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# A strain-specific and a sex-associated STS marker for Asian arowana (*Scleropages formosus*, Osteoglossidae)

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## Abstract

A sex-associated amplified fragment length polymorphism and a strain-specific random amplified polymorphic DNA marker were identified from Asian arowana (dragonfish; *Scleropages formosus* Müller & Schlegel) by screening pooled genomic DNA samples from three different strains as well as males and females respectively. Both markers were cloned, sequenced and successfully converted into sequence-tagged-site (STS) markers. The strain-specific STS marker could be applied to differentiate the Indonesian golden strain of Asian arowana from the green and blood-red strains before the stage when colours become identifiable. Individuals from the green strain could be sexed with an efficiency of 82.7% using the sex-associated STS marker. Thus, populations with preferred sex ratios can be formed without the need of rearing a large number of fish.

**Keywords:** dragonfish, bonytongue, AFLP, RAPD, molecular sexing, strain identification

## Introduction

The Asian arowana (dragonfish; *Scleropages formosus* Müller & Schlegel, Osteoglossidae) is one of the most expensive species in the ornamental fish industry (Scott & Fuller 1976). The number of individuals in the natural habitat had been reduced rapidly due to over-fishing, therefore the species has been listed as one of the most highly endangered fish by Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1975 (Greenwood, Rosen, Weitzman & Myers 1996). Their commercial export, import and sales are normally prohibited in all member countries of CITES, unless the specimens concerned are bred in captivity. Captive breeding of the species was first achieved in the Agri-Food & Veterinary Authority (formerly Primary Production Department) in Singapore as early as 1981. Following the example of Singapore, several other countries in the region have also established captive populations (Dawes, Lim & Cheong 1999).

There are, however, unsolved problems regarding the captive breeding for conservation and production of this endangered species. Our project addresses some of them by using molecular genetic methods. The first problem is that the sex ratio of breeding populations could not be optimally set, because the sex of this fish can only be determined at a sexually mature stage (3–4 years) and the procedure based on visual inspection is not completely reliable. The second problem is the identification of strains. Asian arowanas have three main varieties (red, golden and green), the first two of which are further divided into strains, such as blood red, chilli red, Indonesian gold or Malaysian gold (Goh & Chua 1999). The market price of red and Malaysian golden strains is much higher than that of the others. Because the colour of the young individuals from all strains is very similar, they can be difficult to recognize until the juvenile stage (ca. 8 months). Moreover, while the red and golden strains are thought to be highly endangered in the wild, and there is an urgent need for protection using captive breeding, the green strain is still relatively common at the natural habitat (Luxmoore 1990).

Random amplified polymorphic DNA (RAPD; Williams, Kubelik, Livak, Rafalski & Tingey 1990) and

amplified fragment length polymorphism (AFLP; Vos, Hogers, Bleeker, Reijans, Vandelee, Hornes, Frijters, Pot, Peleman, Kuiper & Zabeau 1995) are both PCR-based methods, which amplify several different regions of a genomic DNA template in parallel. The resulting band pattern is dependent on the template and the primer(s) used. Both methods, together with others, such as representational difference analysis (RDA; Lisitsyn, Lisitysn & Wigler 1993), have been successfully applied for the identification of sex-specific genomic DNA markers from fish (Iturra, Medrano, Bagley, Lam, Vergara & Marin 1998; Kovacs, Egedi, Bartfai & Orban 2001), other animals (Bello & Sanchez 1999; Navin, Prekeris, Lisitsyn, Sonti, Grieco, Narayanswami, Lander & Simpson 1996; Perez-Perez & Barragan 1998) and plants (Reamon-Buttner & Jung 2000). Similar techniques have also been used to find and develop sequence-tagged-site (STS) markers to differentiate various strains (Tilsala-Timisjarvi & Alatossava 1998) or species (Delozier, Peltier & Jalouzot 1999; Erlandson & Batt 1997; Jones, Noble, Lockyer, Brown & Rollinson 1997a)

In this publication, we report the identification of a sex-associated AFLP marker and a strain-specific RAPD marker in the Asian arowana using pooled DNA samples. The two markers were successfully converted into STS markers and are applicable for rapid sexing of the green strain or for differentiation of the Indonesian golden strain from blood red and green varieties respectively. Together with the microsatellite markers isolated from the species earlier (Yue, Chen & Orban 2000), these markers provide improved tools for researchers and farmers for breeding, maintaining and studying Asian arowana.

