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Parentage assignment of a hormonally induced mass spawning in Japanese eel (*Anguillla japonica*)

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ABSTRACT

Anguillid eels are a high value species for aquaculture in East Asian countries, but natural stocks of eels have been decreasing. Mass production of glass eels is one of the most effective ways to solve this problem. Genetic improvements will become important to establish mass production of glass eels in the near future. However, there is a lack of basic information, such as parentage assignments, for implementing an eel breeding program. In this study, we aimed to understand the parentage assignment of Japanese eel (*Anguilla japonica*) using a multiple breeding method and microsatellites from eight loci. Five female and 15 male eels were used for mass spawning, and the progeny were collected at the pre-leptocephalus (2 days post-hatch [dph]) and the leptocephalus stage (30 dph). Almost all progeny (297/301 = 98.7%) were assigned to a parental couple. Only two female eels (F86 and F88) contributed to the next generation, whereas 13 of 15 males contributed to the next generation. Although there were no statistical differences in the paternity ratio between stages, some males changed the ratio in both females. These data are not directly useful for a breeding program, but are useful to maintain pedigree information, which is essential for implementing a breeding program.

1. Introduction

Anguillid eels are one of the most popular food fish for “kabayaki” (grilled eel with soy sauce), which is a traditional dish in Japan, and are a highly valued aquaculture species in East Asian countries. Eel aquaculture is totally dependent on wild glass eels captured in estuaries. However, natural stocks of eels, particularly the commercially valuable temperate species, including the Japanese eel (*Anguilla japonica*), European eel (*Anguilla anguilla*), and American eel (*Anguilla rostrata*) have decreased markedly due to overfishing, environmental destruction, or other factors, such as ocean conditions (Dekker et al., 2003; Miller et al., 2009). Decreases in natural stocks of eels is a major cause of unstable supplies and rising glass eel prices, which are serious problems in the eel culture industry.

One effective way to solve these problems and stabilize management of the eel culture industry is to establish mass production techniques for artificial seedlings and reduce the fishing impact on wild glass eels. Over the last decade, eel aquaculture techniques, such as artificial maturation and rearing techniques for larvae, have been greatly improved (Ohta et al., 1997; Tanaka et al., 2001; Tanaka et al., 2003; Kagawa et al., 2005; Tanaka, 2014). However, large-scale commercial production of glass eels has not been practical to date, because of the high larval mortality rate (Okamura et al., 2014). Thus, further improvements are necessary to commercially produce glass eels in sufficient number to meet the demands of the aquaculture industry due to the low larval survival rate in captivity (Okamura et al., 2007; Okamura et al., 2009).

The life cycle of Japanese eel has been completed under artificial conditions (Tanaka, 2014), which has made it possible to apply a breeding program for artificial eel populations. The draft genome of Japanese eel has been sequenced using the Illumina platform (Henkel et al., 2012) and the genetic linkage map has been constructed (Nomura et al., 2006; Nomura et al., 2011; Kai et al., 2014). Therefore, genetic improvements will gain importance in eel aquaculture in the near future. A coordinated breeding program is needed to domesticate eels and ultimately to selectively breed Japanese eels in captivity, including phenotypic selection, family selection, and marker-assisted selection based on quantitative trait loci.

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Parentage assignment is essential as a basic tool to implement a breeding program. In the last decade, several studies have demonstrated the ability to establish parentage in fish reared communally using highly polymorphic microsatellite markers (García de León et al., 1998; Jackson et al., 2003; Hayes et al., 2005; Porta et al., 2006; Castro et al., 2007). However, no parentage assignment has been developed for Japanese eel, although a large number of microsatellite loci are available for parentage allocation in Japanese eel (Tseng et al., 2001; Ishikawa et al., 2001; Nomura et al., 2006; Nomura et al., 2011). The draft genome of Japanese eel has been published (Henkel et al., 2012), which has made it easier to detect microsatellite loci. The aim of this study was to establish parentage assignments of Japanese eel during multiple breeding using microsatellite loci.

2. Materials and methods

2.1. Maturation and spawning

Five female eels (529–1164 g body weight) and 15 male eels (271–587 g body weight) captured in Taiwan at the glass eel stage were used for mass spawning. Female eels were artificially induced to mature by administering a saline suspension of chum salmon *Oncorhynchus keta* pituitary extract (SPE; 20 mg/kg body weight) once a week following the methods of Yamamoto and Yamauchi (1974), and male eels were induced to mature with an injection of human chorionic gonadotropin (1000 IU/kg). Fully mature females, which had oocytes > 750 μm in diameter had reached the final maturation phase, and were induced to spawn spontaneously as follows. After the weekly injections, fully mature females were transferred to a reserve tank (1000 l) controlled at a water temperature of 20 °C, and the female was again injected with SPE (20 mg/kg) 2 days later. About 24 h later, the female was injected with 17α, 20β-dihydroxy-4-pregnen-3-one (Sigma, St Louis, MO, USA) at a dose of 2 mg/kg to induce ovulation. Then, all females were set in a 10,000 l tank (spawning tank) together with 15 mature males, and the water temperature of the tank was raised to 22 °C to help trigger spontaneous spawning. After spawning, the pectoral fins were clipped from the parental eels and were stored in 100% ethanol for DNA extraction. Both maturation and spawning occurred in full-strength seawater.

