Effects of Body-Color Mutations on Vitality: An Attempt to Establish Easy-to-Breed See-Through Medaka Strains by Outcrossing

Ayaka Ohshima,*,1 Noriko Morimura,*,1 Chizuru Matsumoto,*,1 Ami Hiraga,*,1 Ritsuko Komine,*
Tetsuaki Kimura,†1 Kiyoshi Naruse,† and Shoji Fukamachi*,2

*Laboratory of Evolutionary Genetics, Department of Chemical and Biological Sciences, Japan Women’s University, Tokyo, †Laboratory of Bioresources, National Institute for Basic Biology, The Graduate University for Advanced Studies (SOKENDAI), Aichi, and 2National Institute for Basic Biology, Interuniversity Bio-Backup Project Center, Aichi, Japan

ABSTRACT “See-through” strains of medaka are unique tools for experiments: their skin is transparent, and their internal organs can be externally monitored throughout life. However, see-through fish are less vital than normally pigmented wild-type fish, which allows only skilled researchers to make the most of their advantages. Expecting that hybrid vigor (heterosis) would increase the vitality, we outcrossed two see-through strains (SK2 and STIII) with a genetically distant wild-type strain (HNI). Fish with the see-through phenotypes were successfully restored in the F2 generation and maintained as closed colonies. We verified that genomes of these hybrid see-through strains actually consisted of approximately 50% HNI and approximately 50% SK2 or STIII alleles, but we could not obtain evidence supporting improved survival of larvae or fecundity of adults, at least under our breeding conditions. We also found that four of the five see-through mutations (bg98, i-3, gu, and il-1 but not if) additively decrease viability. Given that heterosis could not overwhelm the viability-reducing effects of the see-through mutations, easy-to-breed see-through strains will only be established by other methods such as conditional gene targeting or screening of new body-color mutations that do not reduce viability.

Body surfaces of vertebrates are pigmented by cells called chromatophores, up to six types of which (melanophore, xanthophore, erythrophore, leucophore, iridophore, and cyanophore) have been identified in fish (Fujii 2000). These intact chromatophores provide a powerful platform by which to study cell proliferation, differentiation, or migration (Hirobe 2011). However, when research focuses on other cells inside the body, these pigmented cells on the body surface become obstacles. This is particularly critical in experiments in which model fish are used (medaka and zebrafish) because their transparent somatic cells enable in situ observations of internal organs without dissection.

The “see-through” medaka no. 3 (STIII) was established from this point of view (Wakamatsu et al. 2001). It does not have any visible (fully differentiated) chromatophore throughout life, and therefore drug effects or fluorescent proteins expressed in the internal organs are maximally visualized (Kashiwada 2006; Deguchi et al. 2012). STIII is a quadruple recessive mutant with the following spontaneous body-color mutations: albino-3 (i-3), leucophore free (lf), guanineless (gu), and iridophoreless-1 (il-1; see Table 1). The i-3 locus encodes the oculocutaneous albinism type 2 (Oca2) protein that is essential for melanin synthesis in melanophores (Fukamachi et al. 2004). Xanthophores in the i-3 mutant are also colorless because of the potential function of Oca2 in carotenoid metabolism. The lf gene is essential for leucophore development, and the lf mutants lack any visible leucophores (Fukamachi et al. 2006). Proteins encoded by the gu or il-1 locus are necessary for iridophore development in the eyes/abdomen or the opercles, respectively. STIII fish develop and grow normally (Iwamatsu et al. 2003) but are rather weak and difficult to breed, which has prevented widespread use of this strain in laboratories.

We previously established another see-through strain, suke-suke (SK3), which is a triple recessive mutant of radiation-induced (bg98) and...
spontaneous (lf and gu) mutations (Fukamachi et al. 2008). The b locus, on which the bg family 45, member 2 (Sle45a2) protein that is essential for melanin synthesis (Fukamachi et al. 2001). The bg mutation does not suppress melanin deposition as strongly as the STIII and SK2 are weakly pigmented, unlike those of STIII.

The body-color mutations introduced into STIII or SK2 were isolated from a southern Japanese population [see (Fukamachi 2011)]. There is another (northern Japanese) population of medaka, which is so distantly related to the southern population that it was recently proposed as a different species [Oryzias sakaizumii (Asai et al. 2011)].

Genome sequences between the northern and southern populations are approximately 3% different, but their hybrids (F1s) are fully viable and fertile. Indeed, it is well known among medaka investigators that the F1 fish are full of vitality and extremely easy to handle. Therefore, we anticipated that outcrossing of the see-through southern strains with the northern strain and restoring F2 fish with the see-through phenotypes would result in hybrid vigor (heterosis) and establish easy-to-breed see-through strains that could tolerate widespread use in laboratories.