## Materials and methods

### Fish samples and DNA isolation

For the identification of sex-associated DNA markers, tissue samples (i.e. fin clips or muscle) were collected from a total of 23 green dragonfish individuals (10 males and 13 females). The sex of all female and some male individuals used in the sex-specific pools was determined by visual inspection of the dissected gonad and by histological analysis (Stiles 1967) of samples preserved in Bouin's solution following dissection. The remaining males were identified by microscopic analysis of squeezed sperm following the injection of human choriogonadotropin (HCG) administered 2 days before squeezing. For the strain-specific marker search, fin clips were collected from 32 live, anaesthetized individuals of each of the following strains: blood red, Indonesian gold and green. DNA was isolated from the tissues according to Miller, Dykes & Polesky (1988).

### RAPD assays

For the identification of sex-associated DNA markers, RAPD analysis was performed on male and female DNA pools, generated by combining the same amounts of DNA from 10 female and eight male individuals respectively. Three DNA pools (i.e. a separate pool for the green strain, the red one and the golden one respectively) were generated for the strain-specific DNA marker search. Each pool was made of equal amounts of DNA from 32 individuals respectively.

RAPD assays were carried out as described previously (Yue, Li, Chao, Lim & Orban 2002). A total of 800 10-mer RAPD-primers (Operon, Almeda, CA, USA) were used for the sex-associated DNA marker search, while 500 primers (Operon and Genemed [San Francisco, CA, USA]) were used for the strain-specific ones.

PCR products of 20 μL were separated in 2% agarose gels (Bio-Rad, Hercules, CA, USA) containing 100 μg $L^{-1}$ ethidium bromide in 1 × TBE buffer. Band patterns were recorded by a Fluor-S$^{TM}$ (Bio-Rad). Putative sex-associated and strain-specific markers were tested on all individuals present in both pools.

### AFLP analyses

The AFLP$^{TM}$ Plant Mapping Kit (ABI/PE, Foster City, CA, USA) was used to screen the DNA pools in both searches. Five hundred nanograms of DNA from every individual was digested with the appropriate restriction endonuclease combination (i.e. *Eco*RI/*Mse*I or *Taq*I/*Mse*I). In the search for sex-associated markers, both male and female pools contained equal amounts of pre-amplified DNA from five green individuals. The two pools were screened using 64 *Eco*RI/*Mse*I primer combinations supplied with the kit and another 48 *Taq*I/*Mse*I primer combinations designed according to Knorr, Cheng & Dodgson (1999). In the strain-specific search, three pools each containing equal amounts of pre-amplified DNA of 32 individuals from each strain, were tested using 64 *Eco*RI/*Mse*I primer combinations.

PCR amplification, separation of PCR products on ABI 377 sequencers (ABI/PE) and analysis of gels

  

were carried out as described previously (Yue *et al.* 2002). Only fragments between 50 and 500 bp were scored. Similar to the RAPD assay primer combinations, putative markers were re-tested on all individual samples comprising the pools.

### Isolation, cloning and sequencing of markers

Bands amplified by RAPD assays were directly cut from the 2% agarose gel and then purified using the GeneClean II kit (Bio 101, Carlsbad, CA, USA). As AFLP patterns were analysed on the ABI 377 automated sequencer, it was not possible to recover the marker bands directly. Therefore, selected AFLP products (2–3 μL) with putative markers were separated on 8% polyacrylamide gel containing 1 × TBE buffer under the following conditions: 500 V and 115 mA for 2–3 h. Gels were stained using a Silver Staining Kit (Bio-Rad) according to the manufacturer's protocol. The selected band was cutout using a sterile blade, put into 50 μL sterile water, and then heated to 100 °C for 10 min to release the DNA. The resulting solution was centrifuged (1 min, 14 000 rpm), then 1 μL eluant was used as a template for PCR amplification (25 μL) with the same selective primer combination. PCR products were cleaned using the GeneClean II kit (Bio 101), and then cloned into pT-Adv vectors (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol. Colony PCR was performed to determine the insert length of the white clones by using M13-20 and M13 reverse primers. Clones with desired insert length were sequenced using the ABIPrism Big Dye Terminator Cycle Sequencing kit (ABI/PE) on an ABI 377 sequencer.

