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Parentage assignment in hatchery population of brown sole *Pleuronectes herzensteini* by microsatellite DNA markers

SANG-GYU KIM,[1a] KAGAYAKI MORISHIMA,[1b] NOBUKAZU SATOH,[2c] TAKASHI FUJIOKA,[2d] SETSUO SAITO[2c] AND KATSUTOSHI ARAI[1]*

[1]*Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, and* [2]*Hokkaido Institute of Mariculture, Shikabe, Hokkaido 041-1404, Japan*

ABSTRACT: Five loci (*Phz2*, *Phz6*, *Phz7*, *Phz12*, and *Phz14*) of microsatellite DNA markers developed in a previous study for parentage assignment in the hatchery population generated by mating among 61 broodstock fish (35 females and 26 males) in a spawning tank, were selected. After natural spawning in the same tank, larvae collected at three different times were categorized into early phase (EP), middle phase (MP), and late phase (LP) groups. In the parental broodstock, the mean number of alleles per locus was 21.8 and expected heterozygosity (H_E) was 0.813. In the progeny, the mean number of alleles per locus decreased to 11.6 (EP), 14.4 (MP), and 6.4 (LP) and H_E to 0.796 (EP), 0.833 (MP), and 0.681 (LP). Parental assignment determined eight dams and six sires as major parents for the EP group. In the MP group, 13 dams and ten sires genetically contributed to spawning, but only three dams and two sires were involved in LP group progeny. In the hatchery population produced from a limited number of parental fish such as the LP group, genetic variability was apparently decreased.

KEY WORDS: **effective population size, genetic diversity, hatchery population, heterozygosity, microsatellite DNA, parentage assignment, *Pleuronectes herzensteini*.**

INTRODUCTION

Microsatellites consist of multiple copies of tandem repeats of di-, tri-, and tetranucleotide sequences and are distributed throughout the regions of the genome.[1] Microsatellites are ideal molecular markers for various genetic studies because they are highly polymorphic, codominant in the manner of inheritance, and easy to genotype by polymerase chain reaction (PCR).[2] Thus, these markers have been used extensively in various aspects of aquatic organisms including gene mapping, allocation of parentage, kinships, and stock structure in the field of fisheries science.[3] Recently, microsatellite DNA markers have also been actively developed and widely used for genetic studies of natural populations,[4–6] parentage assignment,[7–12] and the management of resources of various kinds of commercially important aquatic animals.[13–18]

Brown sole *Pleuronectes herzensteini* is an important species as an inshore fishery resource in Japan. Recently, stock enhancement by release has been expanded, because optimal conditions for rearing and feeding brown sole larvae have been clarified, and artificial production of seedlings has been technically established by the Hokkaido Mariculture Fisheries Experiment Station (formerly Hokkaido Institute of Mariculture), Japan.[19,20] In the resource rehabilitation project, the genetic impact of artificial release of the hatchery population should be considered from the viewpoint of conservation of the genetic diversity of the wild population. In the artificial production of seedlings, the available number of parent fish is generally limited as a result of space in the hatchery and unconscious selection

*Corresponding author: Tel: 81-138-40-5535. Fax: 81-138-40-5537. Email: araikt@fish.hokudai.ac.jp

[a]Present address: Korea Inter-University Institute of Ocean Science, Pukyong National University, 599-1, Daeyeun, Namgu, Busan 608-737, Korea.

[b]Present address: Fisheries Laboratory, Kinki University, Shirahama, Wakayama 649-2211, Japan.

[c]Present address: Hokkaido Mariculture Fisheries Experiment Station, Muroran, Hokkaido 051-0013, Japan.

[d]Present address: Hokkaido Hakodate Fisheries Experiment Station, Hakodate, Hokkaido 042-0932, Japan.

