# Identification of a sex-linked SCAR marker for *Plecoglossus altivelis* and its application for identifying gender in cultivated and wild populations

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**Abstract**

Ayu (*Plecoglossus altivelis*), one kinds of valuable cultured fish species, show almost no morphological difference between male and female until sexual maturity. Here, we report the identification of sex-linked markers for the ayu, based on Amplified Fragment Length Polymorphism (AFLP) generated from cultured fish (15 males and 15 females) by using 63 different primer combinations. Genomic fragments ($n$ = 3733) were produced with a mean frequency of 59 bands per primer pair. A male-specific of 139 base pair band was amplified and converted to a sequence-characterized amplified region (SCAR marker) designated as Ayu102. Six distinct genomic fragments were produced in 12 wild samples (6 males and 6 females). The fragments designated *a, b, c, d,* and *e* were detected only in males, and one fragment (*f*) was detected in both genders. Nucleotide sequence analysis showed that fragments *e* and *f* were 96.67% identical. Ayu102 marker was detected in 45-d-old larvae, in both cultured and wild fish populations, and in offsprings generated by gynogenesis. Fragment *e* was detected in all males, and in only 6.7% of females in cultured fish. Data suggest that Ayu102 marker is a male-specific marker linked to the sex-determining locus of the male ayu and can be used for gender identification.

**Keywords:** AFLP, *Plecoglossus altivelis*, Sex-linked marker.

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## Introduction

Ayu is a popular commercial fish found on Chinese mainland, Taiwan, and in Japan where it is cultured in freshwater farms. In these countries, ayu is widely consumed because of the sweet taste, high nutrient content, and light scent. Ayu is sexually dimorphic, and after maturation, body color of males darkens and they lose weight. In contrast, females mature later, grow faster, and are considered more delicious than the males. For these reasons, females are sold for twice the price of a mixture of males and females. In the animal kingdom, sexual size dimorphism is a widespread phenomenon (Fairbairn and Blanckenhorn, 2007; West, 2009; Kavanagh *et al*., 2011). Sexual allometry follows Rensch's rule in different species (Rensch, 1960). The mechanism that regulates the relationship of body size to shape is highly complex (Horppila *et al*., 2011). And in some commercial fish such as *Perca flavescens* (Lynn *et al*., 2008), *Psetta maxima* (Aydın *et al*., 2011), *Kajikia audax* (Kopf *et al*., 2012), size of the females' body are larger than the males. In contrast, males of *Oreochromis mossambicus* (Oliveira and Almada, 1995), *Lamprologus callipterus* (Schütz and Taborskym, 2000), etc. are larger than the females.

The average ratio of males to females is 1:1 in most commercial fish (Devlin and Yoshitaka, 2002). Allometric properties of cultivated fish of both genders may result in wasting resources. Without considering drawbacks, a unisex population generally possesses virtues such as rapid growth, controlled breeding, and high economic value (Viñas *et al*., 2012). Therefore, much attention has been focused on gender regulation and developing unisex breeding populations. Biological basis for sex determination is quite complex and differs among fish species; it is known for a few species (Herpin and Schartl, 2009; Piferrer *et al*., 2012) but is poorly understood for most commercial species. Thus, screening for sex-linked markers is the most effective and feasible method for generating unisex breeding populations.

Sex-linked DNA markers have been successfully applied to screen several kinds of species used in aquaculture (Liu and Cordes, 2004). Three sex-linked markers have been screened using amplified fragment length polymorphisms (AFLPs) and bulked segregant analysis (Michelmore *et al*., 1991) in *O. niloticus* (Lee *et al*., 2011). Two female-specific AFLP markers were reported for *Verasper variegatus* (Ma *et al*., 2010). Chen *et al* reported a female-specific marker based on AFLP in *Cynoglossus semilaevis* and successfully used it to generate a SCAR marker that was applied to breed an all-female population (Chen *et al*., 2008). However, AFLP is not always effective in all species (Gao *et al*., 2010; Yarmohammadi *et al*., 2011). As an alternative to using AFLPs, Kovács *et al* obtained 2 male-specific markers in *Clarias gariepinus* by using randomly amplified polymorphic DNA (RAPD) technology (Kovács *et al*., 2000). Waldbieser *et al* screened 7 simple

sequence repeat markers that were closely linked with the sex determination locus of *Ictalurus punctatus* (Waldbieser *et al*., 2001). Combining homologous clones with AFLP, Watanabe *et al*. detected one ayu male-linked DNA marker, D7-141*, from Japanese ayu populations (Watanabe *et al*., 2004). There are significant morphologic differences between Chinese mainland and Japanese ayu. Based on these differences, Wu and Shan designated the Chinese mainland Ayu as a new subspecies called *P. altivelis chinensis* (Shan *et al*., 2005). It is not known whether D7-141* can be used as male-specific marker for Chinese mainland Ayu.