### Amplification of the sex-associated and the strain-specific STS markers

In order to identify the sex-associated markers from the two inserts cloned, we used stepwise primer extension. We synthesized four extended AFLP primers by adding one base (A, T, G or C) to the 3′ end of the unlabelled *Mse*I selective primer and performed AFLP analysis with male and female samples. The primer yielding differential amplification in the two sexes was then further extended by one more base, and the procedure described above was repeated. Altogether, a four-base extension was enough to select the sex-associated marker. Specific PCR primers complementary to the ends of markers were then designed by using software PrimerSelect (DNASTAR). The specific primers for the sex-associated marker were: DSX66A: 5′-6Fam-TAACTCAAAGTAGAATAGAACAATG-3′, DSX66B 5′-AATTCAAGGGAACTGATGACTCTA-3′, while for the strain-specific marker: OPAO3F 5′-GTTCTCGGACTGAG-3′ and OPAO3R 5′-GTTCTCGGACCCTT-3′ (sequences corresponding to the AFLP/RAPD primers are underlined). As the ends of fragments were selected for primer design, the primer pair for each objective band contained some of the AFLP primer sequences or RAPD primer sequences at the 5′ end. The STS-PCR reaction contained 30-ng genomic DNA, 1 × PCR buffer (Finnzymes, Espoo, Finland) with 1.5 mM $MgCl_2$, 0.2 μM of each primer (see Results for sequence), 0.2 mM of each dNTP and 1 U of DyNAzyme II DNA-Polymerase (Finnzymes). The cycling conditions were: an initial denaturation at 94 °C for 2 min followed by 34 cycles of 30 s at 94 °C, 30 s at optimal annealing temperature, 30 s at 72 °C, and then a final extension step of 5 min at 72 °C. The optimal annealing temperature (56 °C for the sex-associated marker and 52 °C for the strain-specific marker) for each STS marker was obtained using a temperature-gradient PCR machine (Eppendorf, Hanburg, Germany). PCR products (0.25 μL) were separated on the ABI 377 sequencer as described previously (Yue *et al.* 2002).

### Duplex PCR

In order to improve the efficiency of molecular strain differentiation, we developed a duplex-PCR according to Yue, Beeckmann, Bartenschlager, Moser & Geldermann (1999). The reaction mix consisted of two primer pairs: one (OPAOF3/R3; see Results for sequence) for the strain-specific band and the other (DSX06A/B; A: 5′-ACACAGTGCAGGTAACAGAATCT-3′; B: 5′-GAAACCAGAGTTGGGACAGGTC-3′) designed from a sequence we isolated from dragonfish (GenBank Accession No.: AF390197). The master mix consisted of 30-ng genomic DNA, 1 × PCR buffer (Finnzymes) with 1.5 mM $MgCl_2$, 0.2 μM of each primer (OPAOF3/OPAOR3 and DSX06A/B), 0.1 mM of each dNTP and 1 U of DyNAzyme II DNA-Polymerase (Finnzymes). PCR cycling conditions on a PTC-100 (MJ Research, Waltham, MA, USA) were: an initial denaturation at 94 °C for 2 min, followed by 30 cycles: 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were separated on 2% agarose gel and patterns were analysed as described above.

