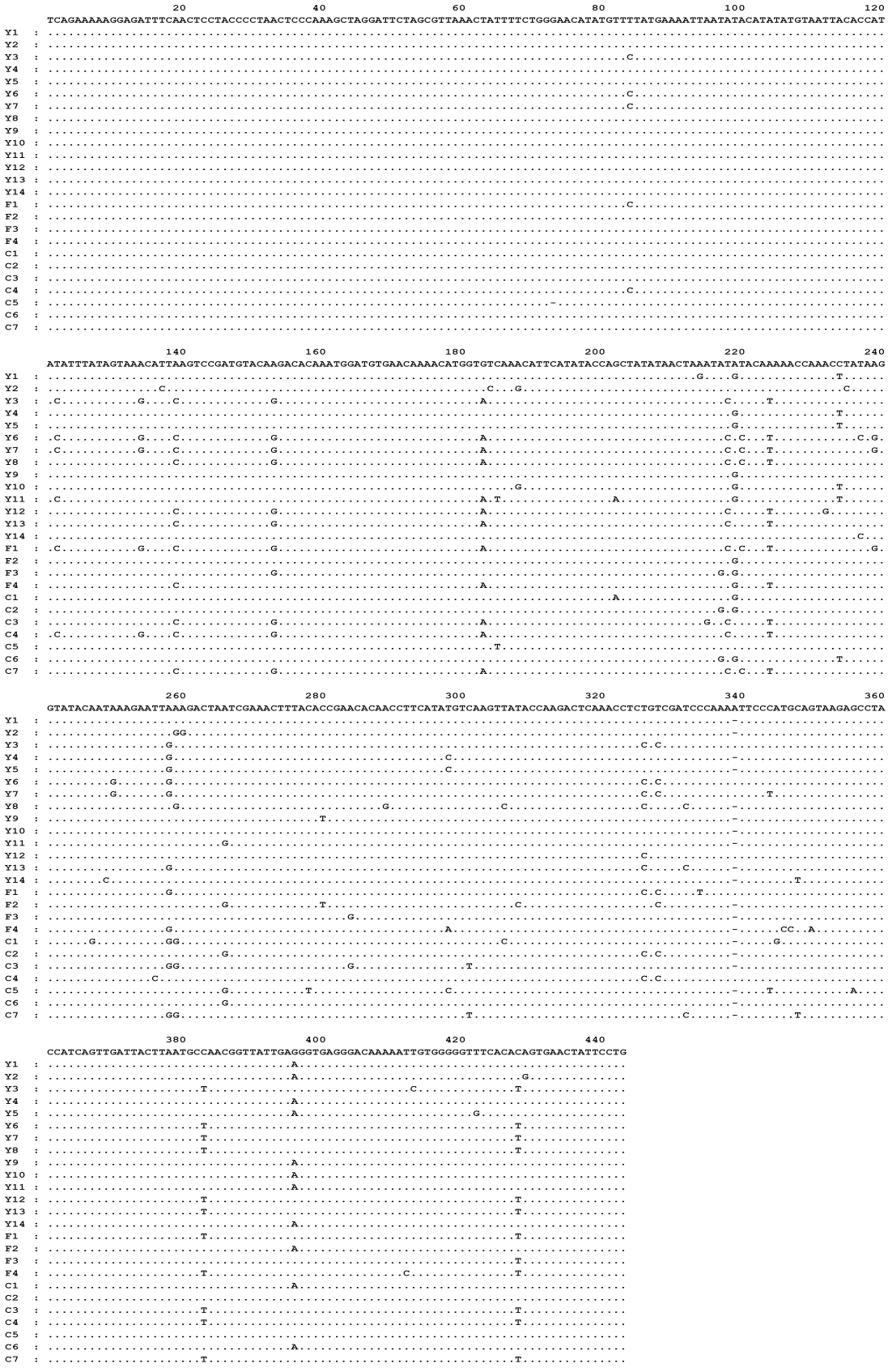

Fig. 5. Sequences of 25 mtDNA-haplotypes identified among the 300 individuals in the 3 hatchery strains (HY strain: 14 haplotypes, Y1-Y14; HF strain: 4 haplotypes, F1-F4; HC strain: 7 haplotypes, C1-C7). Identity with the reference sequence is indicated by dots, and a dash (-) represents a single nucleotide deletion/insertion.

Table 15. Number of mtDNA haplotypes commonly shared between the hatchery strains and wild populations

Haplotypes[*1]	Hatchery strains			Wild populations		
	HY	HF	HC	HKD	TTR	CHB
Y5	1	0	0	0	0	1
Y10	1	0	0	1	0	0
Y11	4	0	0	0	0	1
F1	0	45	0	0	2	0
C3	0	0	12	0	0	1

[*1] Sequence of each haplotype is shown in Fig. 5

Table 16. Estimates of F_{ST} (Φ_{ST}) value based on microsatellites and mtDNA sequences in several combinations of samples

Sample combinations	Microsatellites		mtDNA	
	F_{ST}	p	Φ_{ST}	p
Global (no subdivision)	0.055**	0.000	0.123**	0.000
Among hatchery strains	0.088**	0.000	0.187**	0.000
Among wild populations	0.004*	0.003	0.005	0.134
Hatchery *vs* wild group[*1]	0.019**	0.000	0.046**	0.000
HY *vs* HKD	0.026**	0.000	0.084**	0.000
HF *vs* TTR	0.086**	0.000	0.150**	0.000
HC *vs* CHB	0.034**	0.000	0.079**	0.000

[*1] Allele (mtDNA haplotype) frequency estimated for the 3 hatchery strains and the 3 wild populations were pooled into a hatchery group and a wild group, respectively

The F_{ST} (Φ_{ST}) values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=$p<0.005$ and **=$p<0.001$

females, it can be concluded that only 25% of the candidate female broodstock for both strains (HY strain: 14/50; HC strain: 7/30) were effective to found each strain, although it was not possible to estimate the number of effective males.

Gene diversity (H_e) associated with the microsatellite loci did not show pronounced differences between the hatchery strains and wild populations, although it is subject to one exception: a significant reduction of the H_e value was observed in the HF strain (see also below). The Kruskal-Wallis rank sum test, which is a conservative and non-parametric analysis, indeed revealed that there was a significant difference of the mean H_e values between the hatchery strains and wild populations, however, a previous genetic assessment of Japanese flounder using four microsatellite loci (Yoshida *et al.*, 2000), and several studies for other fish species (reviewed in O'Connell and Wright, 1997; Coughlan *et al.*, 1998; Norris *et al.*, 1999; Desvignes *et al.*, 2001) demonstrated that allelic diversity was substantially reduced in hatchery strains without significant differences of H_e values between hatchery strains and wild populations. In so far as a first generation hatchery strain, these results are hardly surprising since an estimate of heterozygosity could be inflated if the strain of interest was founded using heterozygous parents. A population bottleneck of short duration may possibly reduce the number of alleles without significant losses of heterozygous individuals (Allendorf, 1986; Allendorf and Ryman, 1987). While, it is likely that significant reductions of both microsatellite and

mtDNA variabilities detected in the HF strain might be caused by a population bottleneck together with occurrences of inbreeding events when this strain was founded. This is because the HF strain was founded using both wild caught fish and broodstock maintained in this hatchery, although the level of inbreeding, if any, seems not to be high since homozygote excess was not evident in this strain ($H_o/H_e=0.97$), and moreover, the F_{IS} value estimated for this strain was indeed higher compared with other samples, but not significant ($F_{IS}=0.018$, $p=0.20$).

Strain/population differentiation

The high F_{ST} and Φ_{ST} values estimated for between the hatchery strains, and between the hatchery strains and the wild populations, exhibit that there was pronounced genetic differentiation between these samples. This is in contrast to the results obtained by the previous mtDNA-RFLP approach (Sugaya *et al.*, 1999), which revealed that about a half of the 15 haplotypes detected in four Japanese flounder hatchery strains were commonly shared between at least two strains. These authors also reported that genetic heterogeneity between a hatchery group into which four hatchery strains were pooled and a wild group into which four wild populations were pooled was not significant. The results presented here do not support their findings since there was significant evidence of genetic heterogeneities between the hatchery and the wild group (Table 16: $F_{ST}=0.019$, $p=0.000$; $\Phi_{ST}=0.046$, $p=0.000$). The limited types of restriction endonucleases available for the use in the RFLP method (*Hpa* Ⅱ, *Hae* Ⅲ, and *Hind* Ⅲ) may not necessarily be satisfactory to detect genetic differentiation between strains on a fine scale, it is not clear however as regards the foundation and maintenance of the strains for which they screened.

Summary

This chapter demonstrated that the simultaneous use of the 11 microsatellite loci and the sequences of the mtDNA control region is a powerful approach to monitor genetic diversity within and between Japanese flounder hatchery strains. This chapter also revealed that there was pronounced genetic differentiation between the hatchery strains and the wild populations, and the loss of microsatellite alleles and mtDNA haplotypes in hatchery strains was typical due most likely to the limited number of effective parents. It should be noted that further massive stocking of hatchery-reared fish into the natural environment might possibly result in irredeemable losses of alleles (haplotypes) in natural stocks. The only way to minimize genetic impacts of stocking to natural stocks is to improve the genetic management of hatchery broodstock.

Chapter 5. Microsatellite-based pedigree reconstruction of a Japanese flounder hatchery strain

As seen in the preceding chapter, the genetic variability of Japanese flounder hatchery strains to be stocked into the natural environment is typically reduced. Rate of loss of genetic variability in a population is based on the effective population size (Primack, 1998), and the extent of the effective population size is affected by several factors such as a small number of founders, unequal sex ratio in breeding populations, and family-size variations (Gall, 1987; Hedrick, 2000). It is thus recommended that hatchery managers should strive to minimize the effects of these possible causes of reduced effective size, and such factors can be accurately evaluated by an examination of the pedigree structure in the strains of interest.

Almost all of the flounder hatcheries employ the mesocosm spawning method to produce flounder-seedlings available for stocking practices. Using this method however it is difficult to control the mating behavior of the broodstock in the spawning tanks, and thereby it is complex to predict the pedigree structure of reproductive output. In practice, hatchery-reared flounder are usually bred without any

consideration to the mating system, and consequently the pedigree structure of flounder hatchery strains seem to deviate far from the ideal conditions (*e. g.*, equal number of male and female parents, random distribution of family size) (Chapter 4; Fujii, 2001). While, intentional culling practice, usually for larger size in total length, is commonly operated in flounder hatcheries to encourage the survival of released flounder-seedlings in the natural habitat. Culling for particular traits however has the potential to give rise to a large family-lineage bias through selection of siblings (Allendorf and Phelps, 1980; Allendorf and Ryman, 1987), possibly resulting in the reduced effective size of released fish due to a large variance in family size. The pedigree structure therefore should ideally be monitored in both breeding and release programs as a routine hatchery operation in order to improve flounder hatchery management.