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may decrease genetic variability, because seed production procedures are normally designed for the convenience of hatchery workers and the efficient use of spawning and rearing facilities in the hatchery. Thus, release of the hatchery population is being reconsidered so as not to damage the genetic integrity of the natural population. The understanding of genetic variation through parentage assignment is an important step for the management and conservation of fishery resources.

In the present study, genetic variability was compared between hatchery populations from different spawning times and parental broodstock using microsatellite DNA markers previously developed.[21] Each progeny population was also investigated for genetic variability. Parentage assignment was then examined between parental broodstock and hatchery progeny.

MATERIALS AND METHODS

Flatfish samples

Parental fish of brown sole *Pleuronectes herzensteini* broodstock were caught by trawling in the coastal area of Erimo district, Hokkaido, Japan in 2003. They were transported alive and then reared as broodstock at the Hokkaido Institute of Mariculture, Shikabe, Hokkaido, Japan. Progeny were produced by natural spawning using 61 parental fish (35 females, 26 males) in an 8-t round fiber-reinforced plastic tank. To examine the number of parental fish that contribute to production of progeny in different phases during the spawning period, fertilized eggs collected on 18 May, 25 May, and 6 July 2004 were designated as early phase (EP), middle phase (MP), and late phase (LP) groups, respectively, and reared separately. Approximate numbers of fertilized eggs were 864 000 in EP, 1 150 000 in MP, and 60 800 in LP. Approximately 25 000 hatched fry from each group were moved to an independent rearing tank. The survival rate of 3-month-old progeny was 29.5% in EP, 44.0% in MP, and 10.1% in LP. Sixty-four progeny per phase were randomly collected for microsatellite DNA genotyping.

Parentage assignment and data analysis

Genomic DNA was extracted from fin clips of parents, and whole 3-month-old progeny belonging to each of the spawning groups (EP, MP, and LP). PCR was performed in 10 μL reaction volumes containing a 50–100 ng DNA template. The reaction mixture contained 0.05 μL (5 U/μL) *r*Taq polymerase (TaKaRa, Shiga, Japan), 1 μL 10 × PCR buffer, 0.8 μL dNTPs (200 μM), 0.33 μL (1 pmol/) M13 tailed forward primer, 0.33 μL (10 pmol/μL) reverse primer, and 0.33 μL (10 pmol/μL) labeled M13M1 primer (5′-CACGACGTTGTAAAACGAC-3′).[22,23] The labeled M13 primer was labeled at the 5′ end with VIC, NED or PET fluorescent dyes (Applied Biosystems, Foster City, CA, USA). The PCR condition was 32 cycles of denaturing for 15 s at 94°C, annealing for 15 s at 56°C and extension for 30 s at 72°C, and final extension for 30–60 min at 72°C. Genotypes were determined as approximate allele sizes (base pairs) by a 3130xl Prism genetic analyzer (Applied Biosystems) and GENEMAPPER software v.3.7 (Applied Biosystems) using com-LIZ 500 as the size standard. Five microsatellite loci (*Phz2*, *Phz6*, *Phz7*, *Phz12*, and *Phz14*) were amplified using primers developed by Kim *et al.*[21]

Parentage allocation using genotypes was performed using PAPA v2.0 software[24] based on breeding likelihood.[25] For the purpose of characterizing variation at the five microsatellite loci, data from the parent and progeny samples were analyzed. Allele frequencies and expected heterozygosity (H_E) in parents and progeny groups were calculated for each locus. The observed heterozygosity (H_O) was calculated directly from observed genotypes. These statistical analyses were conducted using GENEPOP v3.4.[26]

The effective number of parents contributing to the progeny was estimated by effective population size (N_e) in each phase according to Lande and Barrowclough:[27]

$$N_e = 4N_{ef}N_{em}/(N_{ef}+N_{em})$$

$$N_{ef} = (N_f\bar{K}_f - 1)/[\bar{K}_f - 1 + (\delta^2{}_{Kf}/\bar{K}_f)],$$

where N_{ef} and N_{em} are the effective number of breeding females and males, respectively, N_f is the census number of females, $\bar{K}_f$ is the average number of progeny dammed by each female, and $\delta^2{}_{Kf}$ is the variance in female progeny number. The same formula was used to calculate the effective number of breeding males, N_{em}.