## Results

### Isolation and characterization of a sex-associated DNA marker

The comparative RAPD assays performed with 800 10-mer primers failed to yield a sex-associated marker; on the other hand, the AFLP analysis identified one. A 292-bp band – amplified by the *Eco*RI-AAG/*Mse*I-CTC primer pair – appeared in the male DNA pool and was absent from the female one. Further analysis of the 10 ($2 \times 5$) individuals contributing to the two pools proved that this band appeared only in the males (Fig. 1). Sequence analysis revealed that the band in question contained two fragments with the same length. One of the two sequences (GenBank Accession No.: AF391095) contained a $(CA)_9$ microsatellite sequence in the position between 201 and 218 bp,

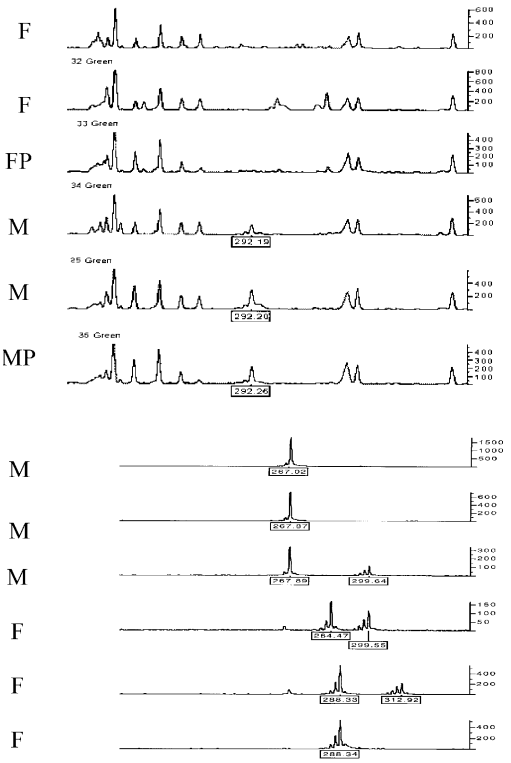


**Figure 1** A male-specific band (292 bp) was amplified from the genome of the green strain of Asian arowana using the AFLP primer combination: *Eco*RI-AAG/*Mse*I-CTC. The upper panel represents the electrophoretogram of the AFLP analysis, while the lower panel represents that of the STS-PCR, both produced by an ABI 377 sequencer. M: male, F: female, FP: female pool and MP: male pool. Numbers under the peaks indicate the allele size in base-pair. Numbers on the vertical axis represent the relative intensity of the fluorescent signals.

while the other did not contain any repeat. By adding four bases to the 3′ end of the unlabelled *Mse*I selective primer in stepwise fashion and re-amplifying the products, we determined that the repeat-containing fragment was the marker associated with sex. The sequence of the sex-associated marker did not show similarity to any known gene when BLAST-ed against GenBank accessions. On the basis of the sequence; a specific primer pair (see Materials and methods for sequence) was designed to the ends of the marker. The primers contained the *Eco*RI and *Mse*I recognition site sequences and amplified a fragment of 268 bp. PCR amplification results with DSX66A/B on all the five males and five females were consistent with the one obtained using the *Eco*RI-AAG/*Mse*I-CTC, the 268-bp band appeared in all five males, and it was absent from all five females. At the same time, one to two longer bands were displayed in females (Fig. 1). Using this primer pair, all 23 individuals of the green strain with known sex were screened. The genotypes of all the 10 phenotypic males were fully consistent with the phenotypic sex; whereas nine out of 13 phenotypic females showed a female genotype, the other four exhibited a male genotype. Thus, the total accuracy rate of molecular sexing is 82.7%.

### Isolation and characterization of the strain-specific DNA marker

The comparative RAPD assay of the three Asian arowana strains with 500 10-mer primers yielded one differentially amplified band (278 bp, amplified by primer OPA-019), whereas the AFLP analysis did not yield a strain-specific marker. The strain-specific RAPD marker was present in the pattern amplified from both the blood red and green pools, but absent from that of the Indonesian golden one. When all 96 ($3 \times 32$) individuals from the three varieties were genotyped with the primer, all patterns from the red and green varieties showed the band in question, while none from the Indonesian gold did (Fig. 2A). The sequence of the cloned marker (GenBank Accession No.: AF390196) was BLAST-ed using GenBank acessions, but did not identify homologous sequences. A specific primer pair (see Materials and methods for sequence) was designed to the ends of the marker. Using this primer pair, a band was amplified both from green and blood red individuals, but not from the golden ones. The method was further improved to duplex PCR by including a primer pair, which amplified a 125-bp genomic fragment from all