The microsatellite profiling technique has been acknowledged as an efficient approach to examine pedigree structure in mixed family tanks of several aquatic organisms (Herbinger *et al.*, 1995; O'Reilly *et al.*, 1998; Herbinger *et al.,* 1999; Perez-Enriquez *et al.*, 1999; Norris *et al.*, 2000; Huvet *et al.*, 2001; Selvamani *et al.*, 2001). This chapter illustrates an example of microsatellite-based pedigree reconstruction in a Japanese flounder hatchery strain, and describes several implications for further hatchery management options. The possible impacts of the stocking practices to the wild populations are also discussed. The number of broodstock parents used was relatively small (18 wild captives), however, the hatchery procedures by which the strain was created is essentially the same to those operated in other flounder hatcheries.

Materials and methods

Samples of reproductive output

Hatchery production was initiated in May 2001 at the Miyako Hatchery Station of the Japan Sea-Farming Association. A total of 18 fish including 12 ripe females (FM＃1-＃12) and six ripe males (M＃1-＃6), which were wild captives caught in Miyako Bay (142°E, 40°N), were allowed to spawn in a spawning tank. Sexual maturity and health conditions of these fish were examined through the hatchery operations prior to the mating event, and all fish were regarded to be in suitable condition for reproduction. Approximately one million eggs were produced during one night (24 May) without the aid of artificial stripping, and the eggs were transported to a 0.5 ton incubation tank. The hatching success of eggs was estimated by counting the number of eggs and larvae per unit volume (volume cubic method), and it turned out that almost all the collected eggs successfully hatched out (*i. e.,* approximately 100% success of fertilization and hatching). Out of which 600,000 F_1 offspring were randomly collected and communally reared in a 50 ton tank for four months. At four months of the age, the offspring were size-sorted for larger size in total length through a routine hatchery operation, and then the selected fish were tagged and stocked into Miyako Bay (60,000 fish).

Samples of offspring for pedigree reconstruction were collected at three stages during the rearing period. An arbitrary sample of 113 larvae was collected within 24 hours after hatching (sample abbreviation: OP0), and 216 fish were randomly collected at the age of one month (abbr., OP1). A further 407 fish were sampled at four months of the age (abbr., OP4) which was timed with the stocking practice, and this sample was size-sorted (see above): just before the stocking event, 207 individuals were sampled from the fish that were selected for the release (total length, 53.8-97.2mm; mean±s.d. 78.0±7.3mm), and 200 individuals were sampled from the fish selected against (*i. e.*, not to be released) (total length, 37.2-59.4mm; mean±s.d. 50.1±3.7mm). These subsets of non-arbitrary fish were sampled to address the question of whether routine culling operations (size-dependent) in hatcheries would generate a significant skew of family size between the

released and non-released fish.

Parentage determination and statistical analysis

Four microsatellites (*Po1, Po13, Po42*, and *Po91*; Chapter 2) were profiled for all fish samples. Once the microsatellite genotypes of all candidate broodstock and offspring were profiled, each offspring was assigned to its parents by looking at whether the potential parental couples would exactly generate the offspring's genotypes for all loci.

The contribution of each family to the released fish was evaluated using the size-sorted sample (OP4), as the ratio of the number of released fish divided by the number of released fish plus non-released fish. The estimated value was compared with the expectation in each family (released fish: non-released fish=1:1) by using the *G*-test for goodness of fit (Sokal and Rohlf, 1997) with the Williams's correction by which the *G* value can be approximated better to the chi-square distribution (Williams, 1976). The significance level for all multiple tests was adjusted according to the method described by Rice (1989).

The effective population size was calculated based on the formula described by Lande and Barrowclough (1987). First, the effective number of female parents (N_{ef}) and male parents (N_{em}) were calculated according to the formula: $N_{ef}=(N_f k_f\text{-}1)/(k_f-1+V_{kf}/k_f)$ and $N_e m=(N_m k_m-1)/(k_m-1+V_{km}/k_m)$, where N_f and N_m is the census number of female and male parents, k_f and k_m is the mean number of offspring per female and male, and V_{kf} and V_{km} represents the variance in family size for each sex, respectively. This approach assumes non-Poisson distributions of gametes for each sex. The N_{ef} and $N_e m$ value were then combined to calculate the overall effective size (N_e) as follows: $N_e=4N_{ef}\,N_e m/(N_{ef}+N_e m)$.

Possible stocking impact on the effective size maintained in a wild population (N_w) was approximated on the basis of the formula: $1/N_{et}=x^2/N_c+(1-x)^2/N_w$, where N_{et} is the total effective size of the wild population and released hatchery fish, N_c is the effective size of the hatchery fish, and x is the relative contribution from the hatchery fish (Ryman and Laikre, 1991). The effective size of 7,900, which was estimated for a wild population described in Chapter 3 (IWT population), was used as the N_w value.

Results

Allelic variations of the four loci proved to be extensive in 18 potential broodstock (Table 17), and the number of alleles varied depending on the locus (minimum 13 at the *Po42* locus, maximum 19 at the *Po91* locus). Unique alleles, that is, alleles unique to just one individual, were detected for all loci. At the three loci (*Po1, Po13*, and *Po91*), the number of unique alleles was more than a half of the total number of detected alleles. That is, 15 of the 18 broodstock had unique alleles for at least one locus. These unique alleles were efficient indicators to trace pedigrees.

The parentage determination successfully assigned all the offspring back to a single parental couple. Table 18 shows the family size of each family observed in each of the three samples (OP0, OP1, and OP4 samples). A total of nine families were identified across the three samples, and there was an apparent difference of family size among the families. Six families were attributable to one male (M#2): more than 99% offspring were sired by male M#2, and two other males (M#1 and #6) sired only a very few offspring. Three males (M#3, #4, and #5) and six females (FM#1, #2, #3, #5, #6, and #8) did not contribute to the next generation.

A significant difference of the proportion of family size between samples was observed in three families after correction of the significance level for 18 simultaneous test ($p<0.0028$), in family E (between OP0 and OP1 samples, $z=3.68$, $p=0.0002$; OP0-OP4, $z=3.46$, $p=0.0005$), family G (OP0-OP1, $z=4.13$, $p=0.0000$), and family I (OP1-OP4, $z=3.03$, $p=0.0024$, marginal significance). Although the causes of the significant fluctuations in family size can not evidently be determined, these results might be

due to differences in the initial family survival performance. Given the fact that all families identified in the OP0 sample (0 day offspring) appeared in both OP1 (1 month) and OP4 (4 months) samples, the differences of family survival performance, if any, did not result in the demise of any particular family.

The *G*-test revealed that the contribution of offspring to the released fish significantly departed from the expectations in three families, family E, F, and G (Table 18). In families E and G, the contribution to the released fish was significantly lower than that to the non-released fish (family E, $G_{adj}=8.26$, $1df$, $p<0.008$ after correction for 6 simultaneous tests; family G, $G_{adj}=17.55$, $1df$, $p<0.008$, 6 simultaneous tests), while the contribution to the released fish in the family F was significantly high ($G_{adj}=38.19$, $1df$, $p<0.008$, 6 simultaneous tests), suggesting that culling operations would have the potential to select for particular family lineages. The remaining three families, family D, H, and I, did not show any significant deviations from the expectations ($1df$, $p>0.008$, 6 simultaneous tests).

The effective size of this strain was estimated to be of 3.3 (average of the 3 samples) after correction of unequal sex ratio coupled with unequal family size, and it was decreased by 80% of that calculated assuming an ideal situation, that is, all the potential broodstock could equally contribute to the offspring pool ($N_e=16$). Total effective size (N_{et}: wild population and hatchery strain) was also estimated to see the extent of the stocking effect to the total effective size (Fig. 6). The N_{et} value was drastically decreased even though the relative contribution from the hatchery strain was minimal: 20% contribution from the hatchery strain was sufficient to diminish 99% of the ini-

Table 17. Genotypes of 4 microsatellites determined for the 18 candidate broodstock

Candidate broodstock		Genotypes			
Males	TL(cm)[*1]	***Po1***	***Po13***	***Po42***	***Po91***
M#1	50.0	162/196	208/214	172/174	148/156
M#2	49.5	162/180	224/234*	174/236*	156/158
M#3	43.6	198*/222*	212/228*	178/182	160/160
M#4	42.7	164/188	208/210*	178/194*	160/170*
M#5	38.2	162/164	218/232	172/192*	150/160
M#6	45.9	162/162	214/216	172/182	162/166*
Females					
FM#1	53.2	164/208*	232/238*	172/178	160/222*
FM#2	49.5	162/186*	216/236*	172/178	156/182*
FM#3	54.6	162/162	212/216	182/190	162/198*
FM#4	53.8	180/206*	208/212	172/180	190*/194
FM#5	51.0	184/188	208/216	172/176	148/148
FM#6	50.0	162/178*	216/260	172/172	150/194
FM#7	51.7	162/184	216/242*	180/196*	160/176
FM#8	50.5	190/204*	220*/250*	190/190	162/164*
FM#9	54.3	164/196	208/218	176/202*	150/178*
FM#10	50.0	162/162	208/212	176/180	156/156
FM#11	48.0	190/194*	208/212	186/186	146*/184*
FM#12	48.0	162/188	216/224	178/178	154*/158
No. of alleles		15	16	13	19
No. of unique alleles		8	9	5	11

Unique alleles, alleles unique to one individual, are denoted on the individual genotypes by adding an asterisk
[*1] Total length

tial effective size of the wild population (N_{et} =83). It is generally recommended that an effective size of 50 should be maintained in a short-term hatchery production, and 500 be desirable for a long-term self-sustainable hatchery production (FAO/UNEP, 1981). As seen in Fig. 6, the reference figures (N_e=50 and 500) indeed slowed the rate of loss of total effective size, however, a considerable reduction of the initial effective size of the wild population could be occurred as the increase of the contribution from the hatchery strain. The effective size of 500 is also considered as a minimum viable population size (MVP) in the context of genetic conservation (Franklin, 1980; Lande, 1988). The MVP value could be maintained when the contribution from the hatchery strain with N_e=3.3 is of less than 10%, and less than a 30% contribution from the hatchery strain with N_e=50 could maintain the total effective size above the MVP value.