RESULTS

Parentage assignment

Parentage assignment was successfully determined in 92.2% (59/64), 84.4% (54/64), and 100% (64/64) progeny of the EP, MP, and LP groups, respectively. Parentage assignment succeeded for 92.2% of all the progeny examined. Eight dams and six sires, 13 dams and ten sires, and three dams and two sires were identified as predominantly participating in

Table 1 Dams and sires contributing to spawning in early, middle and late phases

Dam #	Progeny EP(%)	MP(%)	LP(%)	Total	Sire #	Progeny EP(%)	MP(%)	LP(%)	Total
2F	0	3 (5.6)	0	3 (1.7)	33M	10 (16.9)	14 (25.9)	0	24 (13.6)
7F	8 (13.6)	6 (11.1)	0	14 (7.9)	34M	7 (11.9)	0	0	7 (4.0)
9F	5 (8.5)	0	0	5 (2.8)	35M	18 (30.5)	0	0	18 (10.2)
11F	19 (32.2)	1 (1.9)	0	20 (11.3)	42M	0	1 (1.9)	0	1 (0.6)
16F	6 (10.2)	1 (1.9)	0	7 (4.0)	45M	8 (13.6)	5 (9.3)	47 (73.4)	60 (33.9)
17F	0	2 (3.7)	0	2 (1.1)	47M	7 (11.9)	10 (18.5)	0	17 (9.6)
18F	9 (15.3)	6 (11.1)	0	15 (8.5)	49M	0	1 (1.9)	17 (26.6)	18 (10.2)
20F	0	1 (1.9)	0	1 (0.6)	50M	0	8 (14.8)	0	8 (4.5)
21F	0	14 (25.9)	0	14 (7.9)	51M	0	4 (7.4)	0	4 (2.3)
22F	0	5 (9.3)	0	5 (2.8)	54M	0	3 (5.6)	0	3 (1.7)
24F	0	0	20 (31.3)	20 (11.3)	57M	9 (15.3)	6 (11.1)	0	15 (8.5)
26F	6 (10.2)	2 (3.7)	0	8 (4.5)	58M	0	2 (3.7)	0	2 (1.1)
28F	4 (6.8)	8 (14.8)	0	12 (6.8)	Total	59 (100)	54 (100)	64 (100)	177 (100)
29F	2 (3.4)	4 (7.4)	0	6 (3.4)					
30F	0	1 (1.9)	0	1 (0.6)					
31F	0	0	27 (42.2)	27 (15.3)					
61F	0	0	17 (26.6)	17 (9.6)					
Total	59 (100)	54 (100)	64 (100)	177 (100)					

EP, early phase; LP, last phase; MP, middle phase.

spawning in the EP, MP, and LP groups, respectively. As shown in Table 1 and Figure 1, 17 of 35 dams and 12 of 26 sires were determined to participate in the production of progeny.

In the EP group, only eight dams were determined to contribute to spawning and approximately 60% of larvae were the progeny of three dams (7F, 11F, and 18F, Table 1). Seven of eight dams contributing to EP produced approximately 50% of the MP, but they were not spawners in the LP group. In the LP group, most larvae were estimated as the progeny of only three dams (24F, 31F, and 61F). They did not contribute to spawning in EP and MP. Overall, 11F, 24F, and 31F predominantly participated in the production of the hatchery population and more than 37% of larvae were estimated to be their progeny. Six, ten, and two sires contributed to spawning in the EP, MP, and LP groups, respectively, but only one sire (45M) continued to mate during all three periods examined, and about 34% larvae were estimated as the progeny of the 45M sire (Table 1). In the MP group, ten sires contributed to spawning.