  

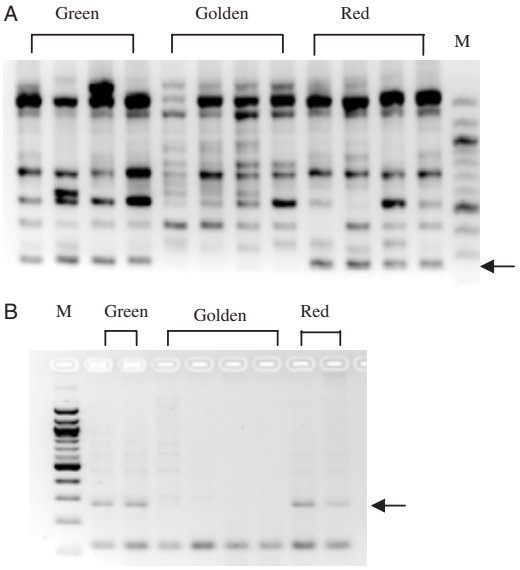


**Figure 2** A strain-specific RAPD-band (278 bp) was amplified using the OPA-019 10-mer primer (Operon). The upper panel (A) shows RAPD patterns, while the lower one (B) shows the bands amplified by STS-duplex-PCR using the primer pairs OPAOF3/R3 (278 bp) and DSX06A/B (125 bp). Labels above lanes indicate colour varieties, M: 100 bp ladder (Bio-Rad). The arrows denote the position of the strain-specific band.

individuals, independently from their colour, providing a positive control. Using this duplex PCR, all 32 individuals from the gold strain could be differentiated from the 32 greens and the 32 blood reds as templates from all the latter amplified two bands, while the golden ones amplified only one (Fig. 2B). The efficiency of golden vs. green/blood red differentiation was perfect (96/96 individuals correctly identified); however, the blood red and green individuals could not be differentiated from each other.

## Discussion

RAPD and AFLP have been used to search for sex-specific genomic markers in many species (Bello & Sanchez 1999; Iturra *et al.* 1998; Reamon-Buttner & Jung 2000). It was reported that AFLP was the most efficient method in finding sex-specific genomic DNA markers in plants (Reamon-Buttner & Jung 2000) and RAPD was not effective in detecting markers very tightly linked to the sex locus of asparagus (Jiang, Lewis & Sink 1997). On the other hand, comparative RAPD assays were used successfully for the identification of sex-associated markers in birds (Bello & Sanchez 1999), rainbow trout (Iturra *et al.* 1998) and in African catfish (Kovacs *et al.* 2001). In the present study, RAPD and AFLP analyses were performed on two sets of pooled samples, yielding a sex-associated AFLP marker and a strain-specific RAPD marker. As AFLP and RAPD analyses reflect different polymorphisms of genomes, they both should be used as complementary methods for screening the differences between genomes.

The identified sex-associated AFLP marker and the strain-specific RAPD marker were directly applicable for molecular analysis of Asian arowana populations. Their usefulness was, however, further improved by converting them into STS-PCR markers as RAPD is rather sensitive to reaction conditions, while the AFLP analysis is relatively complicated (Jones, Edwards, Castaglione, Winfield, Sala, van de Wiel, Bredemeijer, Vosman, Matthes, Daly, Brettschneider, Bettini, Buiatti, Maestri, Malcevschi, Marmiroli, Aert, Volckaert, Rueda, Linacero, Vazquez & Karp 1997b). When the sex-associated AFLP band was isolated and cloned, it yielded two different fragments with the same length. In asparagus, a cloned sex-specific AFLP band also contained a heterologous population of fragments, among which only one corresponded to the original marker (Reamon-Buttner & Jung 2000). In such cases, first the true marker must be identified from among the bands cloned. It was necessary to extend the selective (unlabelled) *Mse*I primer by several base pairs at the 3′ end and re-amplify the pre-selective amplification products. As the anchored primer sequences were extended further and further away from the enzyme cutting sites, only a subset of the original pattern was amplified and detected with each of them. Only the primer that is fully complementary to the sequence in question would amplify the band showing difference in two sexes. Because the sex-associated marker contained a CA-microsatellite, the amplification of the STS marker showed only difference of allele length between the two sexes. The allele of 268 bp was present in all males, but absent in the majority of females. In plants, male-specific DNA fragments containing repeat sequences were also reported (Ruas, Fairbanks, Evans, Stutz, Andersen & Ruas 1998). Microsatellite sequence $(GACA)_4$ was used as a primer to identify sex-specific DNA markers in vultures (Wink, Sauer-Gurth, Martinez, Doval, Blanco & Hatzofe 1998).