Discussion

In most hatchery strains, an unequal contribution of broodstock to the next generation seems to be typical, particularly in cases that the mesocosm spawning method is employed (Taniguchi *et al.*, 1983; Sugama *et al.*, 1988; Perez-Enriquez *et al.*, 1999; Fujii, 2001). The hatchery strain screened in this study is an extreme case, especially regarding the broodstock males: more than 99% of the offspring turned out to have been sired by a single male (M#2) (Table 18). This is somewhat surprising given empirical observations of the mating behavior in Japanese flounder: one female is followed by several males before the release of eggs, and then she mates with multiple males simultaneously. Possible factors leading to the unsuccessful reproduction of other males, such as a poor sperm quality and gametic incompatibility, are very unlikely since the hatching success of the eggs was approximately 100%. There are no records available regarding the mating behavior in the spawning tank in which the strain was founded; a scenario can be drawn such that this male simultaneously (or alternately) mated with multiple females, and there might have been severe reproductive competition among

Table 18. Parental couples of the offspring determined on the basis of the microsatellite profiles, and the number of offspring in each family (family size)[*1]

Families		A	B	C	D	E	F	G	H	I
Male parents		M#1	M#1	M#6	M#2	M#2	M#2	M#2	M#2	M#2
Female parents		FM#4	FM#11	FM#11	FM#4	FM#7	FM#9	FM#10	FM#11	FM#12
Offspring samples	(*N*)									
OP0[*2]	113	0(0.0)	0(0.0)	0(0.0)	29(25.7)	14(12.4)	33(29.2)	22(19.5)	4(3.5)	11(9.7)
OP1[*3]	216	1(0.5)	0(0.0)	0(0.0)	39(18.1)	44(20.4)	41(19.0)	68(31.4)	13(6.0)	10(4.6)
OP4[*4]	407	0(0.0)	1(0.2)	1(0.2)	79(19.4)	71(17.4)	100(24.6)	105(25.9)	18(4.4)	32(7.9)
#Released	207	0(0.0)	1(0.5)	0(0.0)	48(23.2)	24(11.6)	78(37.6)	31(15.0)	7(3.4)	18(8.7)
#Non-released	200	0(0.0)	0(0.0)	1(0.5)	31(15.5)	47(23.5)	22(11.0)	74(37.0)	11(5.5)	14(7.0)
Percent of each family in the released group		NA	NA	NA	60.8	33.8	78.0	29.8	38.9	56.3
G_{adj} value(1*df*)[*5]		NA	NA	NA	3.8	8.3*	38.2*	17.6*	1.5	0.5
p		-	-	-	0.051	0.004	0.000	0.000	0.215	0.475

[*1] Proportion of the family size in each sample is shown in parenthesis(%)
[*2] Random sample collected within 24 hours after hatching
[*3] Random sample collected at 1 month after hatching
[*4] Size-sorted sample collected at the age of 4 months (For details, see text)
[*5] Significance was tested by using the *G*-test(Sokal and Rohlf, 1997) with the Williams's correction (Williams, 1976). Null hypothesis was assumed as the ratio of the released fish and non-released fish was of 1: 1. Significance was denoted by adding *=$p<0.008$ after correction for 6 simultaneous tests. The *p* value is also shown below the G_{adj} value. For family A, B, and C, the *G*-test was not conducted(NA).

males at the mating event.

The effective size of this strain was of just 3.3, and taken this situation, 15-fold or larger number of founders and spawning tanks needed to be prepared to achieve the effective population size of 50, which is a reference figure generally recommended for a short-term hatchery production related to stock enhancement programs (FAO/UNEP, 1981). It is evident that the successful contribution of this strain to the natural environment will result in a highly destructive impact to the total effective size (Fig. 6), and the recommended figure for short-term hatchery production (N_e=50) do not promise to overcome the risk of significant loss of the total effective size; nevertheless hatchery managers should strive to reach the guideline to relieve the effects of the potential stocking impact as much as possible.

One of the most effective hatchery options to increase the effective size in hatchery strains would be to employ the stripping method with one-to-one crossing using a large number of broodstock, which is expected to lead to more homogenous contribution of the broodstock parents. The stripping method is however difficult to practically adopt in routine flounder hatchery procedures due to several constraints such as the nocturnal spawning behavior and the handling-vulnerability of this species. Therefore hatchery management should be improved so as to achieve a large effective size on the premise that the hatchery production will continue to rely on the use of the mesocosm spawning method. The strain screened in this study originated from the fertilized eggs produced in a single night with a relatively small number of candidate broodstock, and thus it can not be set aside the possibility that other potential broodstock would produce offspring in the other days; Hirano and Yamamoto (1992) reported that ripe females released eggs intermittently but irregularly during the spawning period that lasts for as long as three months (individual spawning days per spawning period was estimated to be of 66-88% in the five females they examined), although there have been limited data available as to the reproductive rhythm of males. In such a case, temporal collections of the fertilized eggs produced by variable-in-time parental couples could encourage the increase of the effective size. Alternatively, assuming that the skewed contribution of broodstock was largely caused by an interaction which arose among the potential broodstock such as a reproductive competition (see above), the effective size could be increased by subdividing the broodstock into multiple spawning tanks to minimize the effects of detrimental interactions.

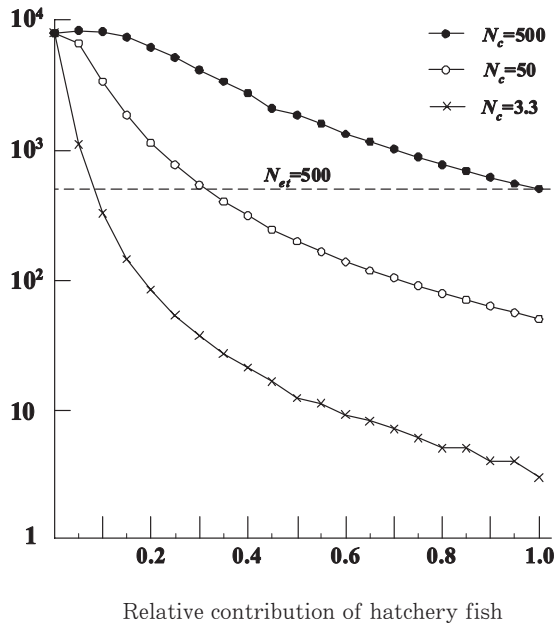

Fig. 6. Total effective size (N_{et}) of wild and hatchery (released) fish (Ryman and Laikre, 1991). The x-axis indicates the relative contribution of released hatchery fish to the total fish (wild plus hatchery fish). The y-axis (N_{et}) is scaled in common logarithm. N_c represents the effective size of hatchery fish, and the initial effective size of wild fish is assumed as of 7,900 (see text). The minimum viable population size of 500 (Franklin, 1980; Lande, 1988) is shown by dotted line.

Another important finding in this study is that the culling operation for larger size fish generated significant deviations of family size between the released and non-released fish. The noticeable variance in the early growth of offspring might be related to the family growth performance. It should be emphasized that even

if an idealized strain with a large number of founders is initially created, culling operations might cause a large family size variance of released fish through selection in favor of a few family lineages. The present author therefore suggests that culling operations for particular traits be avoided to minimize the risk of unexpected pedigree structure to be a possible cause of the reduced effective size.

Summary

This chapter demonstrated that the microsatellite profiling technique would provide the most efficient means to trace pedigrees in mixed family tanks of Japanese flounder. The contribution of broodstock parents to the next generation in the strain screened in this study highly skewed, and such a skewness would be frequently seen in every hatchery production. The present author thus suggests that monitoring of pedigree structure ideally be implemented in every case of hatchery productions. At the same time, the hatchery management options recommended above should be adopted to encourage the contribution of broodstock parents as much as possible.

Chapter 6. Management strategy to minimize loss of genetic variations in closed hatchery strains

It is generally recommended that a large number of unrelated wild fish should ideally be used as the founders of hatchery strains to be reintroduced to the natural habitat so that the genetic variability can be maximized, and at the same time, that the genetic impacts upon the wild fish populations can be minimized (FAO/UNEP, 1981; FAO, 1993). Such recommendations may sometimes be difficult to adopt in practical hatchery-production procedures due to several constraints involving the labor and budget intensity associated with the collection and maintenance of wild fish, and thereby hatchery managers may be constrained to use a subset of hatchery-based fish with a few wild fish as founders to create the descendent generations. This surely enhances the decay of genetic variability in the subsequent generations through a severe genetic drift coupled with inbreeding. In such cases, it is crucial to explore ways that the further loss of founders' genetic variations can be minimized.

Maximal retention of genetic variations from captive populations to the descendants could be achieved through the selection and mating of genetically important individuals in the focused populations, rather than through random mating (Ballou and Lacy, 1995). Several measures to evaluate the genetic importance of individuals have been proposed such as the founder importance coefficient (*fic*: Ballou and Lacy, 1995), genome uniqueness (*gu*: MacCluer *et al.*, 1986), and mean kinships (*mk*: Ballou and Lacy, 1995). A computer simulation study conducted by Ballou and Lacy (1995) suggested that the *mk* provides the most efficient measure to rank genetically important individuals. The *mk* measure is calculated based on the kinship coefficient (f_{ij}: Falconer and MacKay, 1996), which is the probability that alleles possessed by two individuals at the same locus are identical by descent. The mean kinship of individual i is defined as the average f_{ij} value between individual i and all other individuals including itself, and the individuals with low *mk* values are ranked as genetically important individuals (Ballou and Lacy, 1995). The breeding system using the *mk* estimator (mean kinship approach, Ballou and Lacy, 1995) is proceeded as the lowest-ranked male is mated with the lowest-ranked female, and after removing these lowest-ranked individuals for both sexes, the *mk* value is then re-calculated for all the possible combinations of the remaining individuals, and again the individuals with the lowest rank are paired. This procedure is continued until the required number of offspring is obtained depending on the husbandry's capacity. This strategy, in other words, gives a high priority to the individuals with the lowest degree of relatedness to other members in the population, whose alleles have a risk to be undelivered to

the next generations (Rodriguez-Clark, 1999; Lacy, 2000).

It appears that the breeding strategy based on the mean kinship values, which is conceptually called as minimal kinship selection (Doyle *et al.*, 2001), can serve as an efficient hatchery management option to slow the loss of genetic variations in captive populations (Ballou and Lacy, 1995; Montgomery *et al.*, 1997; Rodriguez-Clark, 1999; but see Caballero and Toro, 2000; Fernandez and Caballero, 2001), the practical application of the minimal kinship selection (*mk* selection) however needs the knowledge of the genealogies to estimate the f_{ij} value, which is not available in most cases in Japanese flounder hatchery strains. An alternative measure to clarify the relatedness between individuals is thus required to apply the *mk* selection approach to non-pedigreed strains. A currently available statistic to estimate inter-individual relatedness without pedigree information is the pairwise relatedness coefficient r_{xy}, devised by Queller and Goodnight (1989), as the availability to classify individuals by kinships has extensively been documented (*e. g.,* Blouin *et al.*, 1996; Taylor *et al.*, 1997; Fontaine and Dodson, 1999; Norris *et al.*, 2000).

The first purpose in this chapter is to validate the microsatellite-based r_{xy} statistic as a kinship estimator in a Japanese flounder hatchery strain. Eleven microsatellite markers for which the previous chapters demonstrated the utility were employed to calculate the r_{xy} statistic. Secondly, the present author discusses the potential of the *mk* selection approach in preservation of the genetic variations in Japanese flounder hatchery strains, and several management options applicable to the hatchery production are proposed. In this study, a hatchery strain of Japanese flounder founded by wild captives is referred to a source strain for which the generation is denoted as G_1, and the founder generation (wild captives) is regared as G_0. The focus of interest in this chapter is to explore an effective way to retard the loss of the genetic variability from the generation G_1 to the next generation with a few or no available genetic inputs.

