Linkage Mapping of AFLP and Microsatellite DNA Markers with the Body Color- and Sex-Determining Loci in the Guppy (Poecilia reticulata)

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Linkage Mapping of AFLP and Microsatellite DNA Markers with the Body Color- and Sex-Determining Loci in the Guppy (*Poecilia reticulata*)

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ABSTRACT—The guppy is an ornamental fish species that exhibits various phenotypic characteristics, such as body color and fin-shape. Although linkage relationships of a limited number of phenotypic traits have already been investigated, the association between phenotypic and molecular markers is still unknown. We constructed a total of 35 linkage groups for the guppy using 186 polymorphic loci of AFLP and microsatellite DNA. The locus related to the yellow body color was linked with ten markers and the sex-determination locus was linked with five markers.

Key words: AFLP, microsatellite DNA, guppy *Poecilia reticulata*, linkage analysis

INTRODUCTION

Genetic mapping using a large number of molecular markers has been used as a very essential method for analysing biological phenomena in animal genomes, and has provided powerful marker-based information for breeding science. In particular, high polymorphic DNA markers, evenly distributed throughout the wide genome, are effective for linkage analysis. The linkage maps are also efficient to locate quantitative trait loci (QTLs).

The guppy, *Poecilia reticulata*, is a well-known ornamental fish species that exhibits various phenotypic characterisric such as body color, coloration, and body size. This fish was used in an experimental model to assay genetic differences in various physiological conditions among some individuals or strains, subsequent to establishment of several experimental strains with certain quantitative traits such as growth rate (Nakajima and Taniguchi, 2002) and thermal resistance (Fujio *et al.,* 1995).

Among the 23 pairs of chromosomes of the guppy, 22 are autosomes and one pair is the sex chromosome. Although morphologically identifiable sex chromosome has not been reported, a sex-determining system is recognized experimentally; the males are heterogametic (XY) and the females are homogametic (XX) (Winge and Ditlevsen, 1947). The linkage analysis based on the phenotypic traits elucidates the sex-determining gene that was identified by the color determining loci. Some loci determining phenotypic characteristics that are unique in the male of the guppy, such as color patterns and fin shapes, also suggest that to exist on the sex chromosome (Khoo *et al.,* 1999a, b, c).

By contrast, the genes determining background body color such as albino, blond, or yellow (Goodrich *et al.,* 1944; Haskins and Haskins, 1948, Ueshima *et al.,* 1998) are autosomally inherited and recessive to their wild phenotype. These traits appear to be under the control of genes that are associated with the regulation and formation of the chromatophores. Currently, the linkage relationships of these loci have only been investigated by using phenotypic markers, due to the fact the loci associated with these regions on the chromosome and the DNA sequence have not yet been determined. Recently, a lot of microsatellite DNA markers have been developed in the guppy (Parker *et al.,* 1998; Kelly *et al.,* 1999; Watanabe *et al.,* 2003, 2004a). Watanabe *et al.* (2004a) constructed six linkage groups using microsatellite DNA markers, but the linkage groups determined were just portion, a number far smaller than the chromosome set number of this species.

In the present study, a linkage analysis was performed using AFLP (amplified fragment length polymorphism) and microsatellite DNA markers to fill up the result of previous linkage map data. Moreover, the loci related to body color and sex-determination, were attempted to locate by DNA

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markers.

MATERIALS AND METHODS

Guppy mating and samples

The guppy samples used for the linkage analysis were selected from three strains, F, S3, and Y2, maintained in our laboratory as closed colonies based on body color and morphological and physiological characteristics. The S3 strain, similar to the standard wild phenotype, has been characterized by a small body size, a short tail and a dark body color. The F and Y2 strains express a large body size, a long tail, and yellow body color, and were established from ornamental fish.

