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Aquaculture 249 (2005) 95–105

Aquaculture

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Marker-based estimation of heritability for body color variation in Japanese flounder *Paralichthys olivaceus*

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Received 1 November 2004; received in revised form 13 February 2005; accepted 1 March 2005

Abstract

Albinos of Japanese flounder were found in a hatchery population. Their body color was not uniform and a large variation was observed. To examine genetic effects on the albinism and the color variation, this study estimated relatedness among albinos and wild-type individuals and heritability of body color among albinos using marker-based approaches. Significant differences between albinos and wild-type individuals were observed in all color components of red, green, blue, hue, saturation and intensity with large variations within each group. The number of alleles per locus at seven microsatellite loci was significantly lower in the albinos than in the wild-type individuals. The dendrogram of relatedness estimated from microsatellites indicated that most albinos tended to be closely related to each other. The genetic data suggested that the albinos were produced by some, but not one or many, pairs. Marker-based estimates of heritability among all individuals were significantly greater than zero for all color components with estimates of 0.77–0.98. In addition, significantly positive heritability was observed for red, green and intensity in the albinos. These results indicated that the albinism detected in a hatchery population of Japanese flounder might be strongly heritable as observed in other fish species. Furthermore, estimates of heritability suggested that the color variation observed among the albinos was partly caused by additive genetic effects.

Keywords: Heritability; Relatedness; Microsatellite; Albino; Quantitative trait; Japanese flounder; *Paralichthys olivaceus*

1. Introduction

In order to sustain the resources of some fish and shellfish species under high fishery pressure, enormous amounts of seeds are artificially produced by limited numbers of broodstock parents and released to the ocean. Because mass release of the hatchery-produced individuals to the ocean affects the genetic structure of natural populations, attention has been focused on the genetic diversity of hatchery-reared individuals (Taniguchi, 2003). Several studies showed that the genetic variation of hatchery populations was lower than that of natural popula-

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doi:10.1016/j.aquaculture.2005.03.023

tions (Fujio et al., 1985; Taniguchi and Perez-Enriquez, 2000; Yoshida et al., 2000). In addition to genetic diversity, genetic quality of quantitative traits may be important for hatchery populations because fitness-related traits as well as economically important characters are mostly quantitative traits. One of the most important quantitative genetic parameters is heritability, which represents the proportion of total phenotypic variance attributable to the additive genetic effects (Falconer and Mackay, 1996). Heritability is classically estimated from comparison of phenotypes between individuals of known relationship or from selection response. Therefore, estimating heritability generally requires a controlled breeding program, which is time-consuming and costly.

Efforts have been made to use molecular markers for estimating relatedness between individuals of unknown pedigree (Thompson, 1975; Lynch, 1988; Queller and Goodnight, 1989; Ritland, 1996a; Lynch and Ritland, 1999). Queller and Goodnight (1989) were the first to develop a regression-based model. This approach is designed for estimating relatedness, which is the probability that genes are identical-by-descent, without previous knowledge of the size of relationship classes. On the basis of marker-based estimates of pairwise relatedness, Ritland (1996b) has proposed a method for estimating heritability to study quantitative traits in long-lived organisms or organisms that are difficult to culture, as well as in natural populations. The method involves regressing quantitative trait similarity on marker-estimated relatedness between individuals (Ritland, 1996b). Thus, this approach does not require any information on explicit pedigree. In spite of this prominent idea, practical information on the estimation of heritability using the marker-based approach is limited (Ritland and Ritland, 1996; Thomas et al., 2002; Wilson et al., 2003). A critical feature of a marker-based estimation of heritability is the need of actual variance of relatedness in a population of interest (Ritland, 1996b, 2000). Actual variance of relatedness occurs when there is some mixture of relatives, such as full-sibs vs. unrelated individuals. Since hatchery populations are generally produced by a limited number of parents, the genetic structure of hatchery populations may be suitable for the application of this approach. In addition, the marker-based approach makes it possible to estimate heritability under exact hatchery conditions. This point is important because heritability is affected by environmental conditions as well as genetic factors (Falconer and Mackay, 1996).

