Genetic profiling of hatchery-raised Japanese flounder (*Paralichthys olivaceus*) based on DNA markers: an efficient means to track released flounder[*1]

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Abstract This paper illustrates the efficiency of genetic profiling approach to determine a hatchery strain of Japanese flounder *Paralichthys olivaceus*, which was stocked into a natural sea area in a practical stock enhancement program. The hatchery strain tracked in this study was founded by using 18 wild captives in a hatchery station (JASFA hatchery), and the pedigrees of these offspring were identified by means of microsatellite DNA technique. In September 2001, the hatchery-raised fish (JASFA fish) were stocked into Miyako bay (Iwate Prefecture), and at around the same time and within the same area, a different hatchery strain was released by another hatchery station. In this study, microsatellites together with nucleotide sequences of the mitochondrial DNA control region were used as genetic tags to track the released JASFA fish. A total of 1,576 individuals including both wild and released fish were entrapped in a set net during a six month period one year after the release. Of these, 35 fish were retrieved as survivors of the released JASFA fish with the use of the two classes of molecular marker. The results presented here show that the genetic profiling would be of great use to further our understanding of stocking effects in Japanese flounder.

Key words: Japanese flounder (*Paralichthys olivaceus*), stock enhancement, microsatellites, mitochondrial DNA, parentage.

Stocking of hatchery-raised fish into natural environments has widely been acknowledged as an intuitive approach to enhance the exploitable resources, and also this approach offers a possible way to recover threatened or endangered aquatic organisms (Allendorf and Ryman, 1987). As stocking practices have intensively been carried out in many fishery species, a focus of attention from the viewpoints of biology, ecology, and genetics has been paid to the extent of stocking impacts upon indigenous populations in the sense of both positive and negative effects (FAO, 1993; Schramm and Piper, 1995). Besides utmost concern is essential regarding stocking impact in the context of conservation biology, a balance between cost and benefit associated with hatchery practices should also be investigated in order to further our comprehensive understanding of stocking effects (White *et al.*, 1995; Svåsand *et al.*, 2000; Mustafa *et al.*, 2003); a necessary step towards tackling this challenging subject is to trace the fate of released fish.

Japanese flounder *Paralichthys olivaceus* is one of the species for which stocking practices have intensively been made throughout Japa-

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nese coastal areas to increase the harvest yield (Furusawa, 1997). Hatchery-raised flounder commonly exhibit hyper-melanic phenomenon on the blind body side (Haga *et al.*, 2004 and references therein), and thereby it is less complicated compared with other species to track released fish since such abnormal pigmentation serves as an indicator to discriminate released fish from wild ones (Seikai, 1997; Iwamoto *et al.*, 1998). Using hypermelanosis alone as a tracer of released flounder, however, encounters several constraints. For example, when two or more hatcheries release seedlings within neighboring sea areas, how can we assign a recaptured fish to the hatchery of origin? Such information is crucial for appropriate hatchery management decisions concerning budget, space, and labor capacity that are optimal for each hatchery. Tracking released fish using external tagging method entails the risk of loss or under-detection of tags, and this method is not suitable for the application to juvenile fish with handling vulnerability. Tagging with chemical compounds such as alizarin complexone (Blom *et al.*, 1994) also has disadvantages as this method requires much experience to determine tagged fish accurately, and furthermore, it is not possible to assign a tagged fish to the source hatchery if other hatcheries employ this method in a similar manner.

The genetic profiling approach can be used as an alternative tagging method to overcome several constraints involved in the traditional tagging methods. There have been many studies to establish pedigrees of aquaculture species in mixed family tanks through accurate parentage determination, where microsatellite DNA loci have preferably been adopted as a genetic tag (Herbinger *et al.*, 1995; O'Reilly *et al.*, 1998; Herbinger *et al.*, 1999; Perez-Enriquez and Taniguchi, 1999; Perez-Enriquez *et al.*, 1999; Norris *et al.*, 2000; Huvet *et al.*, 2001; Selvamani *et al.*, 2001; Boudry *et al.*, 2002; Rodzen *et al.*, 2004), and as well in Japanese flounder (Hara and Sekino, 2003). It is expected that parental allocation based on genetic profiles would theoretically be applicable to trace released flounder back to the source hatchery even though several hatcheries practiced stocking of flounder at around the same time and within the same area. Another important asset of this approach is that this method would allow analysis of the family survival performance of released fish under natural environmental conditions.

This paper demonstrates an application of parental allocation in direct tracking of released flounder. The hatchery strain screened in this study was created by using 18 wild captives (12 females and 6 males). First, the pedigrees of this strain in a mixed family tank, which was to be released into a natural sea area, were determined with the use of 4 microsatellite markers. The data from individual microsatellite genotypes together with the nucleotide sequence variation of mitochondrial DNA (mtDNA) control region were then used to assign the released fish back to the hatchery of origin.

Materials and methods

Determination of parent-offspring lines based on microsatellite data

Hatchery production of Japanese flounder was initiated in May 2001 at the Miyako Hatchery Station of the Japan Sea-Farming Association (JASFA hatchery)[*1]. 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 ºE, 40 ºN), were allowed to spawn in a spawning tank. Sexual maturity and the health condition of these fish were examined through the hatchery operation 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

[*1]Present organizational name: Miyako Station, National Center for Stock Enhancement, Fisheries Research Agency.