One male of the F strain (genotype *bb* for yellow phenotype; recessive trait) was mated with one female of the S3 strain (genotype *BB* for wild phenotype; dominant trait) to produce the F_1 progeny (F×S3). All F_1 F×S3 progeny expressed the wild phenotype (*Bb*). Subsequently, one male of the F_1 F×S3 and one female of the Y2 strain (genotype *bb* for yellow phenotype; recessive trait) were mated to obtain the F_2 progeny for genotyping.

Body color distinction by observation of the scale melanophores

Scales were firstly isolated from the dorsal trunk and immersed for 5–10 min in a medaka Ringer's solution (128.1 mM NaCl, 2.6 mM KCl, 1.8mM $CaCl_2$ adjusted to pH 7.3 by $NaHCO_3$; Iwamatsu, 1993). Afterwards, the scales were dipped for 5–10 min into 133 mM NaCl and 133 mM KCl solutions. The protocol for the melanophore observation followed that described by Shinohara *et al.* (2001). Finally, the response of the melanophores related to body color was observed using a microscope.

Microsatellite DNA and AFLP marker analysis

Genomic DNA was extracted from fin tissue following the standard SDS-phenol/chloroform protocol. DNA polymorphisms were assayed by microsatellite DNA and AFLP methods.

A total of 56 microsatellite DNA markers, described in Parker *et al.* (1998), Kelly *et al.* (1999), and Watanabe *et al.* (2003, 2004a), were tested in the 38 F_2 progeny. The microsatellite DNA protocol followed was that described in Watanabe *et al.* (2003). The AFLP protocol was carried out essentially that of Vos *et al.* (1995). Genomic DNA was digested with *Eco*RI and *Mse*I prior to ligation of restriction sites with specific adaptors. Preamplification carried out was utilizing the specific adaptor primers with a single selective base on each primer. The preamplification product was diluted, and selective amplification was carried out utilizing primers with one additional selective base on the *Eco*RI primer and two additional selective bases on the *Mse*I primer. AFLP polymorphisms were detected using 32 primer combinations. AFLP loci nomenclature followed that described in Table 1 of Watanabe *et al.* (2004b). Each AFLP locus was named using the selected primer combinations and

Table 1. Linkage analysis using the F_2 progeny

	No. markers detected	No. markers linked		Linkage rate (%)		No. Linkage groups		Total map distance (cM)	
		LOD 3	LOD 2	LOD 3	LOD 2	LOD 3	LOD 2	LOD 3	LOD 2
F×S3 (Male)	138	117	120	84.8	87.0	27	27	420.4	491.6
Y2 (Female)	48	27	27	56.3	56.3	11	12	89.3	115.3

