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Parentage assessment of incomplete ossification in larval Japanese flounder by microsatellite DNA markers☆

Eitaro Sawayama [a], Kiyoshi Asahina [b], Motohiro Takagi [c,*]

[a] R&D Division, Marua Suisan Co., Ltd., 4472 Kamijima-Iwagi, Ehime 794-2410, Japan
[b] Department of Marine Science and Resources, College of Bioresource Science, Nihon University, 1866 Kameino-Fujisawa, Kanagawa 252-8510, Japan
[c] South Ehime Fisheries Research Center, Tarumi Branch, Ehime University, 3-5-7 Matsuyama-Tarumi, Ehime 790-8566, Japan

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ABSTRACT

Skeletal malformations are a serious problem in seed production of Japanese flounder *Paralichthys olivaceus*, and genetic effect is sometimes suggested as one of the causative factors. In this study, we examined a skeletal deformity named incomplete ossification, and studied its morphological character and parentage assessment of the deformity by microsatellite DNA markers in the hatchery produced offspring at a commercial farm. Naturally spawned fertilized eggs from flounder broodstocks (eight dams and 19 sires) were introduced into 50 kl tanks and reared with normal procedures. We conducted two lots of seed production with lot 2 started one-week later under the same rearing conditions as lot 1. Deformed and normal individuals were collected at 35 days post-hatch and used for further analysis. Body shape of deformed individuals was narrower than that of normal individuals. Moreover, alcian blue–alizarin red staining revealed that vertebrae and the cranium were partly ossified and the appendicular skeleton and fin rays were not ossified even if those parts were completely ossified in normal individuals. DNA parentage analysis revealed that one dam and sire in lot 1 and a dam and two sires in lot 2 were significantly related to the deformed individuals. The dam and sire significantly related to the deformed individuals were the same in the two lots, even though parentages related to normal individuals were different in the two lots. Our results suggest that this type of deformity of Japanese flounder is caused by incomplete ossification and probably affected by genetic factors. Therefore, pedigree selection will be useful for prevention of this deformity.

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1. Introduction

Japanese flounder, *Paralichthys olivaceus*, is an economically important fish species that is widely distributed around Japan. About five million juveniles are produced by broodstock parents per year at many private companies in western Japan and sold as seeds for flounder farming (Murata, 2005). Many kinds of deformities have often occurred in seed production of this species, and the incidence of deformities is a serious problem for aquaculture industry. Deformity can affect different aspects of morphology, such as pigmentation (Sawayama and Takagi, 2011; Takeuchi, 2001), skeleton (Afonso et al., 2000; Cobcroft et al., 2001; Hattori et al., 2004; Kihara et al., 2002) and swimbladder (Chatain, 1994; Kitajima et al., 1994; Zilberg et al., 2004). The high incidence of some deformities, e.g. body shape and color, significantly reduces the market value of the species sold as whole fish and implies additional screening effort at farms. The high incidence of deformity also increases costs for seed production companies, and accidental mixing of malformed fish in sold products degrades company reputation.

Several studies have been conducted to clarify the mechanisms of deformities of Japanese flounder in the focus of nutrition (Haga et al., 2002a,b; Sawayama et al., 2012), rearing conditions (Seikai, 1985) and genetics (Hashimoto et al., 2002; Sawayama and Takagi, 2010a, b, 2012a; Sawayama et al., 2012; Shikano, 2005; Tabata, 1991). Recently, we have mainly focused on genetic background of deformities observed in commercial seed production of Japanese flounder based on microsatellite DNA parentage analysis and successfully identified some deformities caused by genetic factor (Sawayama and Takagi, 2010a,b, 2012a; Sawayama et al., 2012). This method involves of normal and deformed offspring from full- or half-sibling reared at the same tank by assessing parentage using microsatellite DNA polymorphisms. The assigned offspring are divided into full- and half-siblings, and incidences of deformed individuals in each sibling are compared with that of normal individuals. Hypothetically, if particular siblings generate significant numbers of deformed individuals, genetic factors are suggested as a possible cause of the deformity. If not, environmental factors such as nutrition and water temperature may be more significantly associated with the deformities.

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* Corresponding author. Tel./fax: +81 89 946 9902.
E-mail address: takagi@agr.ehime-u.ac.jp (M. Takagi).

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In 2010, a new type of deformity of misshapen body was observed in two commercial production lots of Japanese flounder at Marua Suisan Co., Ltd. (Ehime, Japan). This deformity in body shape showed skeletal abnormality with incomplete ossification at 35 days post hatch and was observed when using one parentage population of eight broodstock populations held at the facility (Sawayama and Takagi, 2012b). Therefore, we hypothesized that this deformity had a genetic influence, and some specific broodstocks and pairs in the broodstock population were associated with the deformity. The present study used microsatellite DNA parentage analysis to investigate the genetic factors related to incompletely ossified individuals observed in two commercial seed production lots. We also performed alcian blue–alizarin red staining to analyze the details of skeletal deformations.

2. Materials and methods

2.1. Seed production and sample collection

Naturally spawned fertilized eggs from four year-old flounder parents (eight dams and 19 sires) cultured at Marua Suisan Co. Ltd., were used for the seed productions. All seed productions were conducted in 50 kl concrete octagonal tanks and 1,000,000 fertilized eggs were introduced in tanks. Rearing water was filtered and sterilized by UV. Water temperature was controlled by water chiller and ranged from 20.0 °C ± 1.0. The photoperiod was 12 L: 12 D. Feeding of rotifers to flounder was started at four day-post-hatch (dph), and *Artemia nauplii* was started from 20 dph. Commercial aquaculture diets were used from 30 dph (Love Larvae, Hayashikane Sangyo Co., Ltd., Japan). We conducted two lots of seed production, and lot 1 started on July13 and lot 2 started on July 20.

