Aquaculture 310 (2011) 329–342

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

# A genetic linkage map of the Japanese eel (*Anguilla japonica*) based on AFLP and microsatellite markers

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ARTICLE INFO

*Article history:*
Received 18 June 2009
Received in revised form 6 October 2010
Accepted 6 November 2010

*Keywords:*
Genetic linkage map
Microsatellite (or short tandem repeat [STR])
AFLP
Japanese eel
*Anguilla japonica*
Recombination rate

ABSTRACT

The first genetic linkage map of the Japanese eel (*Anguilla japonica*) was constructed based upon Amplified Fragment Length Polymorphism (AFLP) and microsatellite (STR) markers with an $F_1$ pseudo-testcross strategy. The segregation of 106 microsatellite loci, which included 74 newly developed, and 463 polymorphic loci identified from 56 AFLP primer combinations was studied in 46 $F_1$ individuals derived from a single female and a male. A total of 319 markers (99 STRs + 220 AFLP) are placed on the female map and 314 markers (91 STRs + 223 AFLP) on the male map. The female map spans 1732.4 cM (Kosambi) in 22 linkage groups with an average spacing of 7.2 cM; the male map spans 964.9 cM in 22 linkage groups with an average spacing of 6.3 cM. The average ratio of male:female recombination rates was 1:2.11. The estimated coverage of the genome was 82.4% for the female map and 74.0% for the male map. This map is the first linkage map in the order Anguilliformes and provides a basis for mapping quantitative trait loci (QTL) and for breeding applications.



## 1. Introduction

The Japanese eel (*Anguilla japonica*) is one of the most important aquaculture fish species for freshwater cultivation in the East Asian countries. Since seeds for the present eel culture depend completely on the capture of wild glass eels in estuaries, unstable supplies and rising glass eel prices are serious problems in the eel culture industry. Besides, as natural eel resources, not only Japanese eel but also European eel (*Anguilla anguilla*) and American eel (*Anguilla rostrata*), have been steadily decreasing due to overfishing, environmental destruction, or other as yet unknown factors (Dekker, 2003; Tatsukawa, 2003), the supply of the wild glass eel for seeds of eel culture has become a serious restricting factor. In order to solve these problems for stable management of eel culture industry, it is absolutely imperative to establish techniques of artificial production of glass eels as seedlings for aquaculture to reduce the demand of wild glass eels. These techniques have been improved markedly over the last few decades. We succeeded in obtaining artificially matured gametes by using hormonal treatment (Ohta et al., 1997). Then, viable leptocephali (Tanaka et al., 2001) and glass eels (Tanaka et al., 2003) have been successfully produced by the development of appropriate rearing techniques. Recently, we achieved the completion of the life cycle of the Japanese eel for the first time under artificial conditions when healthy larvae ($F_2$ generation) were obtained from artificial propagated male and female ($F_1$ generation) (unpublished results). Although further improvements are required for large-scale commercial production of glass eels, recent progress of the artificial seed production techniques has made it possible to apply the breeding program in the artificial eel population. Therefore, genetic improvement will be one of the most important subjects in eel aquaculture in the near future. In order to domesticate and ultimately selectively breed Japanese eel in captivity, a coordinated breeding scheme that includes phenotypic selection, family selection, and marker-assisted selection (MAS) based on quantitative trait loci (QTL) needs to be implemented. A key step in such a genetic improvement scheme is genetic mapping.

Genetic maps are essential tools for genomic studies of fishes. In our previous study, we successfully induced triploid individuals for the first time in the Japanese eel by heat shock treatment, which inhibited the second polar body release after normal fertilization (Nomura et al., 2004). The production of triploid families enables the application of half-tetrad analysis for genetic mapping. Thus, we developed 43 microsatellite (or short tandem repeat; STR) loci and mapped 10 of them as well as 16 previously reported loci relative to the centromere of the eel chromosomes, by using four triploid families produced by inhibition of the second polar body release (Nomura et al., 2006). To apply MAS to the improvement of eel strains for

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doi:10.1016/j.aquaculture.2010.11.006

aquaculture, further genetic mapping, especially constructing a genetic linkage map needs to be conducted.

In the last decade, genetic linkage maps have been constructed for many species of model and commercial fish, such as medaka (Naruse et al., 2000), zebrafish (Postlethwait et al., 1994), guppy (Tripathi et al., 2008), rainbow trout (Sakamoto et al., 2000; Guyomard et al., 2006), Atlantic salmon (Moen et al., 2008), channel catfish (Liu et al., 2003), European sea bass (Chistiakov et al., 2008), tilapia (Lee et al., 2005), Atlantic cod (Moen et al., 2009; Hubert et al., 2010), and pufferfish (Kai et al., 2005). These genetic linkage maps would facilitate the analysis of QTL that control commercially important traits such as spawning time (rainbow trout; Leder et al., 2006), embryonic development rate (rainbow trout; Robison et al., 2001), sex and thermotolerance (rainbow trout; Perry et al., 2005), cortisol levels (rainbow trout; Drew et al., 2007), body weight and condition factor (Atlantic salmon; Reid et al., 2005), and sex (tilapia; Shirak et al., 2006).

In this study, we constructed the first genetic linkage map for the Japanese eel based upon segregation analysis of 106 microsatellite loci which included 74 newly developed markers and 463 polymorphic loci identified from 56 Amplified Fragment Length Polymorphism (AFLP) primer combinations in 46 $F_1$ progeny of a full-sib family as a basic infrastructure for the genetic improvement of eel strains.