Materials and Methods

Pedigree reconstruction

A hatchery strain was created at the Miyako Hatchery Station by placing a total of 14 candidate parents (G_0 fish) including six ripe females (F#1-#6) and eight ripe males (M#1-#8) in a spawning tank for four days. Approximately 500,000 larvae were produced, of which 83 fish sampled at the age of one month (G_1 offspring) together with the 14 captive broodstock parents were screened for the 11 microsatellites (*Po1, Po13, Po25A, Po26, Po33, Po35, Po42, Po48*, *Po52*, *Po56*, and *Po91*, see Chapter 2), and the pedigrees were reconstructed in the same manner described in the preceding chapter. The pedigree information was further used to validate the r_{xy} statistic (Queller and Goodnight, 1989) as a kinship-estimator (see below).

Estimation of pairwise relatedness coefficient

The pairwise r_{xy} statistic, which is calculated correcting a bias for the population allele frequencies by which the relatedness value between individuals x and y will possibly be biased (Queller and Goodnight, 1989), was estimated for all possible dyads within the G_1 offspring based on the individual microsatellite genotypes. The r_{xy} values obtained were compared among three categories including full sibs, half sibs, and unrelated pairs by means of the Kruskal-Wallis nonparametric analysis (Sokal and Rohlf, 1997) using the statistical analysis software STATPARTNER version 4.5 (O-ha Inc., Tokyo). Misclassification rate of the r_{xy} estimator, which is the rate of the errors that an individual in a kinship category is falsely assigned to the other categories, was estimated on the basis of the cut-off value defined as the midpoint between the mean r_{xy} values of any two of the three categories (see Blouin *et al* ., 1996). The utility of the r_{xy} statistic to discriminate the kinships between the G_1 offspring, without the pedigree information, was also addressed by estimating inter-individual

genetic distance, which was simply defined in this study as $D_{(r)}=1.0-r_{xy}$. Negative r_{xy} values were tallied as $r_{xy}=0.0$ (*i. e.*, $D_{(r)}=1.0$). Based on the $D_{(r)}$ matrix obtained, an UPGMA tree topology was constructed using the PHYLIP version 3.6 software package (Felsenstein, 2000), and the tree was visualized by using the TREEEXPLORER software package version 2.12 (provided by Dr. K. Tamura, Tokyo Metropolitan University, Hachioji, Tokyo).

Minimal kinship selection

The average of r_{xy} values between one individual and all the other individuals (mean relatedness: mr_{xy}), was calculated for all G_1 offspring. The original *mk* selection approach devised by Ballou and Lacy (1995) is proceeded by mating between the lowest *mk* male and female by one-to-one crossing (see above), this approach however was not possible to adopt in this study without modifications since there were no records available to determine the sex of the G_1 offspring, and thus the *mk* selection procedures were further simplified, as proposed by Doyle *et al.* (2001), as a subset of lower mr_{xy}-scored fish were selected without doing the recalculation (mk_r selection). It was considered, by the present author, that the mk_r selection would conversely be more applicable to the currently operated production-regimen in Japanese flounder hatcheries as this approach will be more applicable to the mesocosm breeding method than is the original (one-to-one crossing).

The effectiveness of the mk_r selection approach was also examined for a hypothetical situation that the G_1 offspring were kept together with some wild captives. In such a case, it was expected that the wild fish could preferably be selected through the mk_r selection procedures since the wild fish were likely to be unrelated from any members of the G_1 offspring, and since the mk_r selection would theoretically be favorable to the individuals who carry rare genotypes (Ballou and Lacy, 1995). The mk_r selection was applied to a hypothetical strain (abbreviation, G_1^+) consisting of the 83 G_1 offspring and nine wild fish that were collected at Miyako Bay (*i. e.*, the wild fish comprised 10% of this hypothetical strain).

The number of fish to be selected was set as eight, just as same as the census number of the actual G_0 founders (see below). The genetic variability in terms of the allelic diversity (number of alleles per locus: *A*) and the gene diversity (unbiased expected heterozygosity: H_e) estimated for the mk_r-selected fish was compared with that of randomly generated subgroups from the G_1 offspring (or G_1^+ sample) through the Monte Carlo procedures with 100 iterations.

Computer programs to execute the mk_r selection (including the r_{xy} statistic calculation) and Monte Calro simulation were written by the Visual Basic, and the programs were run on the Excel 2000 software (Microsoft Co., Redmond, WA).

Results

Relatedness estimator r_{xy} to distinguish kinships

The pedigrees of the G_1 offspring were unambiguously determined (Table 19): two of the six females (F#5, #6) and four of the eight males did not contribute to the G_1 offspring pool, and the remaining four females and four males produced 10 pedigrees. A total of 3403 dyads in the G_1 offspring were obtained, of which 513 dyads were categorized as full-sibs, 1085 were as half-sibs, and 1805 were as unrelated pairs. The pairwise r_{xy} values in the G_1 offspring ranged from -0.551 to 0.782 (mean$\pm$s. d., -0.013 ± 0.249). Percentage distribution of the r_{xy} values calculated for all dyads in each kinship category is plotted in Fig. 7. The mean r_{xy} values within the categories were estimated as follows: full-sibs: 0.369 ± 0.173; half-sibs: 0.081 ± 0.166; unrelated pairs: -0.178 ± 0.134. The Kruskal-Wallis rank-sum analysis revealed that the difference of the r_{xy} values was highly significant among and between the categories (overall: $H=2022.9$, $2df$, $p=0.000$; between full-sibs and half-sibs: $H=616.0$, $1df$, $p=0.000$; full-sibs and unrelated pairs: $H=1156.9$, $1df$, $p=0.000$; half-sibs and unrelated pairs: $H=1208.0$, $1df$, $p=0.000$).

When the threshold values (cut-off value) to

classify kinships between individuals described above was applied (Table 20), full-sibs and unrelated pairs were distinguishable with a high accuracy at the cut-off value of $r_{xy}=0.095$, as just 0.2% full-sibs were misallocated into unrelated category, and 3 % unrelated pairs were erroneously classified as full-sibs. Cut-off value defined for between full-sibs and half-sibs ($r_{xy}=0.225$) yielded approximately 20% misclassification rates reciprocally, and 17% half-sibs and 23% unrelated pairs were misclassified into each alternate category at the cut-off value of $r_{xy}=-0.049$. These misclassification rates were close to those estimated by Blouin *et al.* (1996), who studied the potential of the r_{xy} statistic to discriminate kinships, based on 20 microsatellite markers for a set of simulated genealogical lines of house mouse (misclassification rates between full-sibs and unrelated pairs: 2.5%; full-sibs and half-sibs: 15-16%; half-sibs and unrelated pairs: 15-17%).

The UPGMA tree topology showing the sibships of the G_1 offspring reconstructed on the basis of the r_{xy}-based genetic distance is given in Fig. 8 . The tree topology advocated the validity of the distance measure to portray the genealogies: 96% of fish were clustered well in accordance with the true kinships, although there were a few exceptions such that two fish in family B (OP3 and OP69) were more closely clustered with four fish in family F rather than are with the other members in family B, and one of the two fish in family D (OP22) was positioned in the neighborhood of OP42 (family J) apart from the full sibling (OP5).

Minimal kinship selection

The mr_{xy} values of the G_1 offspring ranged from -0.103 to 0.118 (mean$\pm$s.d., -0.013 ± 0.045), and the mk_r selection procedures retrieved the eight lowest mr_{xy} fish (mk-G_1 sample) including two fish from each of the three families (family A, D, and H) and one from each of the two families (family B and E). None of the members in five families (family C, F, G, I, and J) were found in the mk-G_1 sample. Regarding the G_1^+ hypothetical strain consisting of the 83 G_1 offspring and nine wild fish, the mr_{xy} values ranged from -0.189 to 0.125 (mean$\pm$s.d., -0.011 ± 0.052), and eight fish including six wild fish and two G_1 offspring (family D and E) were drawn through the mk_r selection (mk-G_1^+ sample). Table 21 gives the summary statistics of the genetic variability estimated for the two mk_r samples together with that of randomly selected subgroups (G_1-*rand* derived from the G_1 and G_1^+-*rand* from the G_1^+). The comparison between the mk-G_1 sample and G_1-*rand* subgroups showed that the mk_r selection gave a slight benefit in the retention of the allelic diversity (A) than the random selection (mk-G_1: $A=6.18$; G_1-*rand*: mean$\pm$s. d., $A=5.85\pm0.39$, average of 100 iterations). The advantage of this approach to recover the allelic diversity was further strengthened when the mk-G_1^+ and G_1^+-*rand* samples were compared, as the A value was of 7.18 for the mk-G_1^+, and of 6.03 ± 0.57 (mean$\pm$ s. d.) for the G_1^+-*rand*. The A value estimated for the mk-G_1^+ sample was consistently greater than that of the 100 iterated random samples with just one exception: the highest A value ($A=7.36$) was obtained at one instance in the 100 iterations conducted.

The mk_r selection was also effective to recover the gene diversity (H_e) as the mk-G_1 and mk-G_1^+ samples yielded a higher H_e value (mk-G_1: $H_e=0.78$; mk-G_1^+: $H_e=0.79$) than the average of the arbitrary subgroups (G_1-*rand*: mean$\pm$s. d, 0.76 ± 0.02; G_1^+-*rand*: 0.77 ± 0.03). It should however be stressed that heterozygosity would be insensitive to a population bottleneck for a short duration, and not greatly be affected by the loss of low frequency alleles (Chapter 4; Allendorf, 1986). This can be seen in the results presented here: both the mk_r-selected samples (mk-G_1 and mk-G_1^+) and arbitrary subgroups (G_1-*rand* and G_1^+-*rand*) generated a comparably higher level of gene diversity than the source strain (G_1 offspring: $H_e=0.69$; G_1^+ sample: $H_e=0.70$), despite the fact that the allelic diversity maintained in the source strains were lost in the selected subgroups, suggesting that the gene diversity alone not be used as a measure of the genetic variability in captive populations.