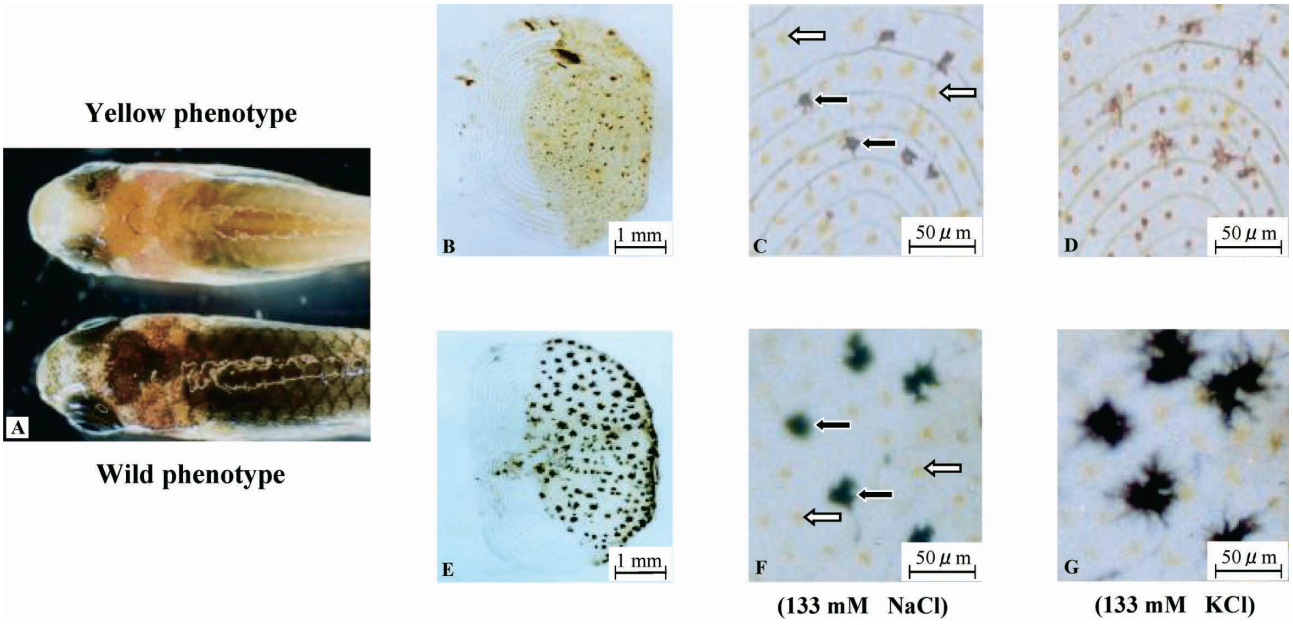

Fig. 1. Two guppy phenotypes discriminated by differences in melanophores formation. (A): Dorsal view of the two phenotypes, (B): The scale taken from the body of the yellow phenotype (small-sized melanophores). (C, D): The responses of the melanophore in a scale from the yellow phenotype. The melanosomes were fully dispersed 5–10 min after soaking in the 133mM NaCl solution, and almost aggregated 5–10 min after the soaking in the 133mM KCl solution. The black arrows indicate the melanophores, and the white arrows the xanthophores. (E): The scale taken from the body of the wild phenotype (large-sized melanophores). (F, G): The responses of the melanophores in a scale from the wild phenotype. The melanosomes were fully dispersed in the 133mM NaCl solution, and almost aggregated in the 133mM KCl solution.

the fragment sizes. For example, *D4-291* was the AFLP locus of molecular size 291bp obtained using the E-AT and M-CAT primer combination.

Each reverse microsatellite primer and the AFLP *Eco*RI primers were 5' end-labeled with biotin. Polymerase chain reaction (PCR) amplification was carried out with a Thermal Cycler MP (TaKaRa, Japan). Following amplification, the samples were mixed with an equal volume of denaturing stop dye, heated for 10 min at 95°C, electrophoresed on 8% polyacrylamide gel (7 M urea, 8% acrylamide), and finally visualized using the Chemiluminescent 'Phototope-Star Detection kit' according to the manufacturer's instructions (New England BioLabs, USA).

Linkage analysis

Genotyping data were obtained using 32 AFLP primer combinations and 56 microsatellite DNA primer sets for the parents and the 38 F_2 progeny. Each band on the gel and was detected as homozygous or heterozygous band as allele. For all allele detected, the approach used was to score only loci for which one parent was scored as band-present (+/–: heterozygotes) and the other parent was scored as band-absent (–/–). Goodness of fit of the observed-to-expected allelic ratios of 1:1 was examined by χ^2 test (P<0.05).

To detect whether the DNA markers linked with the body color- and the sex -determining locus, the phenotypic data associated with body color (yellow phenotype) and sex (male), and the genotyping data from the AFLP and microsatellite DNA markers were analysed for linkage using MAPMAKER/EXP 3.0 software. The presence of alleles inherited from the paternal fish was scored "H" and absence "A", whereas the presence and absence of alleles were scored as "A" and "H", respectively, for alleles inherited from the maternal fish. An initial grouping of markers was performed with the log-odds cut-off (LOD score) 3.0. After, additional markers were added by lowering the LOD score 2.0 to expand the map. The final map distances were calculated using Kosambi map function, and the linkage groups were constructed for each F×S3 and Y2 individual.