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 of 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. Samples of offspring for pedigree reconstruction were collected at three stages during the rearing period. A random sample of 113 larvae was collected within 24 hours after hatching (sample abbreviation: OP 0), and 216 fish were randomly collected at the age of one month (sample abbreviation: OP 1). A further 407 fish were sampled at four months of age (abbr. OP 4) which was timed with the stocking practice, and this sample was size-sorted: 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).

Genomic DNA was extracted from a blood sample of each candidate broodstock parents, and from a fin clip or muscle tissue of each F_1 offspring following Sambrook *et al.* (1989). Four microsatellites (*Po1*, *Po13*, *Po42*, and *Po91*; Sekino and Hara, 2000), for which the Mendelian segregation was verified using several parent-offspring lines (Sekino and Hara, 2001a), were profiled for all fish samples. Polymerase chain reaction (PCR) to amplify each of the four loci was carried out in a 15 μL reaction mixture, which included 10－20 pmols of each primer set, 100 μM of dNTPs, 10mM Tris-HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 0.3 units of AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA), and approximately 50 ng of template DNA using PC-960G gradient thermal cycler (Corbett Research, Mortlake, NSW, Australia) or GeneAmp system 9600 (Applied Biosystems). PCR cycling parameters were as follows: 12 min at 94℃, 30－40 cycles of 30 sec at 94℃, 1 min at a primer-specific annealing temperature, 1 min at 72℃, and final elongation for 5 min at 72℃. The specific annealing temperature of each primer set is available in the previous report (Sekino and Hara, 2000). Polymorphism screening and allele designation were conducted as described elsewhere (Sekino and Hara, 2001b). Once the microsatellite genotypes of all candidate broodstock parents and offspring were profiled, each offspring was assigned to its possible parents. Parent-offspring lines were determined by looking at whether the candidate parents would exactly generate the offspring's genotypes for each locus. A simulated parental allocation analysis based on the Monte-Carlo simulation method was employed using the PAPA version 1.1 software package (Duchesne *et al.*, 2002). This analysis was made in order to ensure the accuracy of parentage determination based on the four microsatellites, and to assess how many numbers of loci would be required for successful parentage assignment. The parental simulation option both under the sexed parents condition (sex of each parent is known) and the non-sexed parents condition (sexes are unknown) was adopted, through which a number of putative offspring's genotypes are generated from possible parental combinations with iterations. The program outputs the results as values for several random variables associated with the accuracy of parentage allocation (*e.g.*, proportion of offspring with correctly allocated parental pairs, for details, see Duchesne *et al.*, 2002) for each locus or various locus combinations. The simulation was done with 1,000 iterations in which 10,000 pseudo-offspring were generated at each iteration.

Assignment of recaptured hatchery-raised fish to the hatchery of origin

At four months of age, the offspring were size-sorted for larger size in total length through a routine hatchery operation. A total of 60,000 fish (JASFA fish) were tagged with alizarin complexone (ALC) and stocked into Miyako Bay on 10 September 2001. Around the same time that the JASFA hatchery stocked the flounder seedlings, 90,000 flounder juveniles

derived from a different hatchery strain were also stocked into the same bay by another hatchery (Iwate Prefectural Stock Enhancement Center) without external or chemical (ALC) tagging operations. Unfortunately, neither the historical records of the hatchery production nor genetic information of the latter strain is available; for this reason, this study was confined to track the released JASFA fish.

One year after the stocking event, a set net was placed in Miyako Bay from September 2002 to February 2003 (6 months), during which a total of 1,576 individuals including both wild and released fish were collected. Based on the empirical observations of the growth rate of Japanese flounder distributed in Miyako Bay, individuals ranging from approximately 18 to 34 cm in total length were sorted as 2001-year-class flounder. Of these, individuals showing hypermelanosis on their blind body side were further selected as a sample of recaptured hatchery-raised fish (149 fish), which was expected to be a mix of fish originating from the two unrelated hatchery strains, and consequently the 149 recaptures were subjected to microsatellite and mtDNA sequencing analysis. Again, this study focused on tracking 2001-year-class (1+) JASFA fish (1+ JASFA fish).

All the 149 recaptures were screened with the four microsatellites in the same manner as described above. In addition to the microsatellite analysis, approximately 435bp segment of Japanese flounder mtDNA including the $tRNA^{Pro}$ gene (71bp) and the left domain of control region was sequenced for the recaptures together with the 18 JASFA broodstock parents, and the identity of mtDNA haplotype between each recaptured fish and JASFA broodstock parent was surveyed. A mtDNA identity although provides information to infer the female parent alone due to the general maternal transmission of the mtDNA genome (Avise *et al.*, 1987), it was expected that knowledge of maternal lineages of the recaptures would strengthen the accuracy of the microsatellite-based parental allocation. Given the fact that a large amount of nucleotide variations are harbored in the Japanese flounder mtDNA control region (Fujii and Nishida, 1997; Sekino *et al.*, 2002; Sekino, 2004), the author considered that all the JASFA broodstock female parents would have different haplotypes from each other. Details of the PCR primer pair to amplify the target region and the PCR profiles are available in Sekino *et al.* (2002). After PCR amplification, leftover dNTPs and primers in the reaction solution were digested by combinational use of exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT Kit, Amersham Bioscience, Piscataway, NJ, USA). Sequencing analysis of PCR products was conducted using BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in combination with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were determined from both directions, and sequence alignment was made with the DNASIS PRO version 2.2 software (Hitachi, Tokyo, Japan).