Japanese flounder, *Paralichthys olivaceus*, is an economically important fish species that is widely distributed around Japan. Enormous amounts of juveniles are produced by broodstock parents at many institutes in most parts of Japan and released to the ocean. There are several quantitative traits of economical importance and biological interest in Japanese flounder (Minami, 1997; Seikai, 1997; Kinoshita et al., 2000). Body color is an economically important trait in Japanese flounder as well as in other flatfish species because pseudo-albinism and hypermelanosis often occur under breeding environments and decrease the desirability of hatchery-reared fish (Seikai, 1997; Bolker and Hill, 2000). Several studies have shown environmental effects on body coloration including hypermelanosis and pseudo-albinism (Seikai, 1997; Bolker and Hill, 2000). Although knowledge of genetic effects will be important for understanding the mechanism of phenotypic determination of body coloration, only a few studies have shown genetic effects on body color (Furutsuka-Uozumi and Tabata, 1999). This may be partly because their large body size and long generation time make it difficult to estimate quantitative genetic parameters using a controlled breeding program.

Albinism is a pigmentation disorder which becomes apparent as a partial or total lack of the wild-type melanin coloration (Hyodo-Taguchi et al., 1997). Albinism is rarely detected in marine fish species. Hundreds of albino Japanese flounder were recently found among several tens of thousands of juveniles in a hatchery population, which was produced by wild-type colored parents. These albinos were characterized by red eyes and brighter body colors than wild-type colored individuals. The body color of albinos was not uniform and a large variation was observed. To examine genetic effects on the albinism and the color variation among albinos, the present study estimated relatedness among albinos and wild-type individuals and heritability of body color using marker-based approaches. Highly variable

microsatellite markers were used for estimation of relatedness and heritability.

2. Materials and methods

2.1. Animals

Albinos were found in a hatchery population at Fukushima Prefectural Sea-Farming Institute, Japan. This population was produced by parental fish originated from aquaculture farms at Kagawa and Kochi Prefectures and domesticated for at least two or three generations at Hakatajima Station, National Center for Stock Enhancement, Fisheries Research Agency, Japan. All parental fish were wild-type colored individuals. Fertilized eggs yielded by a parental stock of 12 females and 33 males by natural spawning were transferred to Fukushima Prefectural Sea-Farming Institute and reared in 75,000-l tanks with flow-through seawater. The water temperature of the tanks ranged from 15.4 to 22.3 °C. Fish were fed *Brachionus plicatilis* from days 0 to 25, *Artemia salina* from days 15 to 60 and dry pellets after day 30. For body color and genetic analyses, 63 albinos and 63 wild-type colored individuals were randomly collected from the stock 210 days after birth. Albinos were distinguishable from wild-type individuals based on bright body colors and red eyes. Total body length ranged between 15.7 and 24.1 cm with a mean of 20.3 cm in albinos and between 17.5 and 27.1 cm with a mean of 22.9 cm in wild-type individuals.

2.2. Body color components

Body color was evaluated using six basic color components: red (R), green (G), blue (B), hue (H), saturation (S) and intensity (I). Body color was examined on the ocular side excluding pseudo-albinism area. The ocular side of the fish body was scanned by a scanner (GT-9300UF, Epson, Tokyo) with 150 dpi resolution in color mode. The color components of each individual were measured at ten points of a 5×5 pixel area using two software programs (Adobe Photoshop 6.0, Adobe Systems, San Jose, CA; GIMP 1.2, written by S. Kimball and P. Mattis, available at http://www.gimp.org/). These components were expressed in values ranging from 0 to 255 in R, G, and B and from 0 to 100 in H, S and I. Statistical comparisons for color components were assessed using *t*-test.

2.3. Microsatellite analysis

DNA was extracted from fin tissue stored in ethanol using Chelex–proteinase K extraction (Estoup et al., 1996) or phenol–chloroform extraction (Taggart et al., 1992). Seven highly polymorphic microsatellite loci (*Pol1*, *Pol3*, *Pol4*, *Po1*, *Po13*, *Po42* and *Po91*) reported by Takagi et al. (1999) and Sekino and Hara (2000) were used in this study. PCR was conducted in a 8-μl reaction volume containing 10–20 ng of template DNA, 20 pmol of each primer set (one fluorescently labeled), 1.5 mM MgCl2, 0.2 mM dNTPs, 0.3 units of Taq DNA polymerase (TAKARA BIO Inc., Otsu, Japan) and 0.8 μl of 10× PCR buffer. The reactions were proceeded as follows: an initial denaturation step at 94 °C for 2 min, followed by 40 s at 94 °C, 40 s at annealing temperature (50 °C for *Pol1*, *Pol3* and *Pol4*, 55 °C for *Po1*, *Po42* and *Po91* and 58 °C for *Po13*), and 90 s at 72 °C for 30 cycles with a final extension at 72 °C for 5 min. PCR products were visualized using automated sequencers (ABI PRISM 310 Genetic Analyzer and ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA) and alleles were scored using GeneScan 2.0 and Genotyper 2.1 (Applied Biosystems, Foster City, CA). The difference in the number of allele per locus and heterozygosity between wild-type individuals and albinos was examined using Mann–Whitney *U* test.