Table 19. Contribution of the G_0 broodstock parents to the G_1 offspring pool

Families	A	B	C	D	E	F	G	H	I	J	
Parents											
Males	M#2	M#6	M#8	M#2	M#5	M#6	M#5	M#6	M#5	M#2	
Females	F#1	F#1	F#1	F#3	F#3	F#3	F#4	F#4	F#2	F#2	Total
Number of offspring	16	10	12	2	21	4	11	5	1	1	83

Table 20. Possible errors to misclassify individuals by relatedness estimator (r_{xy}) and the misclassification rates estimated for between the kinship categories

Type of errors		Cut-off value* (r_{xy})	Misclassification rate (%)
True kinship	Misclassified as#		
	#		
Full-sibs	Half-sibs	0.225	20.7
	Unrelated	0.095	0.2
Half-sibs	Full-sibs	0.225	19.8
	Unrelated	−0.049	22.8
Unrelated	Full-sibs	0.095	2.8
	Half-sibs	−0.049	16.8

* Cut-off value was defined as the midpoint value between any two categories (Blouin *et al.*, 1996)

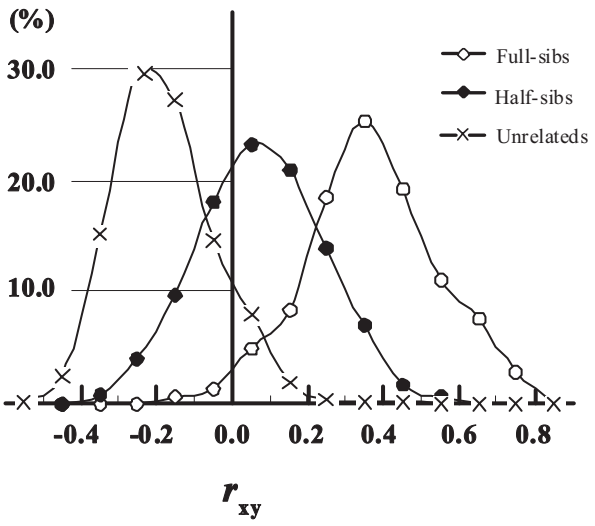

Fig. 7. Percentage distribution of the relatedness estimator estimated r_{xy} for full-sib, half-sib, and unrelated categories in the G_1 offspring. The number of dyads in each category is as follows: full-sibs: 513 dyads; half-sibs: 1,085 dyads; unrelated pairs: 1,805 dyads.

Discussion

Relatedness estimator to resolve kinships

As in a common hatchery-production practice of the Japanese flounder, reproductive outputs are kept and reared in mixed family tanks without any external tags, and unfortunately, it is usual that the historical records of the established strains are not available, and therefore pedigree tracing is difficult, or rather, impossible. This is of a special concern when further generations are constrained to be created by using such non-pedigreed strains, of which there might be highly related individuals. Sib mating should be avoided to minimize the rate of inbreeding in order to counter the threat of inbreeding depression, which may possibly be embodied within a few generations (Princee, 1995 and references therein). Inference of sibships for non-pedigreed captive (or wild) populations in the absence of the pedigree information has been a focus of recent attention

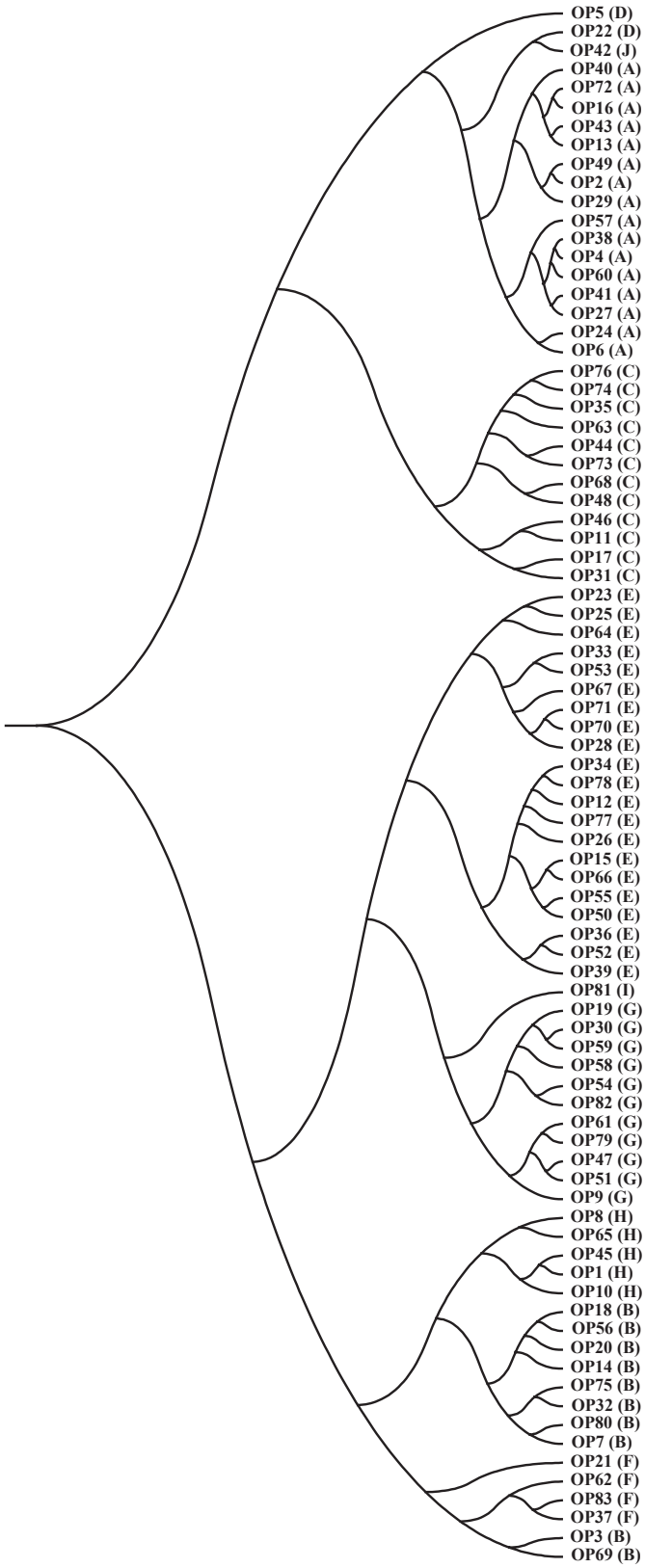

Fig. 8. UPGMA tree topology showing the sibships between individuals in the G_1 offspring on the basis of the genetic distance, $D_{(r)}=1-r_{xy}$ (see text). The tree is rooted by the midpoint rooting method. Labels at branch tips indicate the identity number of offspring, and the pedigree of each offspring is also given in parenthesis.

(Ritland, 2000); the relatedness estimator r_{xy} based on the 11 microsatellite markers employed in this study proved to be useful as a measure of the identity by descent, as the use of the midpoint cut-off value achieved to discriminate the fish in full-sib category from unrelated pairs, or *vice versa*, with few misclassification errors, although there was a relatively high probability of misclassification errors arising between the half-sib and the other categories (approximately 20% misclassification rates). The r_{xy} statistic sometimes entails a wide range of variations even within a kinship category as Queller and Goodnight (1989) pointed out; nevertheless the present author suggests that the microsatellite-based r_{xy} statistic provides an efficient tool as a kinship indicator with the practical uses to minimize the risk of sib mating. The ability of the r_{xy} statistic to resolve kinships was also verified by the attempted tree-topology reconstruction given in Fig. 8. This graphical analysis would be helpful to give a brief overview of sibships between individuals in a non-pedigreed hatchery strain.

Minimal kinship selection

The minimal kinship selection strategy (*mk* selection) following a proposal by Ballou and Lacy (1995) is widely approved as the most efficient and intuitively understandable approach to recover the genetic variations in a captive population. The mk_r selection, which is the simplified version of the *mk* approach (Doyle *et al.*, 2001) employed in this study, also had a benefit to retain the genetic variability compared with the random selection. Given the theory that the *mk* concept will give a focus on the individuals with rare genotypes (Ballou and Lacy, 1995), the efficiency of the *mk* selection in allelic retention would depend on the frequency of rare alleles maintained in the population of interest. As seen in this study, adding a few exotic alleles (wild fish) to the G_1 offspring that had a relatively low level of allelic variations due to the limited number of founders (4 females and 4 males) gave a better performance of the mk_r selection. While, it should be noted that none of the members in several families were found in the mk_r selected subgroup. These results are, however, barely surprising since the *mk* concept aims to find out the genetically important individuals whose alleles are not common in the studied population with the notion that underrepresented founder alleles should be propagated, by which genetic variations could be ensured (Ballou and Lacy, 1995; Lacy, 1995).

Although the high utility of the *mk* selection strategy for the management of captive populations has been accepted in the context of conservation genetics as cited above, there seems to be still room for a measure of disagreement about the *mk* concept: this approach has a drawback upon reducing the rate of inbreeding (Caballero and Toro, 2000). A previous simulation analysis studied by Fernandez and Caballero (2001) concluded that the *mk* selection approach applied to hypothetical populations indeed offered a

Table 21. Genetic variability in terms of the allelic diversity (number of alleles per locus: A) and gene diversity (unbiased expected heterozygosity: H_e; Nei, 1987)

	Parental generation (G_0)[*1]		G_1 offspring			G_1 offspring plus wild fish (G_1^+)[*2]		
	Candidates	Actual parents	Overall	*mk*-G_1	G_1-*rand*	Overall	*mk*-G_1^+	G_1^+-*rand*
Number of fish	14	8	83	8	8	92	8	8
Allelic diversity(A)	10.64	8.27	8.18	6.18	5.85(±0.39)	10.73	7.18	6.03(±0.57)
Gene diversity(H_e)	0.75	0.75	0.69	0.78	0.76(±0.02)	0.70	0.79	0.77(±0.03)

[*1] Parental generation was a subset of wild fish, which were tentatively considered as unrelated fish
[*2] Hypothetical hatchery strain consisting of the 83 G_1 offspring and 9 wild fish
mk-G_1 and *mk*-G_1^+ refer to a subgroup selected through mk_r procedures, and G_1-*rand* and G_1^+-*rand* are random samples selected through the Monte Carlo simulation with 100 iterations (see test for details)
For the random samples, the average of the A and H_e values are shown. Standard deviation of the measures is given in parenthesis.

benefit in retarding the loss of genetic diversity in terms of the allelic diversity and gene diversity, it gave however a poor performance to maintain the overall population fitness compared with other strategies including random mating and compensatory mating approach in which the highest mean kinship male in a population is mated with the lowest female (Santiago and Caballero, 1995; Caballero *et al.*, 1996), resulted from the inbreeding depression. Ballou and Lacy (1995) themselves state in their literature that the *mk* selection strategy would be accompanied by a risk of inbreeding especially in early generations, and they emphasize the necessity to preclude mating among highly related individuals. In this study, the eight mk_r-selected G_1 fish yielded 28 dyads of which three dyads were full-sibs, 10 were half-sibs, and the remaining 15 dyads were unrelateds. Although the sexes of the selected fish cannot be determined, it is very likely that the use of the subset of these fish to create the next generation would result in inbreeding to a greater or lesser extent. The breeding regimen should be thus designed so as to avoid possible sib mating, that is, unrelated pairs should be selected and mated to minimize inbreeding. When the mean r_{xy} value of unrelated pairs in the G_1 offspring (r_{xy} = -0.178) was applied as a cut-off value to discriminate the unrelated pairs from the other categories, one of the 15 unrelated pairs were still misclassified into half-sibs, and three of the 10 half-sibs, of which the smaller r_{xy} values than the cut-off value were yielded, were falsely assigned to the unrelated category; notwithstanding the kinship information derived from the r_{xy} statistic could greatly improve the efficiency in the avoidance of sib mating.