Results

Pedigree tracing in a mixed family tank

Allelic variations of the four loci proved to be extensive in the 18 potential broodstock parents (Table 1), and the number of alleles varied depending on the locus (minimum 13 at the *Po42* locus, maximum 19 at the *Po91* locus). Unique alleles, alleles unique to just one individual, were detected for all loci. At three loci (*Po1*, *Po13*, and *Po91*), the number of unique alleles was more than a half of the total number of detected alleles. Expressing these results in another manner revealed that 15 of the 18 broodstock had unique alleles for at least one locus. These unique alleles were an efficient indicator to trace pedigrees. The results of the simulated allocation analysis in terms of the values for random variables associated with the accuracy of parental allocation are shown in Table 2. Under the situation that the sexes of all the 18 parents were known (i.e., under the actual situation), a successful parentage allocation (99.9%) with negligible probability of

Table 1. Genotypes of 4 microsatellites[*1] determined for the 18 candidate broodstock

Candidate broodstock		Genotypes			
Males	TL(cm)[*2]	*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]GenBank Accession Number: *Po1*, AB046745; *Po13*, AB046746; *Po42*, AB046754; *Po91*, AB046761.
[*2] Total length

failed allocation was achieved by the use of all the four loci. Combinations of three loci could also give rise to a relatively high parentage resolution as approximately 95-99% offspring were correctly assigned to a single parental couple. Using one or two loci however resulted in a poor parentage resolution except for one instance: a combination of *Po13* and *Po91* loci produced a high level of parentage assignment success with a little probability of allocation failure. As was expected, parental allocation performance would overall be depressed if the sexes of the broodstock parents were unknown, as a 4% probability of failed allocation was yielded even though all the four loci were used.

All the offspring samples were successfully assigned back to a single parental couple. A total of nine families were identified across the three samples (OP 0, OP 1, and OP 4; Table 3), and there was an apparent difference of family size among the families. It turned out that 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 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.

Significant difference of the proportion of family size between samples was observed in

Table 2. Values for random variables associated with the accuracy of microsatellite-based likelihood parental allocation method estimated based on Monte-Carlo simulation[*1]

Locus combinations		Sexed condition[*2]				Non-sexed condition[*3]			
		Correct allocation			Failed allocation[*4]	Correct allocation			Failed allocation
		Female parents	Male parent	parental couple	Ambiguity	Two parents	One parent	No parent	Ambiguity
1 locus	*Po1*	0.2293(0.0039)	0.1668(0.0036)	0.1668(0.0036)	0.7707(0.0039)	0.0555(0.0023)	0.0000(0.0000)	0.0000(0.0000)	0.9445(0.0023)
	Po13	0.2084(0.0043)	0.2084(0.0043)	0.2084(0.0043)	0.7916(0.0043)	0.0703(0.0026)	0.0196(0.0014)	0.0294(0.0017)	0.8807(0.0032)
	Po42	0.1146(0.0031)	0.1875(0.0039)	0.1146(0.0031)	0.8125(0.0039)	0.1208(0.0033)	0.1496(0.0035)	0.1445(0.0034)	0.5851(0.0048)
	Po91	0.5034(0.0048)	0.4270(0.0052)	0.3993(0.0050)	0.3854(0.0048)	0.2529(0.0044)	0.1390(0.0034)	0.0932(0.0029)	0.5148(0.0050)
2 loci	*Po1,Po13*	0.6817(0.0044)	0.7093(0.0043)	0.6496(0.0044)	0.2439(0.0040)	0.3542(0.0047)	0.0403(0.0020)	0.0165(0.0012)	0.5889(0.0049)
	Po1,Po42	0.7874(0.0040)	0.7735(0.0038)	0.7196(0.0045)	0.1448(0.0032)	0.5101(0.0050)	0.0599(0.0024)	0.0090(0.0010)	0.4210(0.0049)
	Po1,Po91	0.7753(0.0037)	0.7944(0.0036)	0.7753(0.0037)	0.1943(0.0036)	0.5653(0.0049)	0.0545(0.0022)	0.0059(0.0008)	0.3744(0.0048)
	Po13,Po42	0.6488(0.0052)	0.7069(0.0047)	0.6488(0.0052)	0.2870(0.0046)	0.4746(0.0050)	0.0634(0.0024)	0.0213(0.0015)	0.4407(0.0050)
	Po13,Po91	0.9576(0.0020)	0.9541(0.0021)	0.9523(0.0021)	0.0312(0.0018)	0.7272(0.0044)	0.0419(0.0020)	0.0107(0.0011)	0.2202(0.0040)
	Po42,Po91	0.8498(0.0039)	0.8464(0.0044)	0.8064(0.0047)	0.0989(0.0034)	0.6536(0.0046)	0.0942(0.0030)	0.0480(0.0021)	0.2042(0.0041)
3 loci	*Po1,Po13,Po42*	0.9597(0.0021)	0.9604(0.0019)	0.9391(0.0025)	0.0181(0.0014)	0.8150(0.0038)	0.0239(0.0015)	0.0062(0.0008)	0.1549(0.0035)
	Po1,Po13,Po91	0.9878(0.0011)	0.9878(0.0011)	0.9878(0.0011)	0.0104(0.0010)	0.8535(0.0036)	0.0122(0.0011)	0.0014(0.0004)	0.1329(0.0035)
	Po1,Po42,Po91	0.9483(0.0022)	0.9503(0.0022)	0.9483(0.0022)	0.0482(0.0021)	0.8327(0.0036)	0.0132(0.0012)	0.0039(0.0006)	0.1502(0.0034)
	Po13,Po42,Po91	0.9929(0.0008)	0.9920(0.0009)	0.9902(0.0010)	0.0043(0.0006)	0.9280(0.0026)	0.0136(0.0012)	0.0044(0.0007)	0.0034(0.0541)
4 loci	*Po1,Po13*	0.9991(0.0003)	0.9995(0.0002)	0.9991(0.0003)	0.0004(0.0002)	0.9590(0.0020)	0.0010(0.0003)	0.0003(0.0002)	0.0396(0.0020)
	Po42,Po91								