2.4. Estimation of relatedness and heritability

Pairwise relatedness was estimated by a regression-based model (Queller and Goodnight, 1989). The calculation was performed with the microsatellite data at seven loci using the RELATEDNESS 5.0 program (written by K.F. Goodnight and D.C. Queller, available at http://www.gsoftnet.us/GSoft.html). A dendrogram of relatedness among individuals was constructed using the unweighted pair–group method with arithmetic averages (UPGMA) reported by Sneath and Sokal (1973) with the PHYLIP 3.5

program (written by J. Felsenstein, available at http://evolution.genetics.washington.edu/phylip.html). Statistical comparisons for pairwise relatedness were assessed using one-way factorial analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) multiple comparison test.

Heritability was estimated according to Ritland (1996b, 2000) with the microsatellite data at seven loci using the MaRQ 1.0 program (written by K. Ritland, available at http://genetics.forestry.ubc.ca/ritland/programs.html). The estimation of heritability was on the basis of a simple regression of pairwise trait similarity on pairwise relatedness. The phenotypic similarity of a quantitative trait Y for two individuals i and j was calculated as

$$Z_{ij} = \frac{(Y_i - U)(Y_i - U)}{V}$$

where U and V were the sample mean and variance of Y, respectively, in the population. Among all pairs, the average Z_{ij} equals the phenotypic correlation. Genetic components of similarity were estimated as

$$Z_{ij} = 2r_{ij}h^2 + e_{ij}$$

where r_{ij} was the relatedness coefficient measured by the method of Queller and Goodnight (1989), h^2 was the heritability and e_{ij} was the random error. Heritability was estimated as

$$\hat{h}^2 = \frac{Cov(Z_{ij}, r_{ij})}{2Var(r_{ij})}$$

where Cov(Z_{ij}, r_{ij}) was the covariance between estimated relatedness and phenotypic similarity and Var(r_{ij}) was the actual variance of relatedness. The estimation of the actual variance of relatedness was described in detail in Ritland (1996b, 2000). Significance of heritability estimated from the marker-based method and the actual variance of relatedness were determined by bootstrapping over individuals with a bootstrap number of 1,000. Estimates were deemed significant if 95% of the bootstrap values were found to be greater than zero. Standard errors of genetic parameters were computed with the bootstrap method, where individuals were the unit of resampling.

3. Results

3.1. Color variation

All albinos had red eyes but body color varied among the individuals (Fig. 1). Frequency distributions of six color components are shown in Fig. 2. A significant difference between wild-type individuals and albinos was observed in all the color components ($P<0.01$, t-test). There was overlap between the two groups in the range of B, H and S but not in that of R, G and I. A large variation in each color component was observed within the albinos as well as within the wild-type individuals.

Table 1 shows correlations between color components in the two groups. Significant correlation was observed in many combinations ($P<0.01$ or $P<0.05$). In the combinations that showed significant correlation, Pearson's correlation coefficient varied between 0.122 and 0.998 in the wild-type individuals and between 0.062 and 1.000 in the albinos. Relationship of H with R or I was positive in the wild-type individuals but it was negative in the albinos.

3.2. Relatedness

The total number of alleles detected was 90 with 18 at *Pol1*, 15 at *Pol3* and *Pol4*, 7 at *Po1*, 11 at *Po13* and 12 at *Po42* and *Po91*. The number of allele per locus ranged between 7 and 17 with a mean of 12.0 in the wild-type individuals whereas it ranged between 4 and 10 with a mean of 7.4 in the albinos. The mean number of alleles per locus significantly differed between these groups ($P<0.05$, Mann–Whitney U test). Mean observed heterozygosity was 0.856 in all the individuals, 0.873 in the wild-type individuals and 0.846 in the albinos. There was no significant difference in the mean observed heterozygosity between the wild-type individuals and the albinos ($P>0.05$, Mann–Whitney U test).