## 2. Materials and methods

### 2.1. Mapping family

We created an $F_1$ full-sib family by artificial fertilization between an egg batch from a single female and milt from single male as follows. Wild glass eels were purchased from a commercial farm. Because sex ratio is skewed toward males in eel stocks under ordinary culture conditions (Satoh et al., 1992; Chiba et al., 1993; Tachiki et al., 1997), feminized (by feeding a commercial diet containing estradiol-17β for five months; Tachiki et al., 1997) cultivated eel was used as a dam, whereas the male eel was not treated. These female and male eels were cultivated in freshwater for 2–3 years, and then acclimated to seawater at the National Research Institute of Aquaculture, Mie, Japan. Hormonal treatment was carried out for artificial maturation, as described previously (Ohta et al., 1996; Kagawa et al., 1997). The gametes were obtained by gently stripping an ovulating female and a mature male. Artificial fertilization was performed as described previously (Ohta et al., 1996; Kagawa et al., 1997) in order to construct an $F_1$ mapping family. Muscle samples from parental eels were taken for DNA extraction, and immediately stored at −80 °C until use. Post hatched larvae were kept in a 180-l polycarbonate tank supplied with filtered seawater (1-l/min) at 21.5 ± 0.5 °C until eight days after hatching (DAH). Approximately 250 larvae (8 DAH) were stocked in each of three 5-l round acrylic resin tanks. Rearing regimes of eel larvae were according to the procedure described previously (Tanaka et al., 2001, 2003). Forty six larvae were randomly sampled in total from the three tanks on 48 DAH, fixed with 70% ethanol, and stored at −20 °C until DNA preparation.

### 2.2. Genomic DNA extraction and whole genome amplification

Genomic DNA samples were extracted from parental muscle and 46 larvae using Wizard SV Genomic DNA Purification System (Promega, WI, USA) according to the manufacturer's protocol. The DNA was subsequently stored at −20 °C until use. In order to amplify the amounts of each DNA samples, whole genome amplification was carried out using Illustra GenomiPhi DNA Amplification kit (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's protocol. Briefly, 10 ng of genomic DNA (in 1 μl) was mixed with 9 μl of GenomiPhi sample buffer. This mix was heat denatured at 95 °C for 3 min, then cooled on ice. Nine microliters of GenomiPhi Reaction buffer was then mixed with 1 μl GenomiPhi enzyme, and 10 μl of this mix was added to the denatured genomic DNA. The reaction was subsequently incubated at 30 °C for 1.5 h, then heat inactivated at 65 °C for 10 min. The amplified DNA was used as a template for both AFLP and microsatellite analyses.

### 2.3. Genome size estimation by flow cytometry

For the determination of the number of markers necessary to cover the whole genome of the Japanese eel, flow cytometry (FCM) was conducted using a PA type flow cytometer (Partec, Germany) in order to estimate the genome size. The genome size was determined by the relative DNA content of somatic cells compared to standard samples with known C-values. Pufferfish *Takifugu rubripes* (C-value: 0.40 pg/1 C, Brenner et al., 1993) and both diploid and triploid rainbow trout *Oncorhynchus mykiss* (C-value: 2.69 pg/1 C, Krishan et al., 2005) were used as standard samples in nuclear DNA content determination. Whole blood was sampled from three individuals of each species (Japanese eel, pufferfish, rainbow trout (2n), and rainbow trout (3n)), fixed with 70% ethanol, and stored at −20 °C until use. The method of FCM analysis was performed as described previously (Nomura et al., 2004). C-value of the Japanese eel was determined by simple regression model using both peak values of standard samples and their C-values described previously. Estimated C-value was converted into the base pairs using the following formula described by Dolezel et al. (2003):

$$\text{Genome size (bp)} = \left(0.978 \times 10^9\right) \times \text{DNA content (pg)}.$$

The number of markers which are needed to construct a linkage map that covered the whole genome of the Japanese eel, was estimated by a genome size converted from the C-value using the following formula described by Lange and Boehnke (1982):

$$m = log(1-P) / log(1-2d / L)$$

| | |
|---|---|
| m | number of markers |
| P | probability of the marker existence |
| d | genetic distance (cM) between two adjacent markers |
| L | genome size (cM). |

### 2.4. AFLP analysis

AFLP analysis was performed using the AFLP Plant Mapping kit (Applied Biosystems, CA, USA) according to the manufacturer's protocol. Five hundred ng of total DNA was digested with 5 U of *Eco*RI and 1 U of *Mse*I, and then the fragments were ligated with *Eco*RI adapter and *MseI* adapter using 1 U of T4 DNA ligase at 37 °C for 2 h. The fragments tagged with both adaptors were amplified using PCR Thermal Cycler Dice (TaKaRa, Tokyo, Japan) with the adapter-specific primers and Amplification Core mix contained in the kit. The thermal cycling conditions were 72 °C for 2 min; 30 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min. The amplicon was diluted 1:20 with 20 mM Tris–HCl, 0.1 mM EDTA (pH 8.0) subjected to PCR amplification using the Amplification Core mix and the pair of selective primers added a nucleotide to 3′ terminus of sequence of *Eco*RI or *Mse*I site, or without the additional nucleotide. The selective primers used in this study are shown in Table 2. Primer containing *Eco*RI site is labeled with the fluorescent dye [TET] at the 5′-end. Thermal cycling conditions for the selective PCR amplification were as follows: 94 °C for 2 min; 10 cycles of 94 °C for 20 s, 66 °C for 30 s and 72 °C for 2 min (1 °C touching down from the previous cycle); 30 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min; and 60 °C for 30 min.

The AFLP reaction products were mixed with an equal volume of loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.05% bromophenol