[*1] Proportion of pseudo-offspring whose female or male parent, or parental couple were correctly allocated is shown. Standard deviation generated through 1,000 Monte-Carlo iterations is given in each parenthesis. Simulations were made with 1,000 iterations, and 10,000 pseudo-offspring were produced at each iteration. Parental genotypes were derived from the JASFA broodstock parents.

[*2] A situation where both microsatellite genotypes of all candidate parents and the sex of each parent are known.

[*3] A situation where microsatellite genotypes of all candidate parents are known but the sex of each parent is unknown.

[*4] Proportion of offspring whose parental couple was not specified. An "Ambiguity" is a situation where 2 or more parental couples scored the highest (non-zero) likelihood (Duchesne *et al* ., 2002).

Table 3. Parental couples of the offspring determined on the basis of the microsatellite profiles, and the number of offspring in each family (family size)[*1]

Family	(*N*)	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										
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	1	0	48	24	78	31	7	18
#Non-released	200	0	0	1	31	47	22	74	11	14

[*1] Proportion of the family size in each sample is shown in parenthesis(%)
[*2] Random sample collected within 24 h 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).

three families after correction of the significance level for 18 simultaneous test (Rice, 1989) ($p<0.0028$), in family E (between OP 0 and OP 1 samples, $z=3.68$, $p=0.0002$; OP 0-OP 4, $z=3.46$, $p=0.0005$), family G (OP 0-OP 1, $z=4.13$, $p=0.0000$), and family I (OP 1-OP 4, $z=3.03$, $p=0.0024$, marginal significance). Although this study cannot evidently determine the causes of the significant fluctuations in family size, these results might be due to differences in the initial offspring survival rate. The fact that all families identified in the OP 0 sample (0 day offspring) appeared in both OP 1 (1 month) and OP 4 (4 months) samples suggests that the differences of family survival performance, if any, would not result in the demise of any particular family.

Tracking released fish using DNA markers

The microsatellite-based parental allocation revealed that 114 of the 149 recaptures did not have compatible genotypes with any parental couples retrieved from each sex of the JASFA broodstock parents, and each of the remaining 35 fish was assigned unambiguously to just a single parental couple among the JASFA broodstock parents (Table 4). As cited above, the mtDNA control region of Japanese flounder is known to have an extremely high level of nucleotide variations. This is seen in the fact that the 18 JASFA broodstock parents did not share common mtDNA haplotypes with each other, although there was no variable site in the $tRNA^{Pro}$ gene (Fig. 1 and Appendix). A total of 49 haplotypes were detected among the 149 recaptures, of which 41 fish had mtDNA haplotypes that also appeared in the JASFA broodstock female parents (Table 4 ; two recaptured fish having the same mtDNA haplotype with one male parent are also shown). The female parents of 35 recaptures inferred from microsatellite profiles were compatible to maternal lineages traced on the basis of mtDNA identity, apart from an additional six recaptures each of whose mtDNA haplotype was identical to that of a particular JASFA female parent. These six fish were rejected from 1+

JASFA fish since none of the possible parental couples in the JASFA broodstock parents generated any microsatellite genotypes compatible with those of the six recaptures across the four loci; reamplification and re-typing of microsatellite alleles enhanced the validity of the exclusions of the six fish. Finally, the affiliation of the 35 recaptures determined based on the genetic profiles were confirmed with the ALC information.

The proportion of each family estimated for the recaptured 1+ JASFA fish (family size) is illustrated in Fig. 2. When an expected family size was assumed such as the proportion of each family in the JASFA strain from which the 1+ JASFA fish originate, which was estimated when the JASFA seedlings were released into the natural environment (see above), *G*-test for goodness of fit (Williams, 1976; Sokal and Rohlf, 1997) revealed that there was no significant difference between the family size distribution of the 1+ JASFA fish and the expectation ($G_{adj}=2.691$, $p=0.442$, $3df$, where four families with the expectations less than 5.0 were combined).

Discussion

Unequal contribution of broodstock parents to the next generation

In most hatchery strains, an unequal contribution of broodstock to the next generation has been known to be typical, particularly when the mesocosm spawning method is used to establish hatchery strains (Taniguchi *et al.*, 1983; Sugama *et al.*, 1988; Perez-Enriquez *et al.*, 1999; Fujii, 2001; Sekino *et al.*, 2002). Hara and Sekino (2003) investigated a cultured strain of Japanese flounder, and they found that four of the six females and four of the eight males actually contributed to establish the strain. The strain screened in this study is an extreme case, especially for the broodstock males: a single male sired more than 99% offspring. 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 be reproductive interaction among males at the mating event.