Fig. 3 shows the frequency distribution of pairwise relatedness between wild-type individuals

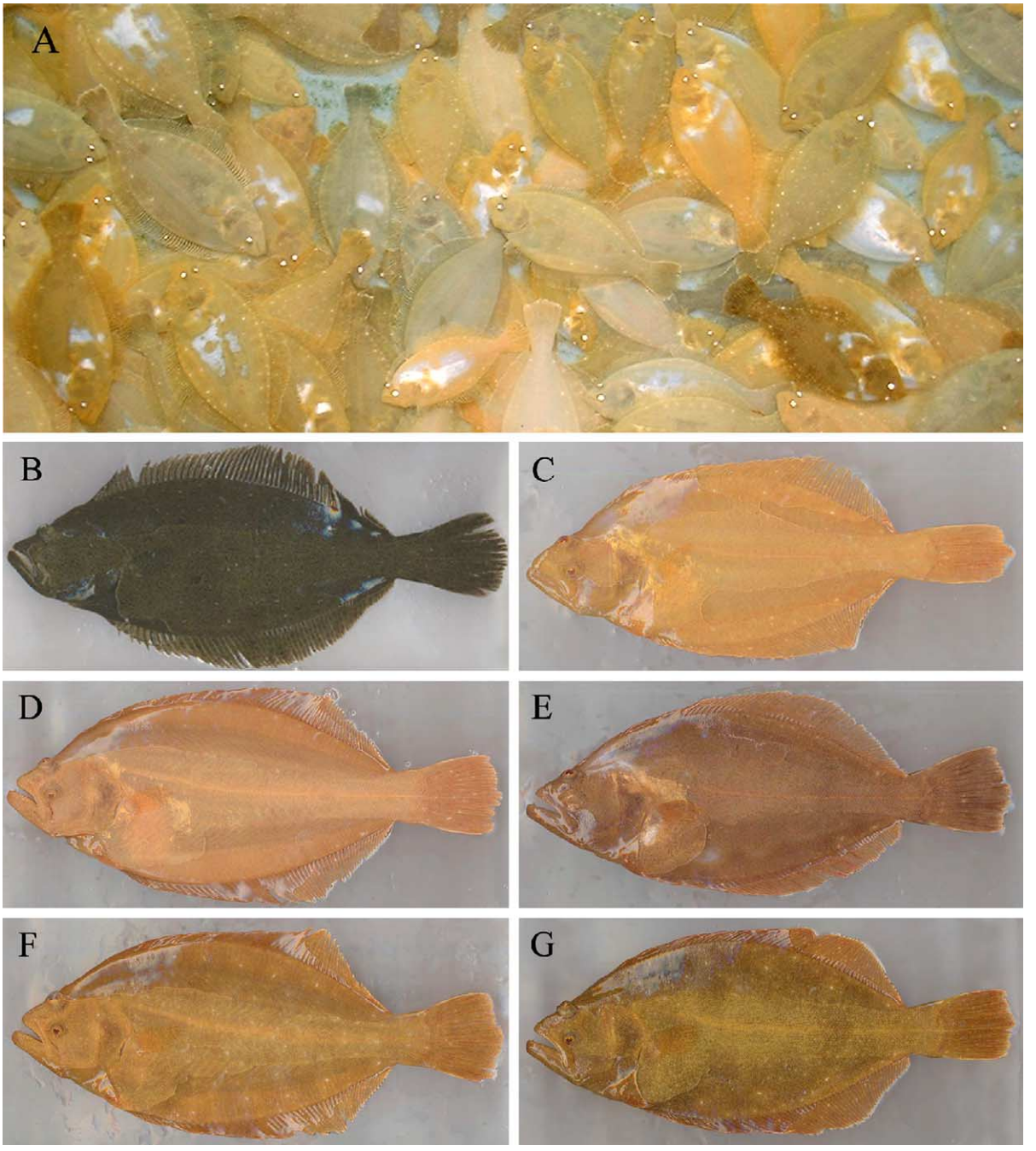

Fig. 1. Pictures of the albino Japanese flounder found in a hatchery population. A: Stock tank of albinos. A large variation in body color is observed among albinos. White areas observed on ocular side of some fish are pseudo-albinism. B: Wild-type colored individual (R, G, B, H, S and I are 78.4, 72.8, 54.2, 12.9, 30.5 and 30.5, respectively). C: Albino (202.1, 151.2, 86.0, 9.0, 57.4 and 78.9). D: Albino (191.2, 145.8, 108.4, 7.4, 43.4 and 74.6). E: Albino (157.3, 113.8, 75.3, 7.8, 52.7 and 61.3). F: Albino (163.4, 123.0, 71.2, 9.4, 56.3 and 63.7). G: Albino (158.0, 117.0, 72.8, 8.6, 53.5 and 61.7).

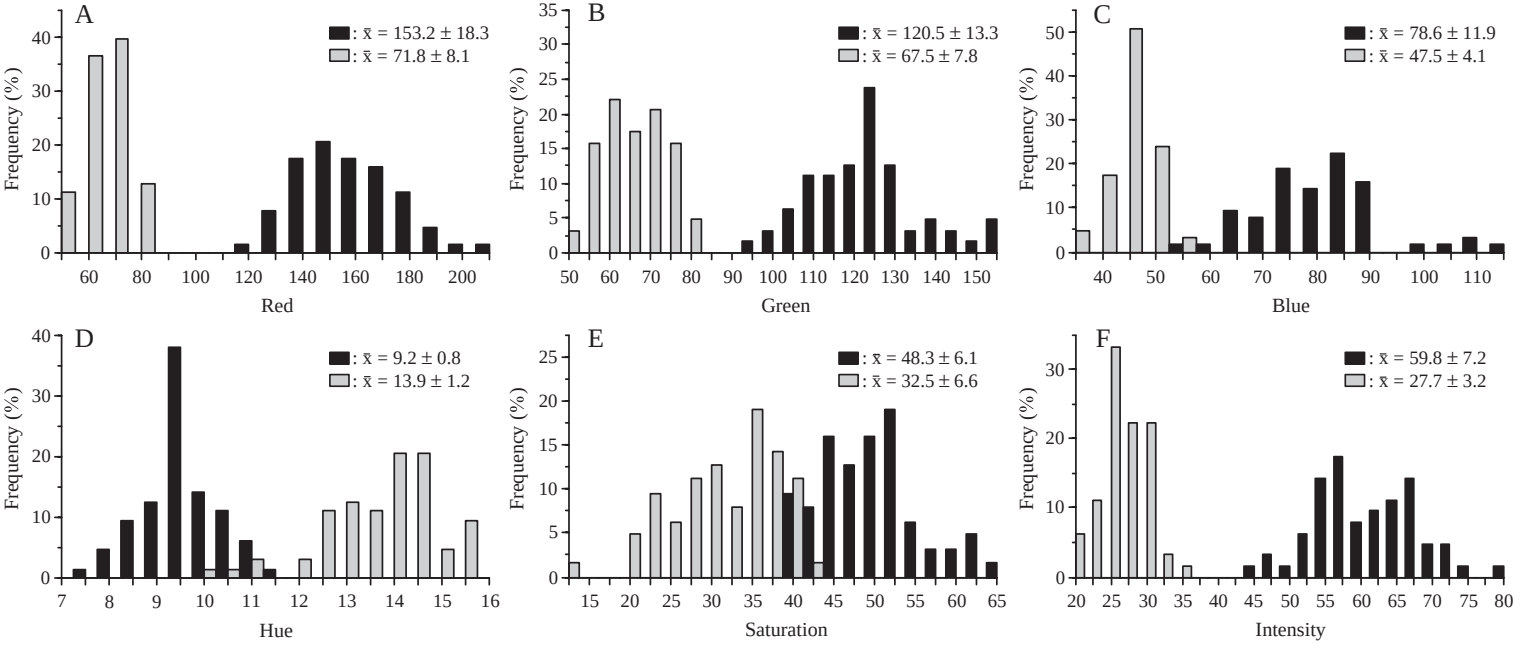

Fig. 2. Frequency distributions of red (A), green (B), blue (C), hue (D), saturation (E) and intensity (F) in albinos (black bars) and wild-type individuals (grey bars). Values represent mean ± S.D.