The number of dams and sires contributing to spawning in the MP group was larger than in the EP and LP groups (Fig. 1). These were nine, 15, and three parental pairs in EP, MP, and LP, respectively. In EP and LP, progeny were clustered around major parents; however, progeny appeared from a relatively large number of parental fishes participating in MP. Single pair mating was two (7F × 45M, 18F × 57 M) in EP, four (2F × 54M, 18F × 57M, 20F × 49M, 28F × 50 M) in MP, and only one (61F × 49 M) in the LP group.

Changes in genetic parameters

In the parental broodstock, the number of alleles per locus varied from ten (*Phz14* locus) to 38 (*Phz12* locus), and the average H_E was 0.834 (Table 2). In EP, MP, and LP progeny, the average H_E was 0.796, 0.833, and 0.681, respectively, and N_a varied from six (*Phz6*) to 20 (*Phz12*), from six (*Phz14*) to 26 (*Phz12*), and from four (*Phz6*) to nine (*Phz2*). Especially in LP, *Phz12* showed reduction of N_a to eight and *Phz6* showed decreased $H_E = 0.341$. These parameters were lower than estimated in the broodstock (parent) population (mean $N_a = 21.8$, $H_E = 0.834$), except for H_E in MP. Thus, genetic variation of hatchery stock, especially in LP, was apparently reduced when compared with broodstock captured wild.

After identifying the pedigree, the effective population size (N_e) was estimated as shown in Table 3. The effective number of females (N_{ef}) was greater than that of males (N_{em}) in each phase. Variance of contributions was higher in male parents (δ^2_{Km}) than in female parents (δ^2_{Kf}) in MP and LP. The N_E was 11.25 in EP, 14.87 in MP, 3.78 in LP, and 15.3 in overall. The N_e/N ratio was 0.18, 0.24, 0.06, and 0.25 in EP, MP, LP and overall group, respectively, while the N_e/N_c ratio was 0.80, 0.65, 0.76, and 0.53, respectively.