The effective population size of this strain was estimated to be of 3.3 (average of the 3 samples) after correction of unequal number of females and males coupled with unequal family size, on the basis of the formula proposed by Lande and Barrowclough (1987), which assumes non-Poisson distributions of gametes for each sex. The effective size was decreased by 80% of that calculated assuming all of the potential broodstock could equally contribute to the offspring pool ($\underline{N}_e=16$). The most effective hatchery option to obtain a more homogenous contribution by broodstock would be to employ the stripping method with one-to-one crossing using a large number of broodstock. 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 flounder 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, and thus there remains a possibility that other potential broodstock would contribute to the next generation in the other days; Hirano and Yamamoto (1992) reported that ripe females released eggs intermittently but irregularly during the spawning period that lasts for three months (individual spawning days per spawning period was estimated to be of 66-88% in the 5 females examined), although there have been limited data available to address the reproductive rhythm of

Table 4. Microsatellite genotypes and mitochondrial DNA haplotypes of 43 recaptured fish, and results of parental allocation of the recaptured fish[*1]

Recaptured fish(ID No.)	Microsatellite genotypes *Po1*	*Po13*	*Po42*	*Po91*	MtDNA haplotypes[*2]	Parental couple[*3]	Recaptured fish(ID No.)	Microsatellite genotypes *Po1*	*Po13*	*Po42*	*Po91*	MtDNA haplotypes	Parental couple
0178	180/184	216/224	174/196	158/176	HF7	FM#7-M#2	1094	164/180	208/224	176/236	150/158	HF9	FM#9-M#2
0337	162/188	224/224	174/178	154/158	HF12	FM#12-M#2	1119	164/180	208/234	174/176	158/178	HF9	FM#9-M#2
0354	180/206	208/234	174/180	158/194	HF4	FM#4-M#2	1154	162/196	208/224	174/202	150/156	HF9	FM#9-M#2
0371	162/184	216/234	174/196	156/176	HF7	FM#7-M#2	1192	164/180	208/234	174/202	150/156	HF9	FM#9-M#2
0414	164/180	208/234	202/236	150/156	HF9	FM#9-M#2	1203	162/190	208/214	172/186	148/184	HF11	FM#11-M#1
0508	162/162	208/224	174/180	156/158	HF10	FM#10-M#2	1224	162/180	208/224	174/176	156/156	HF10	FM#10-M#2
0609	162/180	224/234	178/236	156/186	HF12	FM#12-M#2	1225	162/180	212/224	180/236	156/156	HF10	FM#10-M#2
0610	164/180	218/234	174/202	150/156	HF9	FM#9-M#2	1313	162/180	208/234	180/236	156/186	HF10	FM#10-M#2
0751	162/180	208/234	174/180	156/158	HF10	FM#10-M#2	1424	180/206	212/234	180/236	158/194	HF4	FM#4-M#2
0752	180/196	208/234	174/176	150/156	HF9	FM#9-M#2	1473	162/196	218/234	174/176	150/156	HF9	FM#9-M#2
0949	162/180	212/234	180/236	156/190	HF4	FM#4-M#2	1474	162/164	208/234	174/176	156/178	HF9	FM#9-M#2
0950	162/196	218/224	174/202	150/158	HF9	FM#9-M#2	1492	180/190	208/224	174/186	146/158	HF11	FM#11-M#2
0952	180/196	218/224	174/176	158/178	HF9	FM#9-M#2	1498	180/196	218/224	202/236	158/178	HF9	FM#9-M#2
0953	162/164	218/234	174/176	150/158	HF9	FM#9-M#2	1511	180/196	208/234	174/176	150/156	HF9	FM#9-M#2
1089	162/196	218/224	202/236	156/178	HF9	FM#9-M#2	1549	162/162	208/224	180/236	156/156	HF10	FM#10-M#2
1091	180/206	212/224	174/180	158/190	HF4	FM#4-M#2	1552	162/162	216/234	178/236	158/158	HF12	FM#12-M#2
1092	162/180	208/224	176/236	156/156	HF10	FM#10-M#2	1553	162/180	208/224	172/236	156/190	HF4	FM#4-M#2
							1582	180/196	218/224	202/236	150/156	HF9	FM#9-M#2
No parental couple assigned[*4]													
0179	162/216*	214/222*	172/192	160/192*	HM5	--	0966	162/178	216/224	172/182	150/150	HF6	--
0230	162/232*	214/250	200/206*	148/154	HF12	--	1125	162/162	216/216	172/202	150/160	HM5	--
0750	216*/232*	232*/246*	178/192	156/192*	HF12	--	1173	176*/190	212/224	178/186	156/184	HF11	--
0965	180/190	216/220	178/190	158/162	HF8	--	1513	178/232*	216/246*	192/200*	156/192*	HF12	--

[*1] Of the 149 recaptured fish, individuals having compatibilities of microsatellite genotypes across 4 loci and/or mitochondrial DNA haplotypes with the JASFA broodstock parents are shown.
[*2] For nucleotide sequences of each haplotype, see Fig. 1 and Appendix.
[*3] Parental couples determined based on the microsatellite data.
[*4] Parental couples of these recaptured fish were not found in the JASFA broodstock parents according to the microsatellite data. Alleles not appeared in the JASFA broodstock parents are indicated by adding an asterisk.