Summary

For the closed, or nearly closed hatchery production of Japanese flounder in the absence of pedigree information, the present author proposes several hatchery options as follows: first, genetically important individuals should be explored through the mk_r selection procedures. The mk_r selection would be more feasible in the Japanese flounder hatchery production rather than the original *mk* selection approach. Next, the kinships of the selected fish should be investigated by using the r_{xy} statistic as a guideline. The definition of the cut-off value to determine unrelated pairs depends on hatchery's motivations, it is although expected that applying more stringent cut-off values will achieve more accurate kinship-discrimination. Then the selected fish should be subdivided into multiple spawning tanks minimizing the risk of sib mating. These options may not necessary promise to stamp out the possibility of inbreeding, but will promise better outcomes than blind hatchery practices.

Chapter 7. Conclusions and perspectives

As described in Chapter 5, the minimum effective population size of 50 is widely accepted as a guideline value for the short-term hatchery production related to stock enhancement programs. It should however be noted that even if the reference figure could be achieved, stocking practice have an inherent risk to reduce the effective size of wild populations, especially when the contribution of stocked fish to reproduction in the natural populations is high (Chapter 5). In this regard, the notion of conservation genetics encounters a counterargument brought from the practical hatchery's scope: in the sense of conservation genetics, it may be preferable that the contribution of the stocked fish to the natural populations should be minimized to retain the effective size of wild populations at the current level; from the viewpoint of practical hatchery concept, however, the contribution of stocked fish to the natural populations should be maximized to increase the exploitable biomass. The ideas from the two sides are paradoxical, and it is considered, by the present author, that one of the compromise proposals is to increase the effective size of hatchery strains as much as possible.

Based on the results described in the previous chapters, the present author proposes here

several hatchery options to maximize the effective size of Japanese flounder hatchery strains, and to minimize the genetic impact of the stocking practice to the natural as follows: (1) captive broodstock to create new hatchery strains should be taken from the indigenous wild populations, and then the outcomes should be released back into the natural habitat from which the founders were derived. Because the wild Japanese flounder populations around Japan should not be managed as a single stock unit (see Chapter 3), and such efforts will encourage to preserve the currently detectable genetic stock units; (2) at the mating event, wild fish (unrelated in all likelihood) and spawning tanks should be prepared as many as possible, and the mating procedures should be carried out in separated tanks subdividing the candidate broodstock parents, rather than mating them in a single spawning tank. This mating design enables to minimize unexpected interactions among the candidates, which may allow a skewed family size dominated by a few broodstock parents (Chapter 5). The offspring pool into which the reproductive output derived from each tank are mixed will be available for stocking practices; (3) collection of fertilized eggs should be carried out for several days and on several times during the spawning season; (4) culling for particular traits should be avoided both during the rearing period and at the release event; (5) when the offspring pools to be stocked into the natural environment have to be created by using hatchery-based fish, the genetically important individuals in the strains of interest should be explored, and the kinships should be determined to avoid sib-mating as proposed in Chapter 6. A guideline for hatchery production, recommended by the present author, is summarized in Fig. 9.

To practically carry out the recommendations outlined above however may entail a trade-off between costs (labor, budget, and facility availability) and benefits in hatcheries; notwithstanding it should be emphasized that stock enhancement programs should not aim to yield a temporal harvestable surplus, but rather the perpetual exploitation of resources through the enhancement of natural stocks. Genetic considerations from the standpoint of conservation genetics are therefore essential for this ultimate task.

While, the genetic tagging of hatchery-based fish would provide the most efficient means to examine the genetic effects of stocking practice upon the natural gene pools. This study could not provide concrete evidence of the stocking effects to the natural populations, the present author however considers that it is an important task for future genetic studies related to stock enhancement, as there have been limited studies available to enhance our understanding of the genetic stocking effects to the wild populations in marine fish species (but see Perez-Enriquez *et al.*, 2001; Svasand *et al.*, 2000). A long-term monitoring of stocking effects can greatly enhance our understanding of the reproductive success of stocked fish; it can only be determined with the use of the heritable molecular markers. This will in turn contribute to give more insight into the genetic management strategy for the Japanese flounder resources in the natural environment.

Finally, the present author will continue to stress that stock enhancement practices should aim to encourage the natural productivity that will secure the exploitable resource biomass for the future without the need for artificial help, but should not be directed toward a temporary recovery of resource losses caused by a fishery-related management failures such as over-exploitation.

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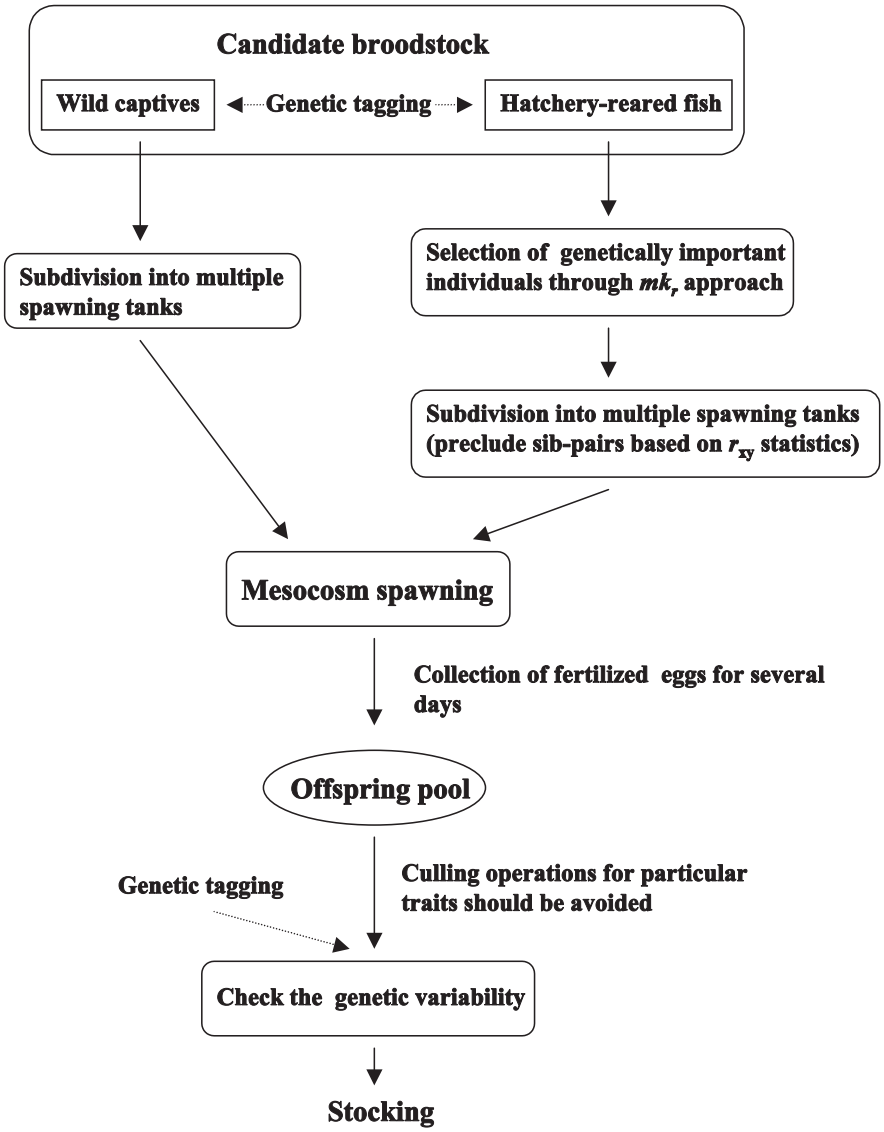

Fig. 9. Hatchery management options to minimize loss of genetic variability in Japanese flounder hatchery strains

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Appendix 1. Allele frequency distributions of 11 microsatellites for the 7 wild Japanese flounder populations

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po1	162	0.330	0.330	0.406	0.424	0.389	0.420	0.423
	164	0.080	0.080	0.094	0.085	0.023	0.083	0.068
	166	0.000	0.020	0.000	0.000	0.023	0.007	0.011
	168	0.000	0.000	0.000	0.014	0.000	0.014	0.000
	170	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	172	0.010	0.040	0.036	0.049	0.057	0.049	0.023
	174	0.020	0.030	0.022	0.007	0.045	0.007	0.011
	176	0.020	0.030	0.015	0.007	0.011	0.007	0.000
	178	0.040	0.040	0.029	0.014	0.068	0.042	0.000
	180	0.050	0.030	0.036	0.056	0.057	0.028	0.045
	182	0.030	0.010	0.000	0.021	0.011	0.000	0.011
	184	0.040	0.040	0.044	0.056	0.034	0.035	0.080
	186	0.040	0.010	0.029	0.007	0.023	0.028	0.034
	188	0.120	0.040	0.051	0.085	0.068	0.042	0.034
	190	0.040	0.050	0.036	0.021	0.011	0.028	0.057
	192	0.010	0.050	0.036	0.042	0.034	0.035	0.023
	194	0.020	0.020	0.015	0.021	0.000	0.000	0.000
	196	0.030	0.050	0.029	0.014	0.034	0.021	0.057
	198	0.010	0.000	0.007	0.021	0.000	0.014	0.000
	200	0.000	0.040	0.007	0.014	0.034	0.049	0.023
	202	0.010	0.020	0.007	0.007	0.011	0.007	0.011
	204	0.030	0.020	0.007	0.000	0.023	0.007	0.000
	206	0.000	0.010	0.015	0.000	0.000	0.007	0.000
	208	0.000	0.000	0.007	0.000	0.000	0.000	0.011
	210	0.010	0.000	0.029	0.000	0.000	0.014	0.000
	212	0.000	0.010	0.007	0.007	0.000	0.000	0.011
	214	0.010	0.030	0.015	0.021	0.011	0.007	0.011
	216	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	218	0.010	0.000	0.007	0.000	0.000	0.014	0.023
	220	0.000	0.000	0.000	0.000	0.011	0.007	0.000
	222	0.000	0.000	0.000	0.000	0.011	0.000	0.011
	226	0.020	0.000	0.007	0.000	0.000	0.000	0.000
	228	0.010	0.000	0.000	0.000	0.000	0.007	0.011
	230	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	232	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	234	0.000	0.000	0.007	0.000	0.011	0.007	0.000
	264	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Po13	206	0.000	0.000	0.030	0.007	0.014	0.000	0.021
	208	0.100	0.100	0.160	0.109	0.111	0.103	0.099
	210	0.010	0.010	0.010	0.007	0.021	0.023	0.014
	212	0.160	0.160	0.070	0.101	0.049	0.068	0.106
	214	0.070	0.070	0.140	0.094	0.042	0.136	0.099
	216	0.090	0.090	0.150	0.174	0.118	0.218	0.136
	218	0.060	0.060	0.060	0.109	0.128	0.045	0.070
	220	0.050	0.050	0.050	0.036	0.042	0.011	0.035
	222	0.030	0.030	0.040	0.044	0.049	0.000	0.014
	224	0.050	0.050	0.040	0.029	0.069	0.080	0.049
	226	0.000	0.000	0.040	0.036	0.049	0.034	0.049
	228	0.030	0.030	0.010	0.015	0.063	0.045	0.042
	230	0.040	0.040	0.030	0.044	0.042	0.034	0.049
	232	0.070	0.070	0.010	0.007	0.035	0.045	0.021
	234	0.030	0.030	0.020	0.007	0.007	0.023	0.028
	236	0.040	0.040	0.020	0.022	0.021	0.034	0.028
	238	0.010	0.010	0.040	0.007	0.028	0.023	0.014
	240	0.020	0.020	0.010	0.029	0.021	0.011	0.014
	242	0.030	0.030	0.010	0.015	0.007	0.011	0.035
	244	0.030	0.030	0.020	0.029	0.007	0.011	0.021
	246	0.010	0.010	0.000	0.007	0.014	0.011	0.007
	248	0.010	0.010	0.010	0.007	0.014	0.011	0.000
	250	0.010	0.010	0.020	0.022	0.014	0.023	0.014
	252	0.020	0.020	0.000	0.007	0.000	0.000	0.014
	254	0.010	0.010	0.010	0.000	0.000	0.000	0.000
	256	0.000	0.000	0.000	0.000	0.014	0.000	0.000
	258	0.000	0.000	0.000	0.007	0.014	0.000	0.000
	264	0.010	0.010	0.000	0.000	0.000	0.000	0.007
	266	0.000	0.000	0.000	0.000	0.007	0.000	0.000
	270	0.010	0.010	0.000	0.000	0.000	0.000	0.000
	274	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	278	0.000	0.000	0.000	0.022	0.000	0.000	0.007
	280	0.000	0.000	0.000	0.000	0.000	0.000	0.007
Po25A	201	0.050	0.010	0.015	0.014	0.034	0.014	0.023
	205	0.020	0.000	0.022	0.000	0.000	0.014	0.023
	209	0.020	0.020	0.022	0.021	0.023	0.021	0.023
	213	0.000	0.000	0.007	0.000	0.023	0.014	0.011
	217	0.070	0.122	0.116	0.127	0.068	0.127	0.125
	221	0.220	0.133	0.174	0.141	0.170	0.183	0.182
	225	0.220	0.235	0.152	0.254	0.205	0.141	0.238
	229	0.250	0.318	0.419	0.282	0.353	0.332	0.205