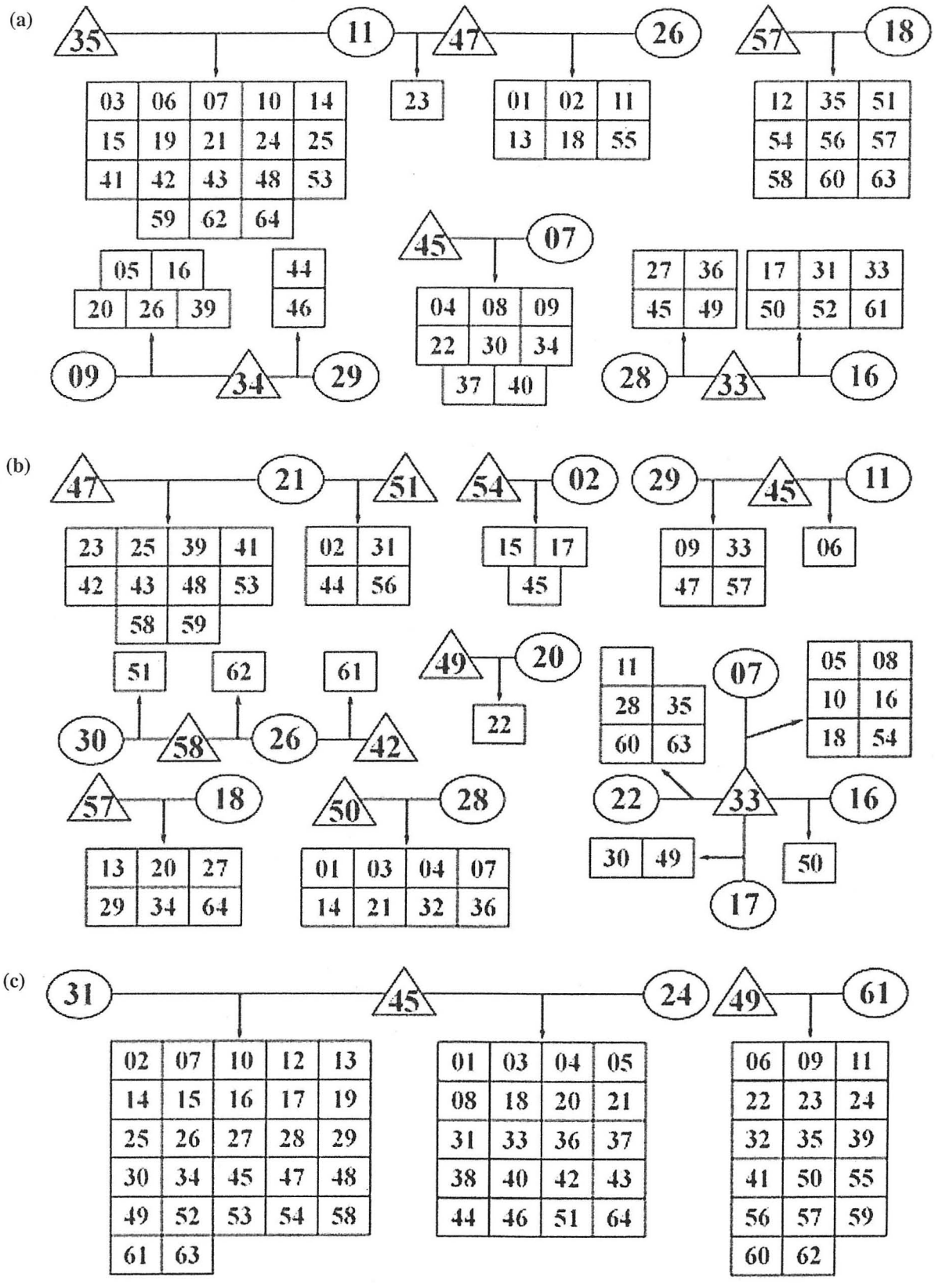

Fig. 1 Parentage assignments using PAPA software based on genetic data using five microsatellite loci. (a) early phase, (b) middle phase, (c) last phase. Sire (△), dam (○), progeny (□).

Table 2 Number of alleles (N_a), observed heterozygosity (H_O) and expected heterozygosity (H_E) at five microsatellite loci of brown sole

	Phz2	*Phz6*	*Phz7*	*Phz12*	*Phz14*	Mean
Broodstock parent (35 females, 26 males)						
N_a	17	17	27	38	10	21.8
H_O	0.770	0.689	0.918	0.967	0.721	0.813
H_E	0.873	0.763	0.886	0.971	0.679	0.834
H_O/H_E	0.882	0.902	1.037	0.995	1.062	0.976
Early phase progeny						
N_a	11	6	14	20	7	11.6
H_O	0.828	0.609	0.859	0.953	0.641	0.778
H_E	0.807	0.727	0.866	0.939	0.639	0.796
H_O/H_E	1.027	0.838	0.992	1.015	1.003	0.975
Middle phase progeny						
N_a	13	11	16	26	6	14.4
Ho	0.953	0.672	0.922	0.875	0.813	0.847
H_E	0.898	0.695	0.908	0.943	0.723	0.833
H_O/H_E	1.062	0.966	1.016	0.928	1.124	1.019
Late phase progeny						
N_a	9	4	6	8	5	6.4
H_O	1.000	0.391	0.641	1.000	0.750	0.756
H_E	0.876	0.341	0.685	0.845	0.660	0.681
H_O/H_E	1.142	1.146	0.935	1.184	1.137	1.109

Table 3 Genetic parameters for EP, MP, LP and overall group

Phase	N	N_c	$\bar{K}_f$	$\bar{K}_m$	δ^2_{Kf}	δ^2_{Km}	N_{ef}	N_{em}	N_e	N_e/N	N_e/N_c
EP	61	14	7.38	9.83	26.84	17.37	5.79	5.47	11.25	0.18	0.80
MP	61	23	4.15	5.40	14.14	17.82	8.08	6.88	14.87	0.24	0.65
LP	61	5	21.33	32	26.33	450	2.92	1.40	3.78	0.06	0.76
Overall	61	29	10.41	14.75	59.38	253.15	11.64	5.69	15.30	0.25	0.53