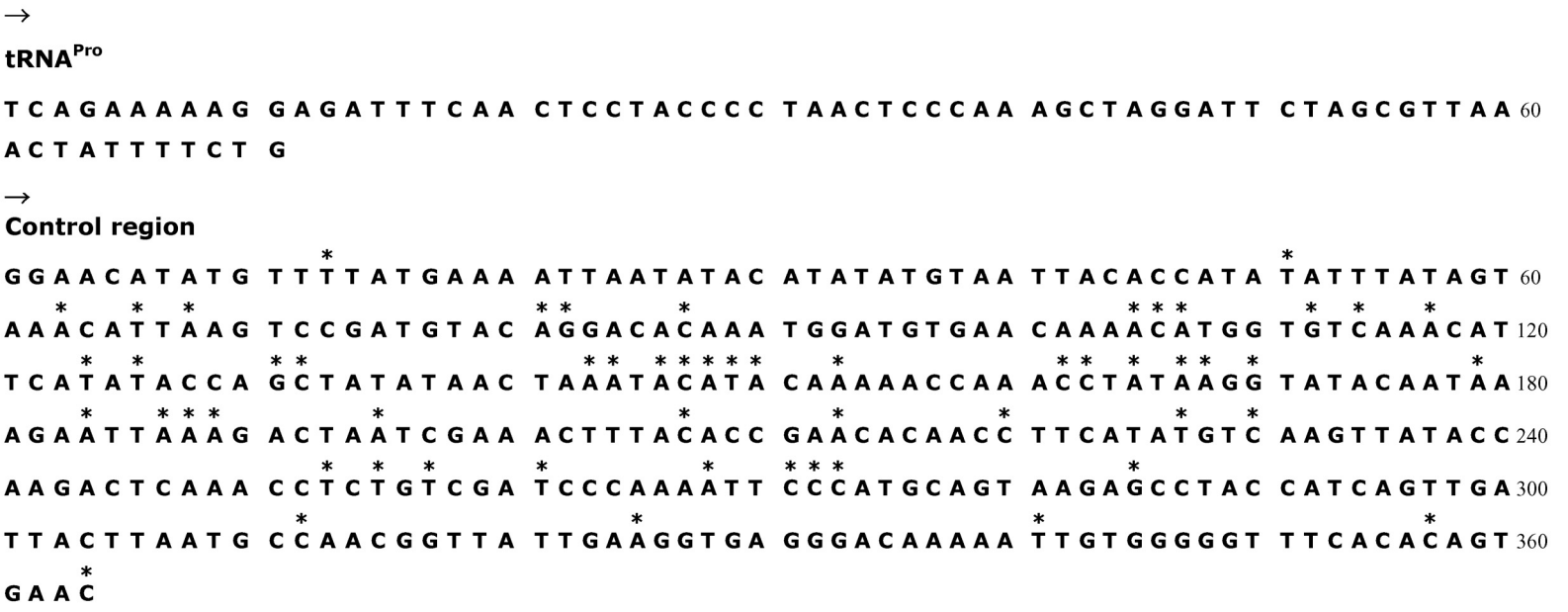

Fig. 1. A reference sequence of Japanese flounder mitochondrial DNA including the tRNA^{Pro} gene and the left domain of the control region. Location of gene refers to Saitoh *et al.* (2000). Asterisks designate variable sites detected in at least one fish of the 18 JASFA broodstock parents (see also Appendix). There was no nucleotide variation in the tRNA^{Pro} gene among 18 broodstock fish.

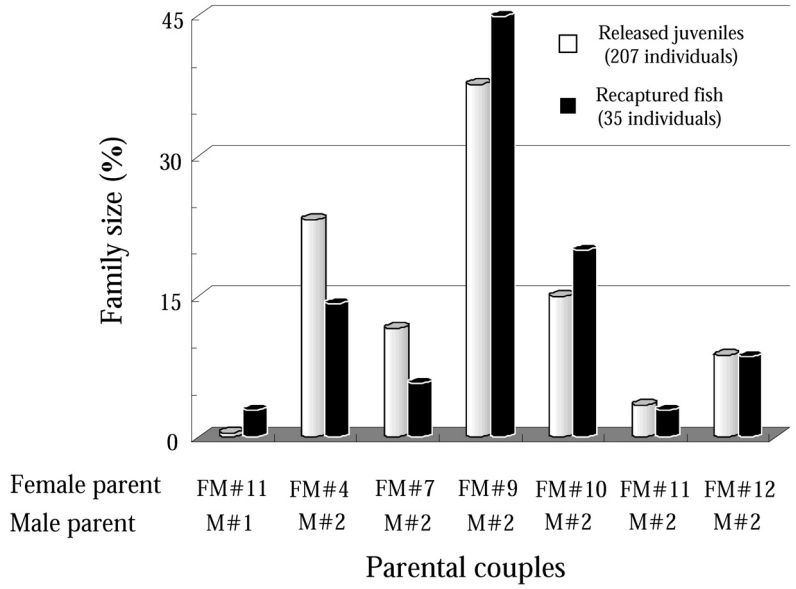

Fig. 2. Family size distribution estimated for the recaptured 1+ JASFA fish (35 individuals). Family size of flounder juveniles to be released (207 individuals) estimated when the JASFA seedlings were released into the sea, from which the recaptured 1+ JASFA fish originate, is also shown. There was no significant difference of the family size distribution between the 2 samples ($G_{adj}=2.691$, $p=0.442$).

males. In such a case, successive collection of fertilized eggs could encourage the increase of the number of actual parents. 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, the effective size could be increased by subdividing the broodstock into multiple spawning tanks to minimize the effects of detrimental interactions.