Table 1
Correlation between color components in wild-type individuals and albinos

Axis		Wild-type		Albino	
X	Y	Regression line	r^2	Regression line	r^2
Red	Green	$y=-3.09+0.95x$	0.962**	$y=20.64+0.65x$	0.810**
Red	Blue	$y=25.94+0.30x$	0.353**	$y=18.09+0.40x$	0.372**
Red	Hue	$y=9.96+0.06x$	0.128**	$y=12.08-0.02x$	0.162**
Red	Saturation	$y=-5.24+0.53x$	0.430**	$y=35.59+0.08x$	0.063*
Red	Intensity	$y=-0.07+0.39x$	0.998**	$y=-0.04+0.39x$	1.000**
Green	Blue	$y=26.48+0.31x$	0.349**	$y=-11.16+0.75x$	0.694**
Green	Hue	$y=8.37+0.08x$	0.264**	–	0.010
Green	Saturation	$y=-3.36+0.53x$	0.404**	–	0.013
Green	Intensity	$y=1.03+0.40x$	0.958**	$y=1.26+0.49x$	0.811**
Blue	Hue	–	0.001	–	0.022**
Blue	Saturation	–	0.042	$y=72.88-0.31x$	0.371**
Blue	Intensity	$y=6.20+0.45x$	0.349**	$y=30.88+0.37x$	0.373**
Hue	Saturation	$y=1.96+2.20x$	0.174**	–	0.042
Hue	Intensity	$y=15.39+0.89x$	0.122**	$y=91.40-3.42x$	0.162**
Saturation	Intensity	$y=17.38+0.32x$	0.434**	$y=45.65+0.29x$	0.062*

Significant at $*P<0.05$ or $**P<0.01$.

and albinos and within each group. The distribution range overlapped among these classifications. However, the peak of the distribution was located at 0.10 in the albinos whereas it was at −0.05 or −0.10 in the other classifications. The mean value within albinos was significantly higher than that within wild-type individuals or between albinos and wild-type individuals ($P<0.01$, Fisher's PSLD). Actual variance of relatedness was 0.030 ± 0.006 (S.E.) in all the individuals, 0.030 ± 0.008 in the wild-type individuals and 0.041 ± 0.018 in the albinos. These values were significantly greater than zero ($P<0.01$). Fig. 4 shows a UPGMA dendrogram of relatedness among individuals. All except four of the albinos tended to be closely related to each other. Some wild-type individuals were closely connected to albinos.

3.3. Heritability

Table 2 shows estimates of heritability using a marker-based method in six color components.

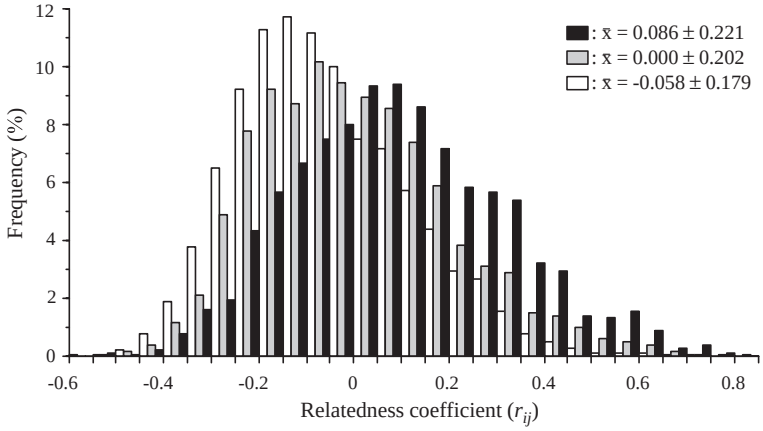

Fig. 3. Frequency distributions of pairwise relatedness within albinos (black bars), within wild-type individuals (grey bars) and between them (white bars). Values represent mean ± S.D.