In the present investigation, we screened the presence of sex-linked markers in genomic DNA isolated from mature gonads of Chinese mainland ayu. To simplify detection, we converted the sex-linked marker into a SCAR marker. The ability of the SCAR marker to identify gender was analyzed in 45-d-old ayu larvae, cultured and wild populations of ayu, and in the offsprings of gynogenesis.

## Material and Methods

*Fish*

Ten 45-day-old ayu larvae were acquired from the Zhejiang Mariculture Research Institute, Qingjiang Station, in December 2010. Cultured fish were acquired from the Zhejiang Ninghai ayu farm in October 2010 and 2011 and the Zhejiang Nihai Huangtan reservoir in October 2011. Fifteen males and 15 females were collected from each source. Six males and 6 females were taken from Zhejiang Wenzhou Nanxi River in October 2011. Fifteen-day-old offsprings obtained via gynogenesis were acquired from the Zhejiang Mariculture Research Institute, Qingjiang Station, in November 2010. The parental fish used to induce gynogenesis were acquired from the Zhejiang Yandang Mountain ayu farm.

*DNA extraction*

DNA was isolated from all samples by using a Genome DNA Isolation Kit (Sangon; no. SK8252). The integrity and purity of DNA preparations were assessed using ultraviolet light to visualize bands that were separated by performed electrophoresis using 1.5% agarose gels. The DNA samples were stored at -20ºC.

*Screening for sex-linked markers*

AFLP analysis was performed according to the method reported by Vos *et al* (1995). The 63 primers synthesized by Shanghai Invitrogen Biotechnology Co. are listed in Table 1. The ayu sex-linked marker was used to screen a cultured population (15 males and 15 females) from the Ninghai ayu farm. Selective amplified products were separated using 8% denaturing polyacrylamide gel electrophoresis and were then detected by silver staining (Sanguinetti *et al*., 1994).

*Generation of a SCAR marker*

One AFLP sex-specific fragment was excised from the 8% denaturing polyacrylamide gel and eluted with 20 µl

sterile water at 4ºC overnight. Reamplification polymerase chain reaction (PCR) was performed with the corresponding primer pair using the same conditions. The PCR products were first detected after electrophoresis was performed using 2% agarose gels and then cloned into the pMD19-T vector (D102B, TaKaRa) for nucleotide sequence analysis. The sequences were analyzed using DNAMAN 6.0 software. Special primers, named Ayu102 were designed using Primer Premier 5.0 software (Ayu102F: 5′-CATTTTTCTGCCAAATCAGC-3′; Ayu102R: 5′-GTCCCATCATCACACTGGTTC-3′). The primers were synthesized by Shanghai Invitrogen Biotechnology Co. The specificity of Ayu102 was first determined in samples taken from wild fish. The PCR conditions were as follows: denaturation at 94ºC for 5 min; 35 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 30 s, and extension at 72ºC for 30 s; and extension at 72ºC for 10 min; finally saved at 4ºC. The PCR products were detected using 12% denaturing polyacrylamide gels and 1.5% agarose gels. Types of gonads of 45-d-old larvae were verified by hematoxylin and eosin staining of paraffin sections (Divers *et al*., 2009). PCR was then performed using Ayu102 marker, and the products were detected as described above. The specificity of Ayu102 marker was further verified in cultured and wild fish and in offsprings obtained via gynogenesis ($n$ = 98).

**Table 1: AFLP-selective primers for sex-specific marker screening of *P. altivelis***

| Primer | Primer sequence (5′-3′) | |
|---|---|---|
| | *Eco*RI | *Mse*I |
| Selective primer | E-AAC | M-CAC |
| | E-AAG | M-CCA |
| | E-AAT | M-CCT |
| | E-ACA | M-CGA |
| | E-ACT | M-CTG |
| | E-AGA | M-CTT |
| | E-AGT | M-CGT |
| | E-ATC | |
| | E-ATG | |

## Results

*Identification of a male-specific marker*

We generated 3733 fragments by using 63 primers, with an average of 59 bands per primer combination. One male-specific fragment was identified using the E-ATG/M-CTG primer combination by screening DNA isolated from 15 males and 15 females (Fig. 1).