At 35 dph, the deformed and normal individuals were collected in both lots and measured body length and weight (72 each of normal and deformed individuals in both lots), and then preserved 99.5% ethanol for further DNA analysis. Some were preserved in 10% formaldehyde for alcian blue–alizarin red staining. Student's *t*-test was used to compare total length and body weight of normal and deformed individuals the differences ($P < 0.05$) using GraphPad Prism 5.0 software (GraphPad software, U.S.A.). Also, occurrence of the deformity was calculated by counting randomly chosen 300 individuals form a tank in both lots at 35 dph.

2.2. Morphological observation

Alcian blue–alizarin red double staining for normal and deformed individuals were conducted according to the method of Dingerkus and Uhler (1977) to understand skeletal formations of the deformity. Five each of normal and deformed individuals from lot 1 were randomly chosen and stained. Morphological observation was conducted under stereomicroscope (SZ61, Nikon, Japan).

2.3. Microsatellite allele detection and parentage analysis

Genome DNA was extracted by High Pure PCR Template Preparation Kit (Roche Diagnosis, Japan) from portions of caudal fin of broodstocks, normal individuals ($n = 72$ in each lot) and deformed individuals ($n = 72$ in each lot) according to the manufacturer's protocol. The primers of four polymorphic microsatellite loci, *Pol-1**, *-3**, *-4** and *-5** (Takagi et al., 1999) were amplified by polymerase chain reaction (PCR). The general PCR protocol was described previously (Sawayama et al., 2012). Forward primers were labeled with fluorescent dyes and reverse primers were tailed (Applied Biosystems, USA). The PCR products were separated by electrophoresis using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems) for fluorescent-labeled PCR products. Alleles were scored using a GeneMapper® Software v4.0 package (Applied Biosystems).

Genetic diversity on the four microsatellite loci, *Pol-1**, *-3**, *-4** and *-5**, was measured by the average number of alleles, allelic richness, observed heterozygosity (*ho*), and expected heterozygosity (*he*). These data were calculated using CERVUS 3.0 (Kalinowski et al., 2007) and FSTAT 2.9.3.2 (Goudet, 1995). Potential parental pairs of each individual were explored using the microsatellite information on the basis of the likelihood-based parental allocation approach, which is available in the CERVUS 3.0. Four microsatellite markers, *Po56** (Sekino and Hara, 2000), *Poli TUF 23**, *121** and *130** (Coimbra et al., 2001), were added for unassigned individuals by first 4 microsatellite loci and manually allocated for the potential parents. The PCR reaction of Po56* was the same as described previously (Sawayama et al., 2012), and the PCR reactions of Poli TUF 23*, 121* and 130* were followed to Coimbra et al. (2001).

Parentage data relative to the deformity were analyzed by contingency table analysis using the χ^2 test. For the deformed individuals, individual numbers in each broodstock were compared to numbers of normal individuals. Statistical analyses were performed using GraphPad Prism 5.0 software. Differences to the equilibrium were accepted as significant when $P < 0.05$.

3. Results

3.1. Morphological features

The occurrence of the deformity was 1.6% and 0.6% in lot 1 and 2, respectively ($n = 250$ and 300 in lot 1 and 2, respectively). Total length of normal individuals and deformed individuals in lot 1 were 17.60 mm ± 1.47 and 11.77 mm ± 0.86, respectively. Body weights of normal and deformed individuals in lot 1 were 60.0 mg ± 17.7 and 21.7 mg ± 8.2, respectively. Total length of normal individuals and deformed individuals in lot 2 were 17.69 mm ± 1.51 and 12.42 mm ± 0.68, respectively. Body weights of normal and deformed individuals in lot 2 were 81.0 mg ± 21.6 and 21.0 mg ± 6.7, respectively. Total length and body weight of normal and the deformed individuals were significantly different in both lots ($P < 0.05$).

The deformed individuals (Fig. 1c) were narrower than normal (Fig. 1a), and had smaller heads. Within the double-stained specimens of normal individuals (Fig. 1b), most skeletal parts were stained by alizarin red, indicating that skeletal formation in normal individuals at 35 dph was almost complete. However, deformed individuals were less-affected by alizarin red and mainly stained by alcian blue. This indicates that skeletal formation of the deformed individuals was incomplete (Fig. 1d).

Skeletal features within each area of the deformity were carefully compared to that of normal individuals (Fig. 2). The head, cranium, jaws, cheek, hydroid arch and opercula, were first compared between normal and deformed individuals (Fig. 2a, b). Within normal individuals, these parts stained well with alizarin red and ossification was normal. On the other hand, the cleithrum was weakly stained by alizarin red in deformed individuals, and ossification was abnormal. In addition, otoliths were not found in the deformed individuals. Among vertebrae (Fig. 2c, d), including the centrum, neural spines, hemal spines and parapophysis, normal individuals stained well by alizarin red, and parts were normally ossified. However, in deformed individuals, the parapophysis was weakly stained by alizarin red, while other parts were well-stained by alcian blue. In the pectoral fin (Fig. 2e, j), the pectoral arch was still cartilage in normal individuals, but no parts of the pectoral arch were observed in deformed individuals. In addition, pectoral fin rays were present and fully ossified in normal individuals, but not formed in deformed individuals. In the pelvic fin (Fig. 2f, k), the pelvic girdle was almost fully ossified and pelvic fin rays were completely ossified and formed in normal individuals. The pelvic girdle was formed within deformed individuals, but was smaller than that of normal individuals and still cartilaginous. In addition, pelvic fin rays were not present in deformed individuals.

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