N, number of broodstock; N_c, contributed number of parents; $\bar{K}_f$, $\bar{K}_m$, averaged progeny count of female and male parents, respectively; δ^2_{Kf}, δ^2_{Km}, variance of female and male contributions, respectively, N_{ef}, N_{em}, effective number of females and males, respectively, N_e, effective population size.

DISCUSSION

Ortega-Villaizan Romo *et al.*[28] reported 85% assignment to 42 parental fishes (24 females and 18 males) for hatchery offspring with three microsatellite loci in spotted halibut. Hara and Sekino[9] succeeded in 86% assignment to 72 possible parental pairs (12 females and six males) using only four microsatellite loci in Japanese flounder; however, the confirmed participating parents in the production of the hatchery population were six females and three males. The success rate of parentage assignment is probably affected by the polymorphism of markers and the number of possible parental pairs. In the present study, from 84.4 to 100% of communal larvae were successfully assigned to a specific parental pair using five microsatellite loci. When compared with the abovementioned studies,[9,28] the rates of assigned parental fishes (dams and sires) were not low despite the more possible combination of parental pairs (35 females and 26 males) and more contribution of parental pairs (eight dams and six sires, 13 dams and ten sires, and three dams and two sires in EP, MP, and LP, respectively) in the resultant hatchery population. In this study, a brown sole (45M) was found to contribute to mating throughout the spawning time, and seven dams and four sires predominately participated in spawning over two spawning periods. Spawning in brown sole was not finished after one mating but continued throughout the period.

Although H_E was not significantly different, H_E was lowest in the LP group. N_a was also lowest in the LP group. Similar reduction in genetic diversity as inferred by microsatellite DNA markers in hatchery populations has been reported in previous works by other authors.[6,9,13,14] Such a decrease might be explained by the limited number of broodstock. It is also suggested that the

contribution of parental fish affects the genetic diversity of progeny according to the spawning time; however, parental fish contributing to LP group progeny were not spawners in EP and MP. MP group progeny were produced by a relatively large number of parental fish, but progeny of the EP and LP groups are also important to maintain the genetic variability of the hatchery, because dams (24F, 31F, and 61F) in the LP group did not contribute to other progeny groups.

The effective number of contributing parents (N_e) was 11.25, 14.87, and 3.78 in EP, MP, and LP groups, respectively, and was much smaller than the number of parental fish ($N = 61$) in a spawning tank ($N_e/N = 0.06–0.24$). However, the N_e/N_c ratio was 0.65–0.80 in three groups and 0.53 overall. The estimation of N_e and N_c in the hatchery population is important for considering management to maintain genetic integrity of the natural resource.

Most progeny (~70% in EP, 56% in MP, and 90% in LP) died during stages from hatching to 3-month-old juveniles and many larvae were also considered to die in the early developmental stages before hatching. Therefore, most of the minority families died before sampling. In the near future, genetic studies on hatchery populations of the brown sole, parental assignment, and pedigree evaluation should be carried out in early developmental stages before hatching.

Microsatellite DNA markers are good molecular tools not only for parentage assignment but also for monitoring genetic variability in hatchery populations. Population genetic studies using polymorphic DNA markers are necessary for the management of commercially important fish species, in order to avoid the reduction of genetic diversity when such a species is utilized for ocean ranching by releasing seeds into wild populations. Parentage assignment will provide information that the genetic diversity may be decreased in the hatchery population when compared with broodstock or the wild population. Therefore, parentage assignment using microsatellite DNA markers is thought to be a basic approach for the genetic management of artificially produced hatchery populations for stocking fish species.

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