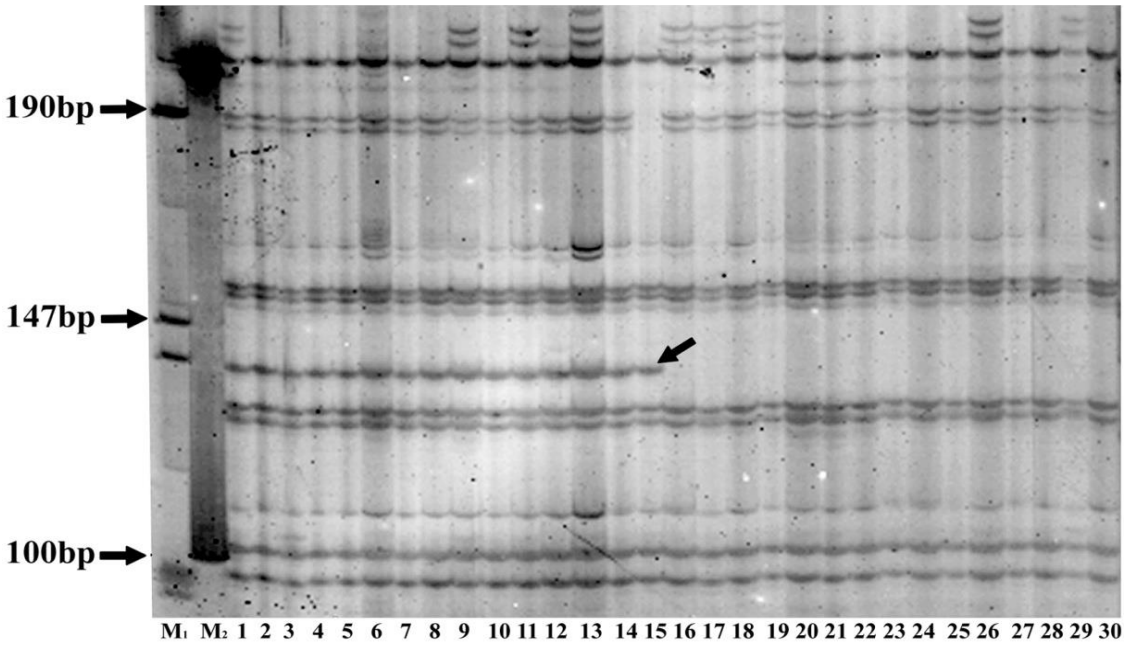


**Figure 1: AFLP analysis performed using the E-ATG/M-CTG primer combination to screen 15 male (lanes 1–15) and 15 female (lanes 16–30), samples were acquired from cultured fish. The arrows point to the male-specific fragment. $M_1$: PUC-19 marker and $M_2$: 100-bp ss DNA marker.**

*Generation of a SCAR marker*

Nucleotide sequence analysis showed that the male-specific fragment was 139 base pairs (bp) long, and its sequence is shown in Fig. 2A. After removing the primer sequences, the core sequence did not return a significant match when used to query the GenBank database (Http://blast.ncbi.nlm.nih.gov). Fig. 3A shows the results obtained using Ayu102 to analyze samples acquired from wild fish (6 samples from each gender). Six distinct genomic fragments were identified, 5 of them (*a, b, c, d*, and *e*) were detected in male samples while fragment *f* was detected in all individuals. After gel extraction, reamplification, cloning, and sequencing, the sequences of the male fragment *e* were found to be identical, while the sequences of fragment *f* were identical for all samples. Other fragments (*a, b, c*, and *d*) could not be sequenced. The only bands detected after agarose gel electrophoresis were approximately 100 bp long (Fig. 3B). Sequence analysis showed that fragments *e* and *f* were 96.67% identical (Fig. 2B and Table 2).