2.2. Larval fish rearing and sampling

Fertilized eggs were incubated at room temperature (22–25 °C) and hatched in 28–32 h; hatched larvae were reared in a 180 l water column filled with seawater (35 psu) and maintained at 25 °C until 5 days post hatch (dph). Larvae were transferred to 50% seawater in a 19 l plankton Kreisel tank at 5 dph. It has been shown that rearing Japanese eel larvae in 50% seawater results in better growth and survival compared to that in full-strength seawater (Okamura et al., 2009). Feeding was started at 6 dph with a slurry-type diet made of shark eggs, krill extract, and egg albumin peptide. Dead larvae were removed periodically to avoid water contamination. Larvae were collected randomly from the rearing tank at the pre-leptocephalus (2 dph) and leptocephalus stages (30 dph) and preserved in 100% ethanol (Fig. 1).

2.3. DNA extraction and microsatellite amplification

Genomic DNA from the breeders was extracted from a small piece of pectoral fin clip using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Genomic DNA from all larvae was isolated and purified according to a standard protocol using phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and twice with diethyl ether and concentrated by ethanol precipitation.

Eight microsatellite markers were used in this study (Table 1). Four markers (AJMS05, AjTR37, AjP1, and AKMS06) were developed in previous studies (Tseng et al., 2001; Ishikawa et al., 2001; Nomura et al., 2006), and the other markers were developed in this study as follows. First, the draft genome of the Japanese eel (Henkel et al., 2012) was downloaded from ZF Genomics (HPADRESS). We identified repetitive sequences in the eel genome using RepeatMasker (Smit et al., 1996–2010). The scaffolds for developing the microsatellite markers were queried against the Repbase-derived RepeatMasker library. The information about the microsatellite repeats in scaffolds was extracted from annotated files that contained the positions and motifs of several kinds of repetitive sequences. Primer 3 (Untergaser et al., 2012) was used to design the appropriate polymerase chain reaction (PCR) primers for the near-detected MS regions that had repeat units ranging from two nucleotides. The size of the amplified PCR product in the reference sequence was set to 100–400 bp.

Multiplex PCR was used to simultaneously amplify four target loci. The universal primer-multiplex PCR method (Blacket et al., 2012) was adapted to amplify the several primer pairs. This method uses multiple universal primers each labeled with a unique fluorescent tag (FAM, VIC, NED, and PET) to co-amplify multiple loci, including size overlapping markers. The sequence information for the universal primers is shown in Table 1. The Qiagen multiplex PCR kit methodology was used to obtain the multiplex PCR products. All eight loci in the multiplex PCR were co-amplified at the same time.

PCR amplifications were performed in a 10 μl reaction volume consisting of 5 μl Qiagen Multiplex Master Mix, 1 μl Qiagen Q solution, 0.04 μM forward primer, 0.16 μM reverse primer, 0.8 μM fluorescently tagged universal primers corresponding to each tailed primer, and 1 μl

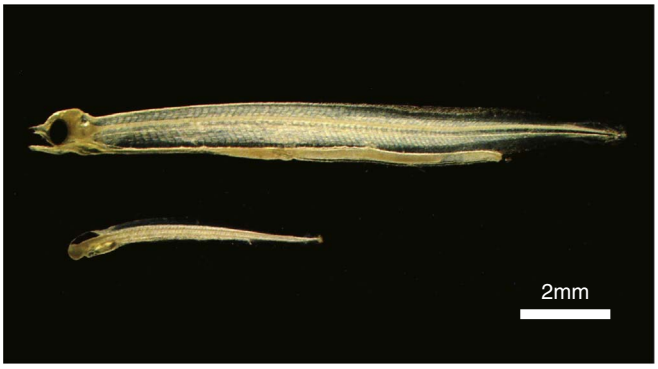

Fig. 1. Japanese eel leptocephalus at 30 dph (upper) and pre-leptocephalus at 2 dph (bottom).

Table 1
Sequence of universal primers and microsatellite markers.

Primer name	Repeat motif	Dye	Primer sequence
RV3		6-FAM	AGCAAAATAGGCTGTCCC
pVP16		VIC	GCCGACTTCGAGTTTGAG
PQE-F		NED	TTGAGAGGATCGCATCCA
M13		PET	TGTAAAACGACGGCCAGT
AjMP3	$(CA)_{15}$	6-FAM	F:TCAGAATGGAACCCTGAACA
			R:AAAACCTCATATTCAAGAAACTCAAGA
AjMP8	$(CA)_{15}$	6-FAM	F:GTGGTGGAGGGAAAGGATTT
			R:TCTCTGGCAGACAGCTTCAA
AJMS05	$(GT)_{15}$	VIC	F:CCTTCAGATTGCTAGCAC
			R:CGGAGTCTAATTGTCTCCTC
AjTR37	$(TG)_{14}$	VIC	F:AGACCTTATGTCACCTTATGCT
			R:AAGATGTTAAATTCAATTGTGC
AjP1	$(CA)_{15}$	NED	F:AATCCATACACATGTACACATG
			R:TAAACAGTGACAGATAACTAGG
AjMP11	$(CA)_{18}$	NED	F:TTGTGGTGCTCACACTTTCC
			R:CACAATCACGGTCTCTCCAA
AJMS06	$(GT)_{19}$	PET	F:ACAGAGCCAGACAAACAGAC
			R:GGTCAGCAAGCAAAACGAAC
AjMP20	$(CA)_{14}$	PET	F:AAAGAGAGCGTCCACCGTTA
			R:ATGGGTATCCACTGGCTGAA