Detection of ayu sex-linked DNA markers using homologous clones

Tomohisa WATANABE,1,2 Kimio YAMASAKI,3 Shingo SEKI2 AND Nobuhiko TANIGUCHI1*

1Laboratory of Applied Population Genetics, Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, 2Department of Aquaculture, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783-8502 and 3Wakayama Prefectural Freshwater Fisheries Center, Naga, Wakayama 649-6112, Japan

ABSTRACT: In order to detect sex-linked DNA markers, linkage analysis was performed in each of three ayu half-sib families using 32 amplified fragment length polymorphism primer combinations, four microsatellite DNA primer sets and phenotypic sex (male). In each family the sex-determining locus appeared in a distal position on one of the linkage groups in which the D7-141* marker was strongly associated with male gender. The D7-141* marker was amplified in all of the male ayu (100%), and 8.7% of the female ayu. The D7-141* marker was detected in all male ayu at a level similar to that in the 40 wild individuals. These results suggest that the D7-141* marker is a male-specific marker linked with the sex-determining locus of the male in amphidromous ayu.

KEY WORDS: AFLP, ayu Plecoglossus altivelis, linkage map, microsatellite DNA, sex-linked marker.

INTRODUCTION

Various sex-determining systems in fishes (e.g. gynogenetic1 and hermaphroditic2) and sex change induced by environmental factors and body size,3,4 have been reported. However, in the cases of many, sex determination in fish has led to the evolution of genetic mechanisms to ensure a 1:1 sex ratio. Because sex-control measures such as sex-balanced and sex-limited reproduction are very important in fisheries and aquaculture industries, there is need of evolutionary and genetic studies on the mechanisms of the sex-determining system. Nevertheless, the sex-determining system using the genetic techniques was clarified only in a few species. As to the development of sex-linked markers, the sex-determining locus has been identified in a single portion of the sex chromosome in a few commercial fishes, such as rainbow trout and channel catfish,5–8 by applying linkage maps.

DNA markers, which are linked with physiological traits clarified by linkage analysis in a family, are not always valid for other families within the species. This may suggest that the linkage of specific physiological traits should be evaluated in a number of lines and families. In order to select specific physiological trait markers suitable for many lines and families, universal trait-associated markers are needed for genetic selection and improvement. However, species-specific sex-linked markers to distinguish sex in commercial fishes have not been reported yet.

Ayu Plecoglossus altivelis is one of the most important fish species in Japanese freshwater culture farms. A large number of ayu stock is produced every year in many farms. In this species the morphological differences between the X and Y chromosomes have not been cytologically identified;9 additionally, the sex-determining locus has not been detected. However, it has been generally recognized that there is a sex-determining system of X–Y because female ayu (XX) can be produced by gynogenesis in ayu.10 Therefore, detection of DNA markers linked with the sex-determining locus might be important in clarifying the mechanism of sex determination.

In the present study, three half-sib families produced by mating a homozygous clonal female ayu with wild diploids were used to obtain segregation of simple DNA markers derived from sire. First, in order to identify DNA markers linked with sex-determining locus, we performed linkage analysis using amplified fragment length polymorphism (AFLP) markers, microsatellite DNA markers and
phenotypic sex. We then tried to apply the male-specific universal DNA markers to individuals collected from wild individuals in order to ascertain their validity.

MATERIALS AND METHODS

Fish sample

One homologous clonal female ayu11 was mated with three male diploids to produce three half-sib families in Freshwater Fisheries Center of Wakayama Prefecture in November 1999. The three half-sib families, named HF1, HF2, and HF3, were raised communally for 12 months. The 187 individuals were collected in the spawning season of 2000 as samples for DNA analysis.

In addition, 40 samples were captured from the wild individuals of amphidromous ayu in the rivers of Wakayama Pref. (n = 24; male, 12; female, 12), Yamaguchi Pref. (n = 4; male, 2; female, 2), and Kochi Pref. (n = 12; male, 6; female, 6). These individuals were used to examine the applicability of the sex-linked markers to wild individuals. DNA samples were extracted from the fin clip of the sample fish by the standard sodium dodecylsulfate (SDS)-phenol/chloroform protocol and stored at 4°C prior to polymerase chain reaction (PCR) analysis.