**Figure 2: A: Sequence of the male-specific band generated using the E-ATG/M-CTG primer-pair. B: Sequence alignment of fragments *e* and *f*.**

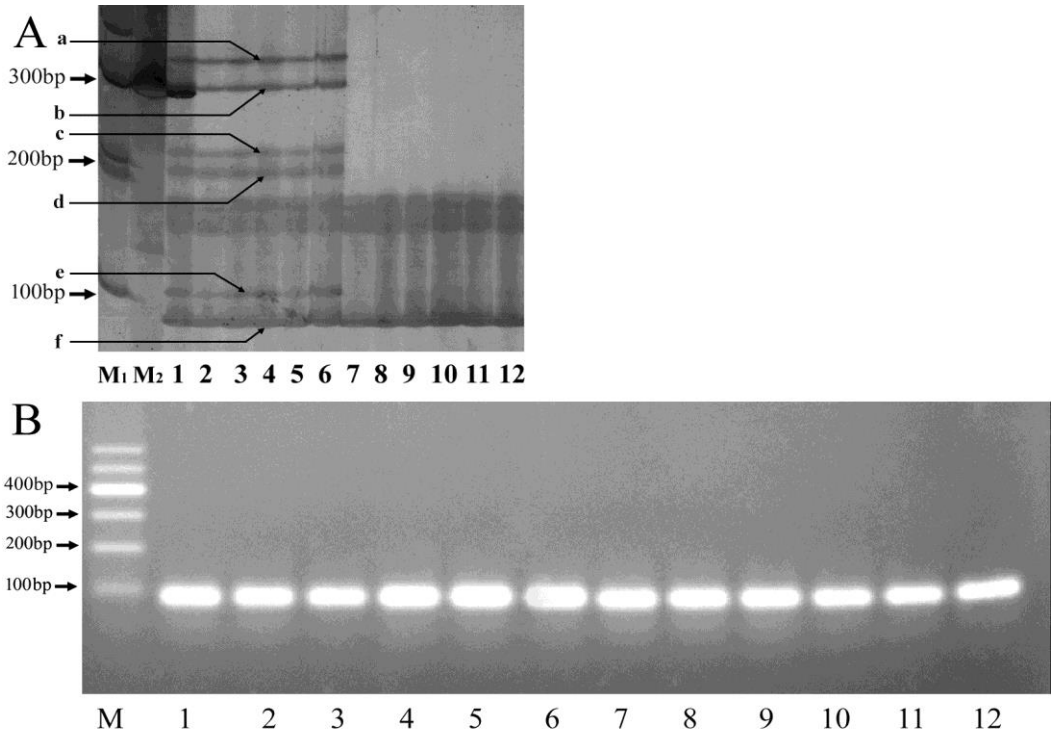


**Figure 3: Analysis of samples acquired from wild fish, using Ayu102 marker. A. Image for 12% denaturing polyacrylamide gel electrophoresis; B: image for 1.5 % agarose gel electrophoresis. $M_1$: 100-bp marker; $M_2$: 300-bp ss DNA marker; M: 100-bp marker; lanes 1–6: males; lanes 7–12: females.**

**Table 2: Summary of sequence alignment of bands *e* and *f*.**

| | **Fragment *e*** | **Fragment *f*** |
|---|---|---|
| Length (bp) | 102 | 90 |
| GC content (%) | 51.0 | 54.4 |
| Transition | - | A→G (33)* |
| Transversion | - | A→C (60, 79)* |
| Deletions | - | TAACCCATA (from 46 to 54)*<br>GTA (from 61 to 63)* |

* The numbers in parentheses indicate the nucleotide position in the sequence

*Application of Ayu102 as a gender-specific marker*

Histological analysis helped detect highly differentiated gonads in all 45-d-old ayu (Fig. 4A, B). Fragment *e* was only detected in males (Fig.4C). Fragment *e* was detected in all males but unexpectedly in 2 females (no.36 and no.46) in samples taken from cultured fish (6.7%). The detection of fragments *a* and *b* coincided with fragment *e* and was sex linked. However, fragments *c* and *d* were not well resolved. Fragment *f* was detected in all individuals (Fig.5). Figure 6 shows the results for samples acquired from wild fish taken from Nanxi River. Fragments *e* and *f* followed the rules mentioned above. Fragment *e*, which was only detected in male parents, was not detected in the offsprings obtained via gynogenesis (Fig.7).