Detection of body color and sex specific-linked markers

For the purpose of identifying the specific markers linked with the body color or the sex determining locus, this research used twelve samples (6 males, 6 females) from four strains, which were: W (wild phenotype), Y2 (yellow phenotype), T (wild phenotype), and Fs (yellow phenotype). They were examined to evaluate the applicability of the yellow phenotype- and the sex-specific linked markers.

RESULTS

Distinction and segregation of the phenotypic markers

The yellow phenotype seems to exhibit a lighter color due to overall smaller melanophore size relative to that of the wild phenotype. The melanophore reaction in the respective scales of the wild and yellow phenotypes was observed using a microscope, after immersing the scales in 133 mM NaCl and 133 mM KCl solutions. Two types of melanophores were distinguished by morphological differences of aggregation and dispersion response size (see Fig. 1). Separately, we also examined part of the head color because the quantity of the melanophores at the surface of the brain was clearly different between the wild and yellow phenotypes.

The wild and yellow phenotypes of body color were segregated distinctly in a 1:1 ratio in the F_2 progeny (wild: 17; yellow: 21). The sex ratio was also scored as 1:1 segregation (male: 21; female: 17). From these results it was shown that the yellow phenotype and the sex are controlled by one single locus.

Linkage analysis

A total of 186 polymorphic markers were detected from the 32 AFLP primers combinations and the 56 microsatellite DNA primer sets. A group of 138 markers included 104 AFLP, 32 microsatellite DNA, and 2 phenotypes derived

Table 2. Segregation and genotypes pattern for each body color- and sex- linked markers in the F_2 progeny

Locus (marker type: band pattern)	Detected no. of marker linked with each phenotype		Distance (cM)	LOD score
	Body color			
Linkage group 5	Wild	Yellow		
A1-109 (AFLP: Positive band)	17	0	0.0	11.44
A4-118 (AFLP: Positive band)	17	0	0.0	11.44
A4-169 (AFLP: Positive band)	17	0	0.0	11.44
C5-146 (AFLP: Positive band)	17	0	0.0	11.44
C5-161 (AFLP: Positive band)	17	0	0.0	11.44
D4-231 (AFLP: Positive band)	17	0	0.0	11.44
Pooc-G53 (microsatellite: homologous)	0	21	0.0	11.44
Pret-32 (microsatellite: homologous)	0	21	0.0	11.44
Pret-35 (microsatellite: homologous)	0	21	0.0	11.44
Pret-45 (microsatellite: homologous)	0	21	0.0	11.44
	Sex			
Linkage group 14	Male	Female		
A2-218 (AFLP: Positive band)	21	0	0.0	11.44
A7-201 (AFLP: Positive band)	21	0	0.0	11.44
D1-302 (AFLP: Positive band)	20	1	2.7	9.14
C7-130 (AFLP: Positive band)	16	5	13.5	5.01