AFLP and microsatellite DNA analysis

The AFLP analysis was carried out as described by Vos \textit{et al.}13 The selective amplification primers used in the present paper are indicated in Table 1. The \textit{Eco}RI primers were designated with a letter from A to D, and \textit{Mse}I primers were designated by a number from 1 to 8. Selective amplification primer combinations were designated by a letter plus a number, with the \textit{Eco}RI primer first. The 32 primer combinations from A1 to D8 were used in the present study (Table 1). The names of loci and alleles of AFLP markers were given using primer combinations and fragment sizes of the bands detected by electrophoresis.

Microsatellite DNA markers were detected by the protocols described in the previous paper14 using four DNA primer sets, \textit{Pal-1*}–\textit{Pal-4*}.

Electrophoresis

Amplified products of both AFLP and microsatellite DNA markers were denatured at 95°C for 10 min, then snap-cooled on ice for at least 5 min prior to loading on an 8% acrylamide gel (7 M urea, 8% acrylamide). Microsatellite DNA products (2 mL each) or AFLP products (3 mL each) were loaded and the gels were run at 55 mA between 2.5 and 3 h, depending on the fragment size for each particular primer set. Amplified fragments were sized by relation to M13 sequence ladder.

Sex and pedigree analysis of the three half-sib families

The phenotypic sex of the offspring was determined based on gametes of the male or female gonad in the spawning season.

The pedigree analysis of the three half-sib families was performed by identifying the offspring that had the same alleles as the parents, by means of microsatellite DNA markers (\textit{Pal-1*} and \textit{Pal-4*} only).

Polymorphic and linkage analyzes

Genotyping data were made using 32 AFLP primer combinations and four microsatellite primer sets for parents and progenies of the three half-sib families. Goodness of fit of observed to expected allelic ratios was examined by \textit{χ²} test (P < 0.05).

Genotyping data of AFLP and microsatellite DNA markers were analyzed for each family using

| Table 1 | Nomenclature by matrix identification of 32 AFLP primer combinations |
|---------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| E-AA (A) | A1 | A2 | A3 | A4 | A5 | A6 | A7 | A8 |
| E-AC (B) | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 |
| E-AG (C) | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 |
| E-AT (D) | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 |

Each \textit{Eco}RI primer was given a letter (A–D) and each \textit{Mse}I primer was given a number (1–8). Thus, for example, the B8 primer combination represents AFLP using E-AC and M-CTT primers.

AFLP, amplified fragment length polymorphism.
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MAPMAKER/EXP 3.0 software. An initial grouping of markers was performed with log odds (LOD) cutoff 3.0, and the linkage group distances were calculated using Kosambi map function.

RESULTS

Pedigree analysis

Of the 187 individuals, 80 individuals (male, 43; female, 37) were assigned to the half-sib family HF1, 69 individuals (male, 38; female, 31), to HF2, and 38 individuals (male, 14; female, 24) to HF3. The sex ratio of each half-sib family was scored as 1:1 (P > 0.05).

Polymorphic analysis

Polymorphic analysis was performed based on the banding pattern for each sire. Genotype for Pal-1*–Pal-4* microsatellite DNA markers, segregated in a 1:1 ratio (P > 0.05), were scored in each of the three half-sib families.

Using 32 AFLP primer combinations from A1 to D8, representative AFLP of between approximately 100 bp and 300 bp were clearly obtained. The total number of AFLP markers detected in each half-sib family was 91 in HF1, 78 in HF2, and 72 in HF3. The genotypes ratios of positive and negative in AFLP markers did not deviate from 1:1 (P > 0.05).

Linkage analysis between DNA markers and sex

The AFLP D7-141* marker from the D7 (E-AT, M-CTG) primer combination was shown to be strongly associated with sex (Fig. 1). The AFLP D7-141* marker appeared in all the male ayu examined, being indicative to male ayu at the 141 bp position; but this band was seen in only a few of the female ayu.

Linkage analysis was performed in each of the three half-sib families by using the polymorphic markers described in Table 2. In each of the three half-sib families, five AFLP sex-linked markers were identified on linkage groups (Fig. 2). Distances from the sex-determining locus ranged from 1.4 cM to 16.9 cM (LOD scores ranged between 8.0 and 18.4). The D1-103* marker was mapped on the closest position to the sex-determining locus, although this marker was not considered to be universal because the D1-103* marker showed a difference by family. The D7-141* marker from the D7 primer combination was closely associated with the sex-determining locus in each of the three half-sib families.

In the wild amphidromous ayu, the D7-141* marker was detected in all male individuals (Table 3). As shown in Fig. 3, it was possible to distinguish male and female ayu using D7-141* markers, except in the case of one individual (no. 24). The rates of agreement between the phenotypic sex and D7-141* marker were 96.3% for HF1, 95.7% for HF2, 94.7% for HF3, and 97.5% for the wild individuals (Table 3).