The feasibility of tracking released fish using DNA markers

The microsatellite profiling approach allocated 35 of the 149 recaptured fish each to a single parental couple generated from the JASFA broodstock parents. The use of four microsatellites proved to be effective to resolve parentage as the accuracy of parental allocation was evaluated in the simulation analysis. A pitfall of the microsatellite-based parental allocation is that this approach however cannot set aside a possibility that a false parental allocation could arise by apparent genotype compatibilities, depending largely on the extent of the allelic variations and/or the relatedness structure in the population of interest; this may be of a special concern especially in its application to field studies. Another obstacle accompanied with this method refers to as transmission errors of microsatellite alleles, which may occur due most likely to typing errors of alleles, PCR errors (e.g., stuttering bands, null alleles, or larger-allele drop out), or mutations which may emerge in parental germ lines, as becoming a major issue in population genetics studies and forensic investigations (O'Connell and Wright, 1997; Marshall *et al.*, 1998; O'Reilly *et al.*, 1998; Norris *et al.*, 2000; Duchesne *et al.*, 2002), and thereby true parents would falsely be excluded, or vice versa, with a certain frequency (Marshall *et al.*, 1998). All the causes described above would prompt an unsuccessful or false parental allocation; using large a number of loci with high allelic diversity (Bernatchez and Duchesne, 2000) coupled with application of parental allocation methods

tolerable to a measure of allele transmission errors (SanCristobal and Chevalet, 1997; Marshall *et al.*, 1998; Duchesne *et al.*, 2002) will achieve a high rate of parentage assignment success. Alternatively, a simultaneous use of other classes of genetic marker, if available, could also allow a credible parental allocation through tracing paternity or maternity of given offspring. This study adopted the nucleotide sequences of mtDNA control region, from which in the case of Japanese flounder a wealth of information to track maternal lineages can be derived. The use of mtDNA haplotype identity alone might result in an overestimation of 1+ JASFA fish; nevertheless the maternal information provides much evidence to improve the reliability of the microsatellite-based parental allocation.

The hypermelanosis phenomenon that typically appears in hatchery-raised flounder enables the discrimination of released hatchery fish from wild ones without much laborious work, but again, the interest of this study lies in the affiliation of released flounder to the source hatchery under the situation that recaptured flounder are from two or more hatchery strains. With the several advantageous properties of the DNA-based parental allocation as mentioned in the introductory section, this method would be currently the most promising approach to meet the purposes on the premise that baseline genetic profiles of seedlings to be released, and ideally, of the parental generation are available. Recent advance of computer-oriented statistical assignment methods based on multilocus genotypes (multiple single locus markers; Davies *et al.*, 1999) has shed a new light on inference of the population of origin of individual fish (reviewed in Hansen *et al.*, 2001a) albeit with missing or incomplete baseline genetic data of source populations (e.g., Hansen *et al.*, 2001b, 2001c). The availability of these methods however depends largely on the extent of genetic differentiation among populations against which an individual assignment is to be tested (Cornuet *et al.*, 1999), and some of these methods assume Hardy-Weinberg equilibrium of marker loci (e.g., Paetkau *et al.*, 1995; Rannala and Mountain, 1997): hatchery strains would not necessarily be under the required conditions, where the potential performance of such statistical assignment methods would be impoverished. The author therefore suggests at this point that genetic profiling should be incorporated as a hatchery management option to elevate the accuracy and efficiency of hatchery fish tracking.

Family size of recaptured fish

The results of the parental allocation analysis coupled with the ALC information allowed concluding that the 35 recaptures were survivors of flounder juveniles produced at the JASFA hatchery in 2001. The recaptured fish screened in this study were entrapped in a set net which effectively only fished a small part of the bay area into which the flounder juveniles were released. Hence it is not evident whether the small number of the recaptured 1+ JASFA fish is attributable to a low survival performance of the released juveniles (60,000 fish) or a low rate of recapture due to dispersal. It is however very unlikely that the released flounder juveniles would have survived without an appreciable mortality on account of the predation pressure upon released flounder juveniles (Yamashita *et al.*, 1994; Furuta, 1996, 1999; Furuta *et al.*, 1998; Saitoh *et al.*, 2003) or due possibly to behavioral deficits ascribable to the long-term captivity of hatchery-reared fish under a protected environment (Olla *et al.*, 1994; D'Anna *et al.*, 2004 and references therein). While, there is little evidence that a significant family size depression would have occurred in particular families of the released JASFA fish since the family size distribution of the recaptured 1+ JASFA fish was consistent with that of JASFA seedlings estimated when the stocking practice was made (Fig. 2). The author considers that the released flounder, which had grown by the released size designed by the hatchery, could have experienced mortality under the natural environment to greater or lesser extent. However, the consistent family sizes between the two samples indicate that such mortality upon the released flounder, if any, would not arise in favor of particular families. The small number of the

recaptures and the sampling period lasted for six months should be taken into account; this leaves room for a more extensive survey to encourage the observations in order to answer the question of family survival performance of released fish under natural environments, which has a significant impact on the effective size of released fish through fluctuations in family size (Gall, 1987). Assessment of family survival performance not only under hatchery conditions but also after the release will provide more insight into better hatchery management including release techniques with which the notion of conservation biology is compatible. The parental allocation approach based on genetic profiles will promise to be of significant use to address this topic.