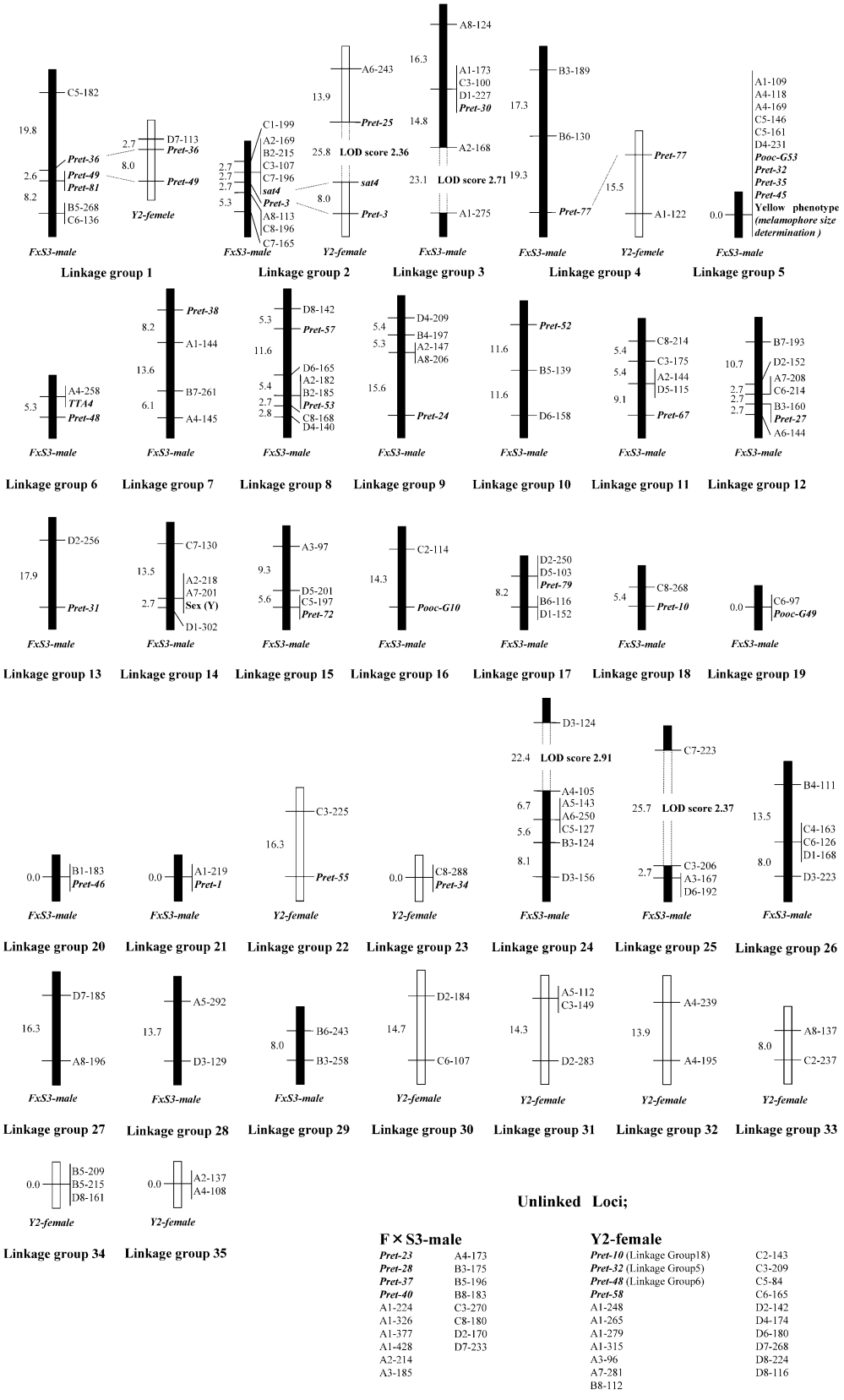

Fig. 2. Thirty-five linkage groups of the guppy (*Poecilia reticulata*) based on AFLP and microsatellite DNA markers with the two phenotypes. The linkage map in black show the result of the F×S3 male, and those in white the result of the Y2 female. Linkage groups nos 1–6 correspond to those of Watanabe *et al.* (2004a). The map distances in terms of centimorgans are described on the left side, the loci names on the right side. AFLP loci names indicate primer combination and approximate size in base pairs of the scored fragment. Microsatellite DNA loci are indicated by the italicized primer name.

from the F×S3 male. The other 48 markers included 36 AFLP, and 12 microsatellite DNA derived from the Y2 female, segregated in a 1:1 ratio. Thirty-five linkage groups were constructed in the F×S3 male and Y2 female (Fig. 2). The linkage map which be grouped by the LOD score 2.0 had a total length of 491.6 cM in F×S3 and 115.3 cM in Y2. Table 1 showed the results for each linkage analysis. The F×S3 male, which was obtained from F_1 of two strains, contained many polymorphic markers.