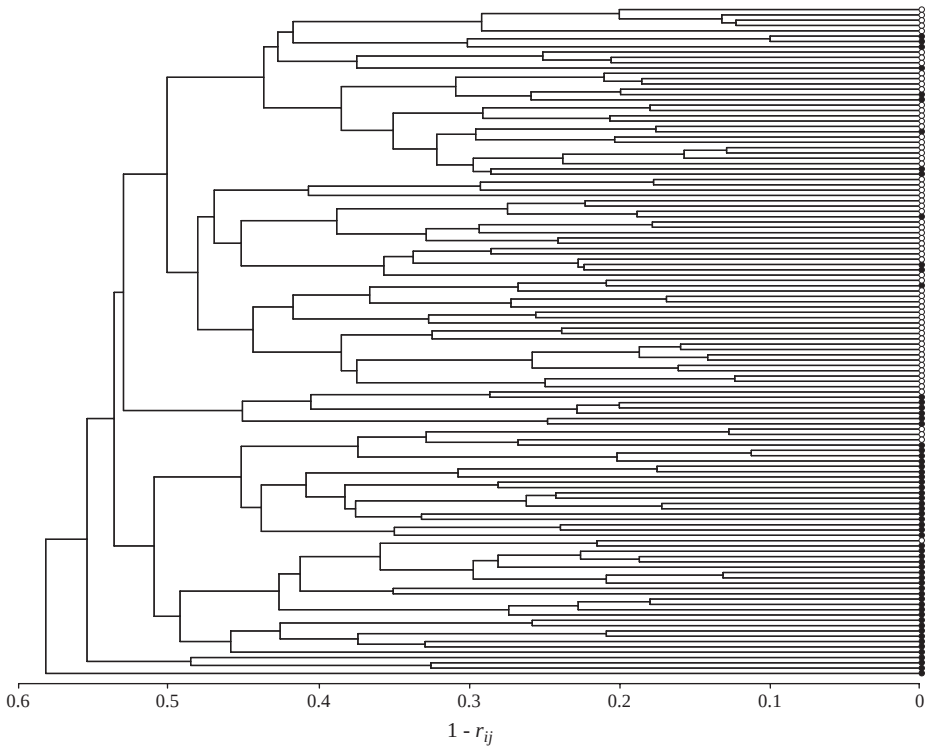

Fig. 4. UPGMA dendrogram of relatedness among albinos (open circles) and wild-type individuals (black circles).

Among all individuals, estimates of heritability were significantly greater than zero in all the color components with estimates of 0.77 to 0.98 ($P<0.01$). Among the wild-type individuals, significantly positive heritability was not observed for any color component ($P>0.05$) except H ($P<0.05$). On the other hand, heritability was significantly greater than zero for R, G and I among the albinos ($P<0.05$). The estimates of heritability were 0.51 for G and 0.44 for R and I.

4. Discussion

Albino Japanese flounder found in the hatchery population were characterized by red eyes and brighter body colors than wild-type colored individuals. Although there was a large variation in body color among albinos, the frequency distributions of color components indicated that albinos are clearly distinguishable from wild-type individuals on the basis of R, G and I. Correlation analyses between

Table 2
Estimates of heritability for color components in wild-type and albinos

Type	Color component					
	Red	Green	Blue	Hue	Saturation	Intensity
All	0.98 ± 0.23**	0.94 ± 0.22**	0.77 ± 0.20**	0.97 ± 0.24**	0.80 ± 0.20**	0.98 ± 0.23**
Wild-type	0.99 ± 0.20	0.11 ± 0.19	0.22 ± 0.24	0.28 ± 0.20*	0.08 ± 0.16	0.09 ± 0.20
Albino	0.44 ± 0.31*	0.51 ± 0.33*	0.31 ± 0.26	0.02 ± 0.21	0.11 ± 0.24	0.44 ± 0.31*

Values indicate heritability and standard error. Significantly greater than zero at *$P<0.05$ or **$P<0.01$.

color components indicated that these components were closely connected with each other, although the strength differed among the combinations as well as between albinos and wild-type individuals. This reflects the complexity of the expression of body color. In addition, relationship of H with R or I was positive in the wild-type individuals but it was negative in the albinos. These results indicated that the albinos have different features of color components from the wild-type individuals.

The hatchery population was produced by a limited number of parents by natural spawning. Therefore, relationship between the offspring may be classified into three categories: full-sibs, half-sibs and unrelated individuals, as observed in hatchery populations of marine fish (Perez-Enriquez et al., 1999; Sekino et al., 2003). Microsatellite analyses showed that the number of alleles per locus in albinos was lower than that in wild-type colored individuals, suggesting that the albinos were produced by specific parents. Pairwise relatedness among albinos was significantly higher than that among wild-type individuals or between albinos and wild-type individuals, although the distribution range was similar among these classifications with a large overlap. Furthermore, the UPGMA dendrogram of pairwise relatedness indicated that most albinos tended to be closely related to each other. The dendrogram also showed that some wild-type individuals were closely connected to albinos, suggesting that these wild-type individuals were produced by the same parents as some albinos. As well as statistical errors generally observed in estimates of relatedness (Ritland, 1996a; Lynch and Ritland, 1999), this may be one of the reasons for the large variation in pairwise relatedness within and between the groups. If albinos were produced by one pair, allele number at each locus would be four or lower. However, the maximum allele number at a locus observed in the albinos was 10. These results suggested that the albinos were produced by some, but not one or many, pairs.