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Appendix. Variable sites detected in the mtDNA control region of each JASFA broodstock parent[*1]

Parents	Haplotypes	Positions[*2] 13	51	63	66	68	81	82	87	105	106	107	112	114	117	124	126	131	132	143	144	146	147	148	149	150	153	162	163	165
	Reference	T	T	A	T	A	A	G	C	A	C	A	G	C	A	T	T	G	C	A	A	A	C	A	T	A	A	C	C	A
FM#1	HF1	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	C	·	·	·	·	G	T	G	·	·	·	·	·	·
FM#2	HF2	C	C	G	·	C	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	C	·	T	·	·	·
FM#3	HF3	·	·	·	·	C	·	·	·	·	T	G	A	·	·	·	·	C	·	·	·	·	·	·	·	·	T	·	·	·
FM#4	HF4	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
FM#5	HF5	·	·	·	·	C	·	·	·	·	·	·	A	·	·	·	·	·	·	·	G	·	·	·	·	·	T	·	·	·
FM#6	HF6	·	·	·	C	C	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	T
FM#7	HF7	·	·	·	·	·	·	A	·	·	T	·	·	·	·	C	·	·	·	·	·	·	T	G	·	·	·	·	·	·
FM#8	HF8	·	·	·	·	C	·	·	·	–	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	T	T	·	·	·
FM#9	HF9	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	G	·	T	·	·	·	·	·	·	·
FM#10	HF10	C	C	G	·	C	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	C	·	T	·	·	·
FM#11	HF11	·	·	·	·	·	·	A	·	·	·	·	·	T	G	·	·	·	·	·	·	·	T	·	·	·	T	·	·	·
FM#12	HF12	·	·	·	·	C	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
M#1	HM1	·	·	·	·	·	·	A	·	·	·	·	·	·	G	·	·	·	·	·	·	·	T	G	·	·	·	·	T	·
M#2	HM2	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	T	·	G	·	·	·	·	·	·	·	·	·
M#3	HM3	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	G	·	T	·	·	·	·	·	·	·
M#4	HM4	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	G	·	·	T	G	·	·	·	·	T	·
M#5	HM5	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	G	T	G	·	·	·	·	·	·
M#6	HM6	·	·	·	·	·	·	A	·	·	·	·	·	T	·	·	·	·	·	·	·	·	T	·	·	·	·	T	·	·

Parents	Haplotypes	Positions 167	168	170	179	184	187	188	189	195	207	213	220	227	230	253	255	257	261	268	271	272	273	285	312	325	341	357	364
	Reference	A	A	G	A	A	A	A	A	A	C	A	C	T	C	T	T	T	T	A	C	C	C	G	C	A	T	C	C
FM#1	HF1	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	·	–	·	·	·	·	·	·	·	·	·
FM#2	HF2	·	G	·	·	·	G	·	·	·	·	·	·	C	·	·	C	C	·	·	·	·	·	·	T	G	·	T	·
FM#3	HF3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	T	G		T	·
FM#4	HF4	·	·	·	G	·	·	·	·	·	·	·	T	·	·	·	·	·	C	–	·	·	T	·	·	·	·	·	·
FM#5	HF5	·	·	·	·	·	G	G	·	·	·	G	·	·	T	·	·	·	·	–	·	·	·	·	T	G	·	T	·
FM#6	HF6	·	·	·	·	C	G	·	·	·	·	·	·	·	·	C	C	·	·	–	·	·	G	·	T	G	·	T	·
FM#7	HF7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·
FM#8	HF8	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	C	·	·	–	·	·	A	·	·	G	·	T	·
FM#9	HF9	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	–	·	·	·	·	·	·	·	·	·
FM#10	HF10	·	·	·	G	·	G	·	·	·	·	·	·	·	·	·	C	C	·	·	·	·	·	·	T	G	·	T	·
FM#11	HF11	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·
FM#12	HF12	·	·	·	·	·	G	·	G	·	·	G	·	·	T	·	·	·	·	–	·	·	·	·	T	G	·	T	·
M#1	HM1	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	–	·	·	·	·	·	·	·	·	T
M#2	HM2	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	C	–	T	T	·	·	·	·	·	·	·
M#3	HM3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·
M#4	HM4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·
M#5	HM5	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	C	C	·	–	·	·	·	·	·	·	·	·	·
M#6	HM6	G	·	·	·	·	·	·	·	·	T	·	·	C	·	·	·	·	C	–	·	·	T	A	·	G	·	·	·

[*1] Identity of each sequence with the reference (Fig. 1) is represented by dots, and a dash designates a single nucleotide deletion.
[*2] Positions of nucleotide sites correspond to the reference sequence of the control region (Fig. 1).