For conversion of the strain-specific RAPD-marker into an STS marker, we had to design specific primers to the end of the RAPD-marker because inner primers would not detect differences restricted to the binding sites of the original RAPD primer. Such

primers complementary to ends can partially or fully contain the binding sites of the RAPD primer. Witsenboer, Kesseli, Fortin, Stanghellini & Michelmore (1995) were able to construct a dominant sequence characterized amplified region (SCAR) marker by shifting the primer position by five to eight bases external to the original RAPD primer, thus relocating unknown nucleotide differences near the 3′ end. However, this could only be carried out by genome walking from the ends of the known sequence.

The sex-associated STS marker derived from the AFLP analysis would sex the green dragonfish with an accuracy of 82.7%. There may be several reasons for the less-than-perfect efficiency of our molecular sexing method. Firstly, the identified marker may not be tightly linked to the sex locus, thus some crossing over may occur between the marker locus and the sex locus. Secondly, the phenotypic female fish may be genetic males, as the phenotypic sex in fish can be determined not only by their genes, but also by endocrine, environmental and even social factors (Baroiller, Guigen & Fostier 1999). Natural sex reversal appears to be a more frequent phenomenon in fish (Sakai & Kohda 1997; Tobin, Sheaves & Molony 1997) than in other vertebrates. Although the accuracy rate for our molecular sexing method is only 82.7%, this STS marker could be useful for improving current breeding programmes of the green strain. Currently, in Asian arowana stocks, the exact sex ratio is often unknown; moreover, some stocks seem to suffer from shortage of males (L.C. Lim, unpubl. obs.). Molecular sexing of broodstock individuals may allow the farmers to set sex ratios according to needs. For example, for purposes of conservation, the sex ratio should be set up one male to one female to reduce genetic drift, whereas for commercial purposes, a different sex ratio may be optimal to raise the efficiency of production. The sex-associated marker could also be used to identify the chromosome where the major sex-determining locus is located and possibly to identify genotypic sex in individuals of the other two strains.

Strain-specific markers could be very useful for those involved in the trading of various strains of Asian arowana. Because the price of the three strains show drastic differences and colours do not develop fully at market size (ca. 8 months and 10–12 cm) in every strain, traders could use our strain-specific markers to confirm the identity of a particular shipment. Such a marker could also be helpful for those working on conservation of Asian arowana. Groups of young fish randomly caught from the wild can be quickly tested for the ratio of various colour varieties and identification of the individuals to be used for maintaining genetic diversity of cultured stocks. Extension of the molecular identification to other varieties by isolating additional markers would be desirable. We performed a more comprehensive RAPD screen with 1500 primers for a DNA marker capable of the differentiation of red from green varieties, without success (G.H. Yue & Q. Zhao, unpubl. results). In a related study using microsatellite and AFLP analyses, we showed that the genetic distance is shorter between green and red than that between red and gold strains (Yue *et al.* 2002). These data together appear to indicate that the isolation of markers able to differentiate between the blood red and green strains or between the blood red and chilli-red 'substrains' may be more difficult and could require more complicated molecular methods. On the other hand, we managed to identify at least one potential RAPD marker, which seems to be efficient in differentiating the Malaysian (crossback) golden and Indonesian golden varieties (K. Ding & L. Orban, unpubl. results).

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