In this study, heritability for body color variation was estimated using a marker-based approach reported by Ritland (1996b). Because this approach is based on regression between pairwise relatedness and pairwise phenotypic similarity of a trait, large variance of the two measures is essential for actual estimation of heritability (Ritland, 1996b, 2000). Large actual variance of relatedness was observed in the population used in this study in agreement with the description that it occurs when there is some mixture of relatives (Ritland, 2000). In addition, considerable continuous variations in pairwise phenotypic similarity were caused by large variations in color components within albinos and wild-type colored individuals as well as between them. Marker-based estimates of heritability among all individuals were high for all the color components with estimates of 0.77–0.98. Since heritability was estimated using all individuals, the high heritability for color components may be caused by a large effect of the albinism gene. In addition, the estimation of heritability may include dominant genetic effects caused by albinism. The high heritability indicates that the albinism found in this population is strongly heritable, suggesting that the phenomenon may be controlled by one or a few major genes as observed in other fish species (Bridges and von Limbach, 1972; Kajishima, 1977; Kirpichnikov, 1981; Rothbard and Wohlfarth, 1993). Furthermore, the fact that albinos were produced by wild-type colored parents indicates that the albinism is a recessive trait. If the albinism is controlled by one or a few autosomal recessive genes, some wild-type colored individuals were produced by the same parents as some albinos as suggested by the UPGMA dendrogram of relatedness. Since the population in which albinos were detected was produced by a limited number of the individuals domesticated for at least two or three generations, inbreeding might have caused an increase in homozygosity of recessive alleles and the expression of albinism.

A considerable variation in body color was observed among albinos. Marker-based estimates of heritability were significantly positive for R, G and I in the albinos. This suggested that the body color variation observed among albinos was partly caused by additive genetic effects. On the other hand, significantly positive heritability was observed for H in the wild-type colored individuals, indicating the possibility of additive genetic effects on body color variation among the wild-type individuals. As significantly positive heritability was observed in different color components, genetic control of body color variation might differ between albinos and wild-type

individuals. Melanophores are one type of important chromatophores which determine body color in flatfish as well as other fish species (Bolker and Hill, 2000; Sugimoto, 2002; Kelsh, 2004). Preliminary data indicated that some albinos did not have melanophores on scales at the ocular side but others had lower numbers of melanophores than wild-type individuals with different numbers among the individuals. Genetic factors might participate in the difference in chromatophore number and the large variation in body color among albinos.

Many albinisms of fish species are controlled by one autosomal recessive gene and show little variation in body color within each group (Bridges and von Limbach, 1972; Kirpichnikov, 1981; Rothbard and Wohlfarth, 1993). Albino Japanese flounder of this study were the first generation albinos produced by wild-type colored individuals and thus potentially contained a large genetic variation in body color among the albinos. Exact inheritance mode of albinism and heritability of body color can be examined through traditional controlled breeding experiments; however, it will take a long time to perform breeding experiments for these analyses in Japanese flounder since their generation time is approximately 3–4 years. This study was one of the few that detected significant heritability using the marker-based method reported by Ritland (1996b). Significant heritability for body color among the albinos suggested the possibilities of selection response for body colors and the establishment of several albino strains with different body colors. Such strains will be useful for the analysis of the body coloration of flatfish including albinism, pseudo-albinism, hypermelanosis and differences between ocular and blind sides. The present study provided important results for further analysis of the genetic control of albinism and for the establishment of albino strains in Japanese flounder.

Acknowledgements

The author thanks all those who kindly assisted in sample collection for this study, particularly Nobuhiro Endo, Fukushima Prefectural Sea-Farming Institute, and Masato Watanabe, Fukushima Prefectural Nursing Research Institute. Thanks also to Yukinori Shimada, Fukui Prefectural University, for assistance in this study.

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