Table 2  No. markers and linkage groups and linkage rate from linkage analysis at each of three half-sib families in ayu

<table>
<thead>
<tr>
<th>Family</th>
<th>No. markers detected</th>
<th>No. markers linked</th>
<th>Linkage rate (%)</th>
<th>No. linkage groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF1</td>
<td>96</td>
<td>71</td>
<td>74.0</td>
<td>23</td>
</tr>
<tr>
<td>HF2</td>
<td>83</td>
<td>34</td>
<td>41.0</td>
<td>13</td>
</tr>
<tr>
<td>HF3</td>
<td>77</td>
<td>34</td>
<td>44.2</td>
<td>13</td>
</tr>
</tbody>
</table>

* Included AFLP, microsatellite DNA and phenotypic sex markers.
DISCUSSION

The three half-sib families produced by mating a clonal female ayu provided genotype segregation of 1:1 in the linkage analysis, and informative markers derived from sire were efficient to detect sex-determining locus. The sex-determining locus was located in a linkage group containing a total of five AFLP markers. Each marker was associated with one sex-determining locus Y. These results suggest that the sex-determining system of ayu is XX for female and XY for male, where the sex is controlled by one locus on one chromosome. Clusters in Fig. 2 show that D7-141* is the male-specific sex-linked marker common to the three half-sib families. Further, direct sequencing of the D7-141* marker is recommended to detect the male-specific marker linked with the sex-determining gene of ayu. The D7-141* marker is also effective as a sex-determining marker in brood stock management and aquaculture.

Although the D7-141* marker is linked with the sex-determining locus and is effective as a male-

**Table 3** No. male and female ayu detected by D7-141* marker at three half-sib families and native population of ayu

<table>
<thead>
<tr>
<th>Detected no. D7-141* markers</th>
<th>LOD score between sex and DNA marker</th>
<th>Agreement rate between sex and D7-141* marker (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (No. male ayu)</td>
<td>Female (No. female ayu)</td>
<td></td>
</tr>
<tr>
<td>HF1</td>
<td>43 (43)</td>
<td>3 (37)</td>
</tr>
<tr>
<td>HF2</td>
<td>38 (38)</td>
<td>3 (31)</td>
</tr>
<tr>
<td>HF3</td>
<td>14 (14)</td>
<td>2 (24)</td>
</tr>
<tr>
<td>Wild individuals</td>
<td>20 (20)</td>
<td>1 (20)</td>
</tr>
</tbody>
</table>

*Agreement rate between the detected sex and the marker is also indicated.

*No. male and female distinguished by phenotypic sex.

LOD, log odds.
specific marker, an exception was observed in one female ayu (no. 24) of the wild amphidromous individuals (Fig. 3). Because this discrepancy was found only in a female individual, it seems unlikely that it was caused by recombination between the marker and the sex-determining locus.

Although recombination may not be excluded as a possible cause of this discrepancy, it is also possible that such discrepancies occur due to sex reversal caused by non-genetic factors, such as water temperature and exogenous endocrine disrupting chemicals. In the present experiment the detected band appeared in all male ayu but disrupting chemicals. In the present experiment as water temperature and exogenous endocrine sex reversal caused by non-genetic factors, such

The discrepancy was shown in only eight of 112 female individuals from the three half-sib families and wild individuals. These eight individuals had a D7-141* marker in spite of being female, similarly with the case of individual no. 24 in Fig. 3. If these eight individuals are secondary female ayu with gonads, we can verify the sex reversion of the sex-reversed female ayu (XY) and normal male ayu (XY). In the offspring, we will find that the sex ratio would be 3 : 1 (XY and YY for male, and XX for female). IFYY is lethal, this ratio would be 2 : 1.

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In the fisheries environment the issue of feminization of organisms by endocrine disrupting chemicals has recently become important.16–18 There are not enough sex-linked markers to prove whether individuals exhibit their true genetic in the natural environments or not. In the future, it will be taken into consideration that genetic linkage mapping allows us to clarify the sex-determining system and to identify the sex-determining locus without directly detecting a sex-determining gene. Further, such mapping will be useful for aquaculture and conservation biology to evaluate, by using common markers such as the D7-141* marker, the changes in phenotypic sex caused by environmental change.

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REFERENCES

15. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L. MAPMAKER. An interactive com-