DNA markers linked with the sex-determining locus

The sex-determining locus was mapped on Linkage group 14 (Fig. 2), constructed with 4 AFLP markers (LOD score 5.01-11.44, map distance 0.0-13.5 cM). *A2-218* and *A7-201* markers were mapped at the closest position to the male sex (LOD score 11.4, map distance 0.0 cM). The band for the four markers associated with sex were clearly displayed only in the case of male sex (Table 2).

DNA markers linked with the yellow phenotype-determining locus

The yellow phenotype-determining locus was mapped on Linkage group 5 (Fig. 2). Ten DNA markers (6 AFLP and 4 microsatellite DNA) showed strong linkage with the phenotype (LOD score 11.4, map distance 0.0 cM). Linkage relationships between the phenotype and the respective

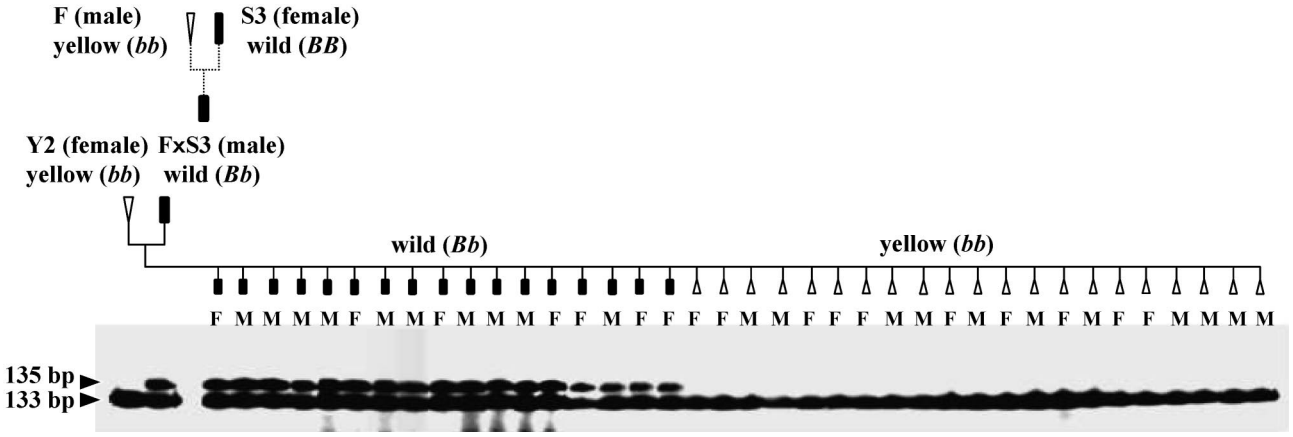

Fig. 3. Representative electropherogram of the *Pret-45* locus, which is linkaged with yellow body color. The left 17 lanes show the wild phenotype with bands 133bp and 135bp, and the right 21 lanes show the yellow phenotype with a single band 133bp. F and M indicate female and male, respectively.