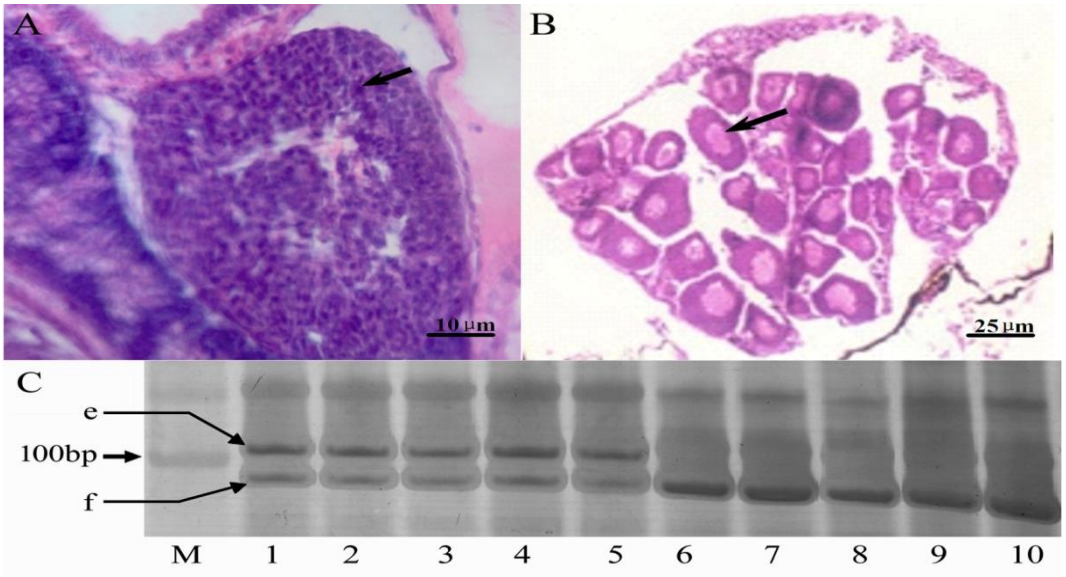


**Figure 4: Histological sections of ayu gonads (A: testis; B: ovary) and the analysis of 45-d-old larvae using Ayu102 marker. The arrows point to spermatocytes in a testis (A) and to oocytes in an ovary (B); M: 100-bp ss DNA; lanes 1–5: males; and lanes 6–10: females. (C).**

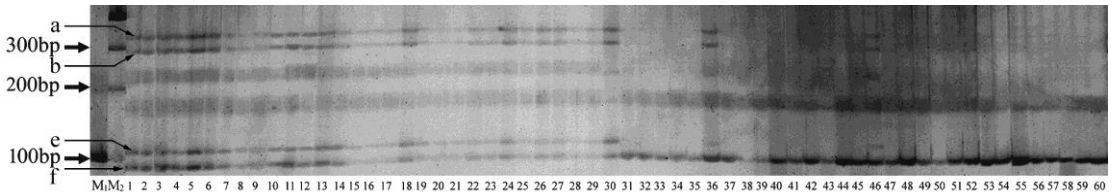


**Figure 5: Identification of male cultured fish using ayu102 marker. $M_1$: 100-bp ss DNA, $M_2$: 100-bp marker; lanes 1–15: males from the Ninghai farm; lanes 16–30: males from the Huangtan Reservoir; lanes 31–45: females from the Ninghai farm; and lanes 46–60: females from the Huangtan Reservoir.**

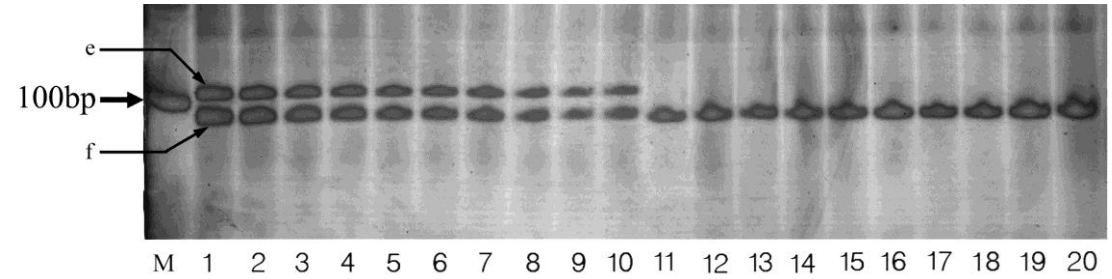


**Figure 6: Identification of wild male fish using Ayu102 marker. M: 100-bp ss DNA; lanes 1–10: males; and lanes 11–20: females.**

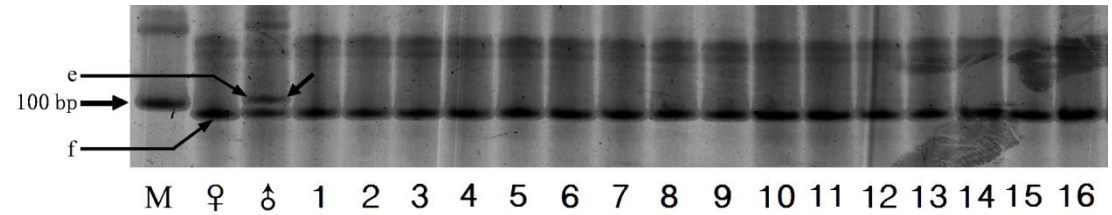


**Figure 7: Identification of males in fish generated by gynogenesis, using Ayu102 marker. M: 100-bp ss DNA, ♀: female parent, ♂: male parent; and lanes 1 – 16: gynogenesis population.**