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po25A	233	0.030	0.051	0.015	0.049	0.045	0.035	0.034
	237	0.060	0.020	0.029	0.021	0.011	0.007	0.011
	241	0.000	0.010	0.000	0.014	0.023	0.014	0.034
	245	0.000	0.000	0.007	0.000	0.000	0.014	0.000
	249	0.000	0.020	0.000	0.000	0.011	0.007	0.000
	253	0.050	0.041	0.022	0.049	0.034	0.070	0.080
	257	0.000	0.010	0.000	0.014	0.000	0.007	0.000
	261	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	265	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	269	0.000	0.000	0.000	0.007	0.000	0.000	0.011
	305	0.000	0.000	0.000	0.007	0.000	0.000	0.000
Po26	141	0.170	0.210	0.250	0.275	0.273	0.188	0.291
	151	0.010	0.000	0.000	0.007	0.000	0.000	0.000
	153	0.010	0.000	0.015	0.000	0.023	0.007	0.012
	155	0.530	0.620	0.485	0.500	0.465	0.486	0.534
	157	0.010	0.010	0.015	0.021	0.034	0.090	0.023
	159	0.270	0.160	0.235	0.197	0.205	0.229	0.140
Po33	257	0.000	0.020	0.015	0.007	0.011	0.035	0.000
	261	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	263	0.600	0.592	0.529	0.500	0.547	0.562	0.559
	265	0.010	0.000	0.007	0.014	0.011	0.007	0.000
	278	0.000	0.031	0.051	0.028	0.011	0.014	0.000
	280	0.100	0.122	0.058	0.070	0.034	0.035	0.034
	282	0.000	0.010	0.029	0.014	0.011	0.000	0.011
	284	0.000	0.000	0.036	0.000	0.000	0.000	0.000
	286	0.080	0.082	0.130	0.085	0.091	0.083	0.011
	288	0.180	0.143	0.138	0.247	0.250	0.215	0.352
	290	0.020	0.000	0.007	0.014	0.000	0.021	0.011
	292	0.010	0.000	0.000	0.014	0.034	0.028	0.011
	294	0.000	0.000	0.000	0.007	0.000	0.000	0.000
Po35	279	0.000	0.010	0.000	0.007	0.000	0.000	0.011
	283	0.000	0.000	0.015	0.000	0.011	0.014	0.023
	285	0.390	0.400	0.421	0.389	0.365	0.368	0.457
	287	0.080	0.020	0.036	0.014	0.011	0.028	0.011
	289	0.190	0.150	0.167	0.176	0.193	0.190	0.136
	291	0.030	0.040	0.007	0.056	0.057	0.049	0.057
	293	0.000	0.020	0.007	0.028	0.011	0.028	0.000
	295	0.080	0.100	0.130	0.120	0.114	0.120	0.125
	297	0.030	0.040	0.022	0.035	0.023	0.035	0.034
	299	0.040	0.030	0.051	0.021	0.068	0.035	0.045
	301	0.040	0.030	0.029	0.014	0.023	0.035	0.045
	303	0.000	0.000	0.007	0.021	0.023	0.000	0.000
	305	0.030	0.010	0.015	0.014	0.023	0.028	0.023
	307	0.000	0.020	0.000	0.014	0.000	0.000	0.011
	309	0.010	0.000	0.007	0.000	0.000	0.000	0.000
	311	0.020	0.020	0.007	0.014	0.011	0.014	0.000
	313	0.010	0.010	0.007	0.000	0.000	0.007	0.000
	317	0.010	0.060	0.036	0.028	0.011	0.014	0.000
	319	0.010	0.020	0.022	0.021	0.045	0.000	0.000
	321	0.010	0.010	0.000	0.014	0.000	0.007	0.011
	323	0.000	0.010	0.007	0.007	0.011	0.014	0.011
	325	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	327	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	331	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	333	0.010	0.000	0.007	0.000	0.000	0.000	0.000
	343	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Po42	152	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	164	0.000	0.020	0.007	0.007	0.000	0.000	0.000
	166	0.020	0.020	0.000	0.007	0.023	0.021	0.000
	172	0.210	0.250	0.218	0.199	0.139	0.149	0.231
	174	0.190	0.070	0.116	0.120	0.068	0.085	0.091
	176	0.030	0.050	0.101	0.106	0.114	0.099	0.091
	178	0.080	0.070	0.101	0.127	0.080	0.141	0.136
	180	0.070	0.040	0.058	0.063	0.068	0.078	0.068
	182	0.080	0.100	0.044	0.042	0.034	0.042	0.068
	184	0.010	0.030	0.058	0.056	0.045	0.028	0.011
	186	0.050	0.010	0.065	0.014	0.045	0.035	0.057
	188	0.050	0.030	0.015	0.021	0.068	0.028	0.023
	190	0.040	0.030	0.015	0.014	0.023	0.035	0.011
	192	0.010	0.010	0.036	0.014	0.023	0.035	0.034
	194	0.000	0.030	0.029	0.042	0.011	0.028	0.000
	196	0.020	0.000	0.015	0.028	0.000	0.007	0.011
	198	0.010	0.040	0.007	0.014	0.034	0.021	0.057
	200	0.010	0.030	0.007	0.007	0.023	0.042	0.011
	202	0.020	0.020	0.029	0.021	0.045	0.021	0.011
	204	0.010	0.030	0.007	0.028	0.011	0.007	0.023

Appendix 1. (continued)

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po42	206	0.000	0.000	0.000	0.021	0.045	0.014	0.000
	208	0.020	0.030	0.036	0.028	0.011	0.028	0.011
	210	0.000	0.010	0.007	0.000	0.011	0.000	0.011
	212	0.030	0.010	0.000	0.000	0.023	0.000	0.000
	214	0.010	0.010	0.000	0.000	0.000	0.007	0.011
	216	0.000	0.020	0.015	0.000	0.023	0.007	0.000
	218	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	220	0.000	0.000	0.007	0.007	0.000	0.007	0.000
	222	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	224	0.010	0.010	0.007	0.007	0.000	0.007	0.011
	226	0.000	0.010	0.000	0.000	0.011	0.007	0.011
	230	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	232	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	242	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	250	0.000	0.010	0.000	0.000	0.000	0.007	0.000
	254	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	270	0.000	0.000	0.000	0.000	0.011	0.000	0.000
Po48	118	0.000	0.010	0.000	0.007	0.000	0.000	0.000
	120	0.010	0.010	0.000	0.014	0.011	0.021	0.023
	122	0.010	0.000	0.000	0.000	0.011	0.007	0.000
	126	0.640	0.740	0.696	0.740	0.740	0.693	0.659
	128	0.000	0.010	0.007	0.014	0.000	0.021	0.000
	130	0.300	0.180	0.239	0.190	0.216	0.188	0.239
	134	0.000	0.020	0.022	0.007	0.011	0.014	0.057
	138	0.030	0.030	0.022	0.014	0.000	0.035	0.011
	142	0.000	0.000	0.007	0.000	0.000	0.007	0.000
	146	0.010	0.000	0.007	0.014	0.011	0.014	0.000
	150	0.000	0.000	0.000	0.000	0.000	0.000	0.011
Po52	153	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	155	0.800	0.760	0.660	0.643	0.774	0.819	0.716
	157	0.010	0.010	0.015	0.014	0.011	0.021	0.011
	159	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	161	0.060	0.160	0.246	0.286	0.136	0.104	0.125
	163	0.130	0.070	0.079	0.057	0.057	0.056	0.148
Po56	139	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	141	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	147	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	151	0.000	0.020	0.000	0.000	0.000	0.000	0.000
	153	0.040	0.000	0.022	0.014	0.011	0.007	0.000
	155	0.000	0.000	0.000	0.000	0.011	0.007	0.000
	157	0.010	0.070	0.015	0.000	0.023	0.028	0.000
	159	0.150	0.060	0.080	0.035	0.116	0.070	0.114
	161	0.050	0.030	0.051	0.085	0.045	0.056	0.068
	163	0.050	0.060	0.051	0.007	0.011	0.000	0.011
	165	0.090	0.120	0.101	0.113	0.068	0.092	0.045
	167	0.080	0.050	0.029	0.028	0.034	0.035	0.045
	169	0.030	0.010	0.015	0.014	0.034	0.042	0.011
	171	0.020	0.010	0.044	0.021	0.034	0.028	0.045
	173	0.100	0.030	0.073	0.085	0.102	0.099	0.128
	175	0.040	0.110	0.105	0.120	0.057	0.149	0.091
	177	0.060	0.120	0.065	0.056	0.057	0.085	0.102
	179	0.060	0.040	0.080	0.128	0.068	0.113	0.102
	181	0.040	0.040	0.051	0.042	0.057	0.021	0.034
	183	0.060	0.080	0.065	0.035	0.045	0.028	0.045
	185	0.000	0.030	0.022	0.042	0.023	0.021	0.023
	187	0.000	0.020	0.029	0.000	0.023	0.014	0.011
	189	0.010	0.000	0.007	0.000	0.023	0.014	0.023
	191	0.010	0.010	0.015	0.056	0.023	0.000	0.023
	193	0.010	0.000	0.000	0.014	0.023	0.007	0.000
	195	0.030	0.040	0.007	0.028	0.011	0.014	0.023
	197	0.010	0.020	0.022	0.014	0.011	0.035	0.011
	199	0.040	0.010	0.007	0.007	0.034	0.014	0.023
	201	0.000	0.020	0.015	0.035	0.011	0.021	0.011
	203	0.000	0.000	0.015	0.000	0.023	0.000	0.011
	205	0.010	0.000	0.007	0.007	0.000	0.000	0.000
	207	0.000	0.000	0.000	0.007	0.011	0.000	0.000
Po91	140	0.000	0.000	0.000	0.000	0.000	0.000	0.023
	142	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	144	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	146	0.070	0.060	0.058	0.042	0.080	0.028	0.034
	148	0.090	0.070	0.094	0.085	0.068	0.056	0.080
	150	0.050	0.050	0.029	0.063	0.034	0.049	0.080
	152	0.000	0.010	0.007	0.000	0.000	0.021	0.023
	154	0.040	0.020	0.036	0.028	0.045	0.049	0.023