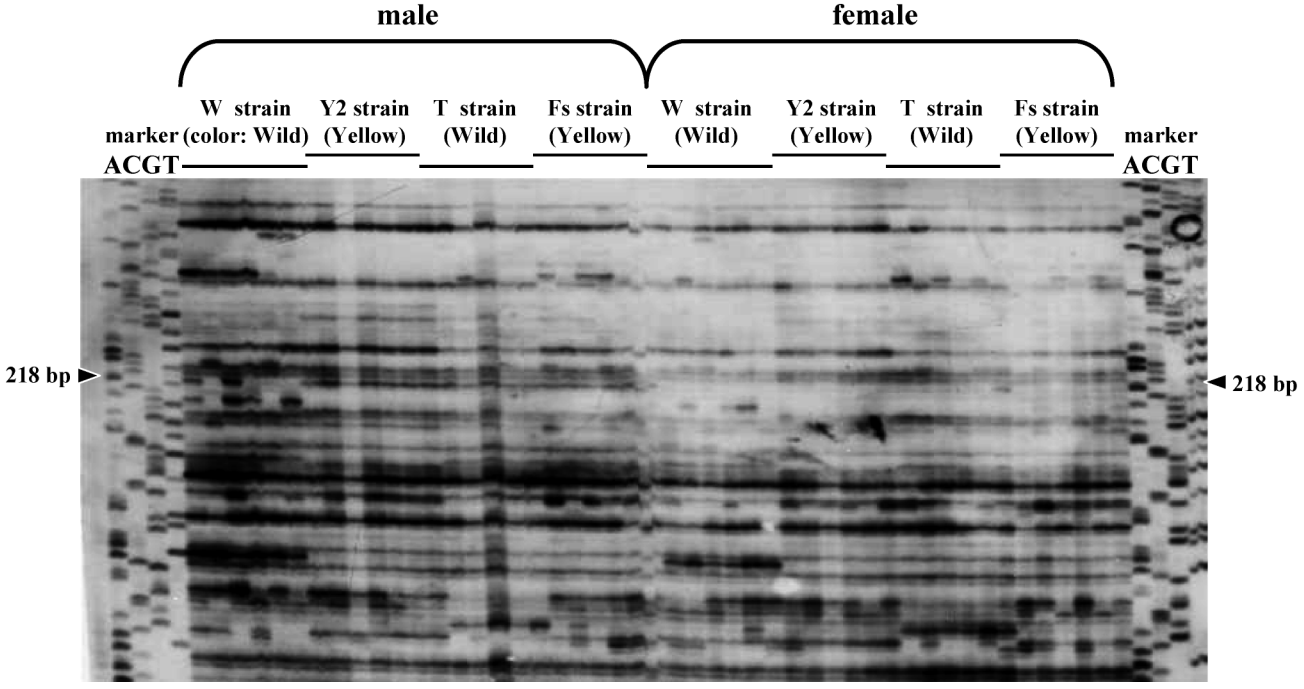

Fig. 4. AFLP electropherogram in four strains [W, Y2, T, and Fs strains; twelve samples (6 males, 6 females) in each strain]. The *A2-218* bands appeared in almost all males; the band did not appear in any females.

DNA markers are shown in Table 2. The other six AFLP markers were linked in repulsion phase to the wild phenotype, being the presence of the band linked to the wild phenotype and its absence to the yellow phenotype. In the case of the microsatellite DNA markers, a heterozygous genotype appeared with the wild phenotype and a homozygous genotype with the yellow phenotype (Fig. 3).

Detection of yellow phenotype- and sex-specific DNA markers

In the present study, a total of nine AFLP markers were mapped close to the body color and sex (map distance 0.0). Therefore, we attempted to detect the yellow phenotype- or sex specific-linked AFLP markers using 4 other strains of 48 samples each (W, Y2, T, and Fs). As a result, although the yellow phenotype- or sex specific-linked markers did not completely agree between genotype and phenotype, the *A2-218* marker could verify an association between the sex phenotype and genotype 90.0% (43/48) of the time (Fig. 4).

DISCUSSION

Linkage map

In the present study, although three strains of cross-breeding were examined, it was not difficult to detect a polymorphic marker efficiently through the crossing of strains of low genetic variability. A total of only 186 markers were detected, with which we constructed 35 linkage groups, and the yellow phenotype- and sex- determining loci were mapped by using AFLP and microsatellite DNA markers. Especially, Linkage group 5 mapping yellow phenotype-determining locus was clustered by multiple DNA markers at a small region. In fish, highly clustered AFLP or microsatellte DNA markers were observed in zebrafish, medaka and channel catfish (Shimoda *et al.,* 1999; Naruse *et al.,* 2000; Liu *et al.,* 2003). But, the reasons for the high level of marker clustering are not known at present. A genomic region with great variation including specific restriction sites and repeat sequences may exist also in the guppy.