## Discussion

Although ayu is one of the most important cultured species in Chinese mainland, its sex determination system and sex-linked marker are unknown. Using AFLP we detected one male specific marker using genomic DNA from cultured population and successfully generated SCAR markers, Ayu102, for ayu gender identification.

Bulked segregant analysis is a useful approach to screen sex-linker markers; however, this cannot be accomplished if gene pools contain genomic DNA from individuals that have undergone sex reversal. Sex determination system of fish is vulnerable to environmental factors, which are known to cause sex reversal (Devlin and Yoshitaka , 2002).Watanabe *et al* reported that the ayu male-specific marker D7-141* could be detected in some female individuals (8.7%), which might have been the result of sex reversal (Watanabe *et al*., 2004). Moreover, genetic diversity could be another factor to influence the process of screening for sex-specific markers (Gao *et al*., 2010). Genetic diversity is relatively low in populations of cultured fish (Frost *et al*., 2006), which could reduce the influence for sex-specific markers screening. Further, a cultured population does not originate from the same family, which overcomes

the complication that causative genetic markers might not be suitable for analyzing different families.

Because the fragments that are selectively amplified using AFLP are uniformly interspersed throughout the genome, AFLP is one of the most effective methods to screen for sex-specific markers. In the present study, we successfully identified 1 male-specific marker (length, 139 bp) generated using AFLP from the genomic DNA of a cultured population of ayu. Our results are in general agreement with the male-specific marker D7-141* reported by Watanabe *et al.*(2004), but the selective primers used in our experiment were all extended with 3 bp at their 3′ ends, which have reduced the number of bands generated in all PCRs and provided a convenient means for separating and detecting the fragments. Because the samples were derived from different populations of the same species, the male-specific marker described here and the D7-141* marker may represent the same locus. However, the male-specific marker is 2 bp shorter than the D7-141* marker, which suggests that genetic differences between Chinese mainland and Japanese ayu is distinguished. Our data support the proposal of Shan *et al* (2005) that Chinese mainland ayu should be considered as a new subspecies.

Although AFLP provides an effective technique for identifying sex-linked markers, not all can be used to effectively generate SCAR markers (Rao *et al*., 2012). This might be caused by mutations at the restriction enzyme cleavage sites or by the possibility that no sex-linked markers exist. Six distinct genomic fragments were amplified by ayu 102 in our studies on ayu. We could not generate sequence data for 4 fragments, and they did not migrate as distinct bands in agarose gels. Moreover, in the lane of 100-bp ss-DNA, other distinguish band was identified together with the main band (Single-stranded 100bp band) during denaturing polyacrylamide gel electrophoresis. This finding may attributable to stripes cross-linking in the loading system.

Sequence analysis showed that fragments *e* and *f* were 96.67% identical. On comparison with fragment *e*, the deletion, transition, and transversion in the sequence of fragment *f* were apparent. Sequence evolution is directly related to evolutionary selective pressure, which is evident from the sequence differences (Dowd *et al*., 2009; Gray, 2012). Our data suggest that selection pressure for fragment *f* might be lower than that for fragment *e*. A specific mechanism could exist to suppress sequence evolution within the region containing sex determination locus.

The results acquired from analysis of 45-d-old larvae, and cultured and wild ayu populations using Ayu102 marker suggest that fragment *e* is closely linked to the sex determination locus. Further, fragment *e* was also amplified from 2 females. Because fragment *e* was detected in all male individuals, its detection in females may have been generated by sex reversal. The sex

reversal rate partly reflects the plasticity of the sex determination system. Evolution generally improves sex determination system in increments, and at the same time the influence of environmental factors gradually diminishes. Ayu has evolved at a relatively low level. The rate of detection of fragment *e* was 6.7% in cultured fish, which is lower than the result reported by Watanabe *et al*. (2004), and might be explained by the genetic differences among populations of ayu as well as culture conditions and environmental changes. Fragment *e* was not detected in the wild samples; although the sample size was small.

In conclusion, the data presented here indicate that Ayu102 marker is useful for ayu gender identification and sex determination system investigation.

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