Locus	Allele	Populations						
		HKD	NGT	TTR	NSK	IWT	CHB	HYG
Po91	158	0.060	0.060	0.094	0.099	0.162	0.104	0.068
	160	0.100	0.200	0.115	0.099	0.034	0.069	0.080
	162	0.110	0.060	0.094	0.085	0.057	0.056	0.057
	164	0.020	0.070	0.029	0.056	0.045	0.090	0.068
	166	0.100	0.020	0.015	0.035	0.011	0.007	0.000
	168	0.020	0.000	0.007	0.014	0.034	0.021	0.000
	170	0.000	0.030	0.007	0.014	0.045	0.049	0.023
	172	0.030	0.020	0.000	0.028	0.023	0.021	0.023
	174	0.010	0.010	0.022	0.042	0.000	0.021	0.011
	176	0.030	0.020	0.036	0.021	0.000	0.028	0.011
	178	0.000	0.030	0.000	0.000	0.011	0.007	0.045
	180	0.000	0.020	0.015	0.007	0.034	0.000	0.023
	182	0.000	0.010	0.000	0.007	0.000	0.007	0.000
	184	0.010	0.010	0.029	0.007	0.011	0.000	0.000
	186	0.000	0.000	0.007	0.000	0.000	0.007	0.034
	188	0.010	0.010	0.000	0.000	0.000	0.021	0.011
	190	0.020	0.010	0.015	0.007	0.023	0.007	0.011
	192	0.000	0.000	0.007	0.014	0.011	0.007	0.023
	194	0.010	0.000	0.015	0.021	0.034	0.035	0.023
	196	0.000	0.010	0.007	0.000	0.023	0.014	0.011
	198	0.000	0.000	0.015	0.000	0.023	0.000	0.011
	200	0.000	0.000	0.029	0.028	0.034	0.007	0.023
	202	0.020	0.050	0.022	0.021	0.011	0.007	0.011
	204	0.000	0.010	0.000	0.000	0.000	0.021	0.011
	206	0.000	0.000	0.015	0.021	0.011	0.000	0.011
	208	0.000	0.000	0.007	0.000	0.011	0.014	0.000
	210	0.000	0.000	0.015	0.014	0.023	0.000	0.011
	212	0.020	0.000	0.000	0.000	0.000	0.007	0.000
	214	0.010	0.000	0.000	0.007	0.011	0.007	0.000
	216	0.000	0.000	0.000	0.000	0.000	0.014	0.000
	218	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	222	0.000	0.000	0.000	0.000	0.011	0.000	0.000
	226	0.000	0.010	0.000	0.000	0.000	0.000	0.000
	230	0.000	0.000	0.000	0.014	0.000	0.000	0.000
	232	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	234	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	236	0.000	0.000	0.000	0.007	0.000	0.000	0.000
	238	0.000	0.000	0.015	0.000	0.000	0.000	0.000
	240	0.010	0.000	0.007	0.007	0.000	0.000	0.000
	242	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	244	0.000	0.000	0.007	0.000	0.000	0.000	0.011
	246	0.000	0.000	0.007	0.000	0.000	0.000	0.000
	248	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	264	0.000	0.000	0.000	0.000	0.000	0.007	0.000
	268	0.000	0.000	0.000	0.000	0.000	0.007	0.000

Appendix 2. Allele frequency distributions of 11 microsatellites for the 3 hatchery strains of Japanese flounder

Locus	Alleles	Sample names			Locus	Alleles	Sample names			Locus	Alleles	Sample names		
		HY	HF	HC			HY	HF	HC			HY	HF	HC
Po1	162	0.550	0.810	0.390	***Po35***	285	0.215	0.445	0.545	***Po56***	173	0.140	0.315	0.160
	164	0.030	0.100	0.045		287	0.035	0.150	0.005		175	0.110	0.000	0.090
	172	0.070	0.000	0.030		289	0.280	0.105	0.055		177	0.185	0.000	0.005
	174	0.040	0.030	0.005		291	0.000	0.080	0.105		179	0.055	0.055	0.025
	176	0.005	0.000	0.000		293	0.105	0.000	0.020		181	0.000	0.000	0.005
	178	0.000	0.000	0.115		295	0.110	0.000	0.155		183	0.020	0.385	0.075
	180	0.030	0.010	0.120		297	0.000	0.000	0.010		185	0.000	0.000	0.020
	184	0.140	0.000	0.045		299	0.030	0.040	0.000		189	0.055	0.000	0.000
	186	0.005	0.000	0.000		301	0.080	0.030	0.015		191	0.030	0.000	0.000
	188	0.075	0.050	0.000		305	0.010	0.000	0.040		193	0.000	0.000	0.115
	192	0.030	0.000	0.165		309	0.005	0.000	0.000		197	0.005	0.025	0.035
	196	0.000	0.000	0.010		311	0.010	0.000	0.000		201	0.010	0.000	0.095
	200	0.000	0.000	0.035		313	0.000	0.110	0.000					
	210	0.005	0.000	0.030		317	0.000	0.000	0.045	***Po91***	146	0.100	0.040	0.060
	222	0.020	0.000	0.010		319	0.115	0.000	0.000		148	0.140	0.000	0.105
						321	0.000	0.040	0.000		150	0.070	0.000	0.040
Po13	208	0.165	0.000	0.210		323	0.000	0.000	0.005		154	0.045	0.025	0.000
	212	0.050	0.225	0.005		329	0.005	0.000	0.000		156	0.265	0.175	0.275
	214	0.060	0.040	0.060							158	0.085	0.055	0.000
	216	0.305	0.600	0.100	***Po42***	166	0.015	0.060	0.000		160	0.100	0.410	0.120
	218	0.130	0.000	0.100		168	0.000	0.000	0.005		162	0.065	0.010	0.015
	220	0.015	0.000	0.035		172	0.335	0.000	0.275		164	0.005	0.000	0.020
	224	0.010	0.005	0.005		174	0.120	0.135	0.050		166	0.005	0.000	0.000
	226	0.080	0.005	0.005		176	0.035	0.465	0.010		168	0.000	0.000	0.045
	228	0.005	0.000	0.015		178	0.020	0.000	0.100		172	0.055	0.100	0.065
	230	0.000	0.015	0.120		180	0.030	0.075	0.000		176	0.000	0.000	0.070
	232	0.020	0.000	0.005		182	0.070	0.000	0.025		184	0.015	0.000	0.035
	234	0.010	0.000	0.075		184	0.035	0.000	0.090		188	0.000	0.140	0.070
	236	0.030	0.000	0.000		188	0.120	0.000	0.135		190	0.000	0.000	0.015
	240	0.070	0.000	0.000		190	0.065	0.000	0.065		192	0.000	0.000	0.030
	244	0.000	0.025	0.065		192	0.010	0.000	0.000		196	0.000	0.000	0.035
	246	0.000	0.085	0.000		196	0.010	0.000	0.100		200	0.015	0.000	0.000
	248	0.000	0.000	0.095		200	0.055	0.185	0.000		204	0.000	0.010	0.000
	250	0.045	0.000	0.000		202	0.000	0.000	0.015		208	0.005	0.000	0.000
	256	0.000	0.000	0.105		204	0.000	0.000	0.085		210	0.010	0.000	0.000
	274	0.005	0.000	0.000		206	0.010	0.000	0.030		224	0.015	0.000	0.000
						212	0.000	0.000	0.010		228	0.000	0.035	0.000
Po25A	201	0.005	0.000	0.010		214	0.000	0.050	0.005		232	0.005	0.000	0.000
	209	0.015	0.000	0.060		218	0.000	0.030	0.000					
	213	0.090	0.000	0.000		248	0.070	0.000	0.000					
	217	0.145	0.000	0.360										
	221	0.135	0.135	0.040	***Po48***	120	0.000	0.000	0.005					
	225	0.200	0.285	0.215		126	0.610	0.740	0.510					
	229	0.250	0.300	0.220		128	0.000	0.000	0.045					
	233	0.015	0.000	0.095		130	0.280	0.260	0.385					
	237	0.030	0.000	0.000		134	0.005	0.000	0.020					
	241	0.050	0.000	0.000		138	0.050	0.000	0.000					
	245	0.000	0.120	0.000		146	0.000	0.000	0.035					
	249	0.055	0.000	0.000		154	0.055	0.000	0.000					
	253	0.005	0.125	0.000										
	257	0.005	0.035	0.000	***Po52***	155	0.970	0.975	0.895					
						161	0.000	0.025	0.070					
Po26	141	0.335	0.205	0.160		163	0.025	0.000	0.035					
	155	0.420	0.365	0.645		165	0.005	0.000	0.000					
	159	0.245	0.430	0.195										
					Po56	147	0.005	0.000	0.000					
Po33	257	0.025	0.010	0.000		153	0.100	0.000	0.045					
	263	0.445	0.470	0.450		157	0.010	0.000	0.085					
	278	0.065	0.000	0.000		159	0.060	0.155	0.035					
	280	0.205	0.150	0.000		161	0.000	0.000	0.070					
	282	0.000	0.035	0.010		163	0.020	0.000	0.000					
	286	0.090	0.025	0.045		165	0.190	0.040	0.040					
	288	0.160	0.075	0.475		167	0.000	0.025	0.080					
	290	0.000	0.235	0.020		169	0.005	0.000	0.005					
	292	0.010	0.000	0.000		171	0.000	0.000	0.015					