The number of linkage groups disagreed with the whole chromosome's number (23 pairs). To construct a detailed linkage map of model fishes corresponding to the haploid number of chromosomes, 634 markers in medaka (*Oryzias latipes*; Naruse *et al.,* 2000) and 2000 makers in zebrafish (*Danio rerio*; Shimoda *et al.,* 1999) were used for analysis. Therefore, the linkage map is a starting line for the mapping of molecular markers and various phenotypic characteristics in the guppy. In order to construct a higher-density map, several hundred markers are required to carry out smooth QTLs analyses such as those of thermal resistance and body size. New genes and polymorphic DNA markers should be developed to fill the gap between the linkage groups and chromosomes.

Sex-linked markers

The guppy is a sex-determining system of X-Y (Winge and Ditlevsen, 1947), Some genes have been believed to be associated with sexually antagonistic traits, and some of them, which link to Y-chromosome, have been mapped based on the sex-determining locus (Nakajima *et al.,* 1998; Khoo *et al*., 1999a, b, c). The *A7-201* and *A2-218* markers, located in a single position of the cluster, have been shown to be linked with a map distance of 0.0 cM. In particular, the *A2-218* marker was confirmed to be associated with the sex, since strong linkage disequilibrium occurred in each strain ($P<0.05$; Fig. 4). However, some discrepancies were obtained in a few exceptional samples, which would suggest a low frequency of recombination between the phenotypic sex and the marker.

Body color determining locus related to yellow phenotype

Goodrich *et al*. (1944) reported four phenotypes composed of four alleles in two loci. The melanophores of blond and cream color, which are smaller than the wild and golden melanophores, were characterized as a recessive phenotype (genotype *bb*) against the dominant phenotype (genotype *B*-) based on the segregation rate in the backcrossed F_2 generation. The physiological responses of the melanophores of the small type (*bb*) were considered relatively sluggish compare with those of the wild type. Also, Ueshima *et al*. (1998) reported that the guppy melanophores of pale yellow color differed in size but not in number, compared with the wild type. In the present study, the wild phenotype showed migration of the melanosomes in the large-sized melanophores. On the other hand, the yellow phenotype was occupied by many punctuate melanophores, and a small number of dendritic cells was observed (Fig. 1). Clear differences in the number of melanophores between the two phenotypes were not observed, and though two melanophore types of different sizes could be clearly distinguished, there was no existing intermediate type. These results suggested that the phenotypic difference was caused by different regulation of the melanophore structure. In addition, the yellow phenotype (*bb* genotype) could be identical to the genotype *bb* of Goodrich *et al*. (1944) and Ueshima *et al*. (1998).

The migration of melanosomes within the integumentary melanophore reveals coloration in the teleost fish, which has been demonstrated to be controlled either by the autonomic nervous system or hormones. The migrations of the melanosomes, which are responsible for the physiological color change, are regulated by the neural system (Sugimoto *et al.,* 1994; Masagaki and Fujii, 1999). By contrast, the melatonin hormones such as melanophore-stimulation hormone (MSH) and melanin-concentrating hormone (MCH) are known to have the ability to cause migration of the melanosomes (Kawauchi *et al.,* 1983). We observed migration of the melanosomes in the wild and yellow phenotypes of the guppy melanophores. Although the nerve systems or melatonins activate the migration of the melanosome, they do not directly regulate the size determination of the melan-

ophore formation. On the other hand, it would be that these different melanophore types exist because of an incomplete formation of the melanosome in the cytoplasm. These cytological differences within melanophores or melanosomes could be influenced by biochemical factors caused by a mutation such as inactivation of an enzyme involved in the metabolism of the melanophores or melanosomes structure.

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