MATERIALS AND METHODS

Fish and breeding conditions

We used STIII and SK2 as the southern see-through strains. As the northern wild-type strain, we used the standard inbred strain, HNI. All fish developed and were grown in the laboratory. Ordinary tap water heated at 27°C was used and was circulated with a central filtration system. Light was provided from ordinary fluorescent lamps for 14 hr per day. We fed <2-week larvae with a few kinds of well-ground flake food (e.g., TetraMin; Tetra), and older fish with live brine shrimps and the flake food, about five times per day (every 2 hr between 1000 and 1800 hr).

Outcrossing and restoring F2 fish with the see-through phenotype

The lf gene is sex-linked and located on both the X and Y chromosomes (Wada et al. 1998). Therefore, we needed to cross the see-through and the wild-type fish reciprocally to obtain males and females with the see-through phenotype at the F2 generation (Figure 1).

As summarized in Table 1, all of the five see-through mutations (bg, i-3, lf, gu, and il-1) are recessive, and all of the mutant phenotypes, except for il-1, appear from embryonic stages. Therefore, we could identify F2 embryos with the SK2 (bg-il-1 triple recessive) phenotype by binocular-microscopic observation and selectively breed these see-through F2s. In terms of STIII, we first selected F2 embryos with the i-3-lf-gu triple recessive phenotype (one-quarter of which should be the i-3-lf-gu-il-1 quadruple recessive embryos) and raised all of the F2 progeny. When the il-1 phenotype became apparent (about 2 mo after hatching), the triple and quadruple mutants were distinguished by intact or binocular-microscopic observations.

Genome-wide genotyping

We randomly chose several adult fish from the original and hybrid see-through strains, and extracted their genomic DNA using a high-salt DNA extraction method (Aljanabi and Martinez 1997). Using each of the genomic DNA as templates, we amplified the M-marker 2009 using polymerase chain reaction and analyzed the bands as described elsewhere (Kimura and Naruse 2010). Because the sizes of all HNI alleles were already known (Kimura and Naruse 2010), we regarded bands at different sizes as the southern alleles.

Vitality comparison

We focused on two characters to compare the vitality of the original and hybrid see-through strains: the survival rate of hatched larvae (viability) and the number of eggs daily spawned by adults (fecundity).

To assess viability, we collected fertilized eggs and placed hatched larvae into tanks (different strains in different tanks). When the number of larvae in the tanks reached 30, 43, or 50 (we could not obtain these numbers of hatched larvae in one day and needed to accumulate larvae hatched on different days but within the same week), we started counting live fish every week.

To assess fecundity, we prepared adult fish that were spawning every day, and we collected eggs attached to the females’ cloaca (spawned eggs are temporarily held at the cloaca by attaching filaments) and those that dropped on the bottom of the tanks every morning. Fertilized and unfertilized eggs were distinguished and counted under a binocular microscope. We incubated the fertilized eggs in methylene-blue-added tap water until they hatched.

Assessment of the see-through mutations on viability

We backcrossed the F1 females (Figure 1) with the original see-through males to obtain embryos with various body-color phenotypes (i.e., wild-type and single/double/triple/quadruple recessive mutants) in the same numbers; the bg, illegal-1, and gu loci are independent on chromosomes (Naruse et al. 2000; Fukamachi et al. 2004), and the il-1 locus seemed not to be linked to any of these loci (see Wakamatsu et al. 2001 and the Results section). It should be noted that this cross
and half (SK2-HH; Figure 2).

We intercrossed the F1 heterozygous for all of these loci, exhibited the wild-type phenotype. All of the SK2 mutations (bg8, i-3, il, and gu loci, and hatched larvae were raised en masse in large containers [51 cm × 36 cm × 24 cm (length, width, height)] without water circulation/filtration. When they reached adult stages, we repheno-typed each fish for all of the five see-through loci, including il-1. The body length (from the snout to the distal edge of the caudal fin) of all the adult fish was also measured.

RESULTS AND DISCUSSION

Outcross of SK2 and STIII

We hybridized the southern see-through strains (SK2 and STIII) with a northern wild-type strain (HNI). HNI is one of the standard inbred strains widely used in laboratories and has wild-type alleles at all of the five see-through loci (b, i-3, il, gu, and il-1). Their F1s, which were heterozygous for all of these loci, exhibited the wild-type phenotype. We intercrossed the F1 fish and collected a large number of F2 siblings, among which we screened individuals with the SK2 or STIII phenotype (Figure 1).

All of the SK2 mutations (bg8, i-3, il, and gu) are independently located on chromosomes (Naruse et al. 2000), and therefore 1/4^3 (= 1/64) of the F2 fish should develop the SK2 phenotype. Among the 4969 F2 eggs that we collected, 7,723 (96.4%) embryos developed normally, 94 of which exhibited the i-3/if-gu triple recessive phenotype (the il-1 phenotype does not appear at this stage). Interestingly, this count was close to, but significantly lower than, the expected value (7,723 × 1/64 = 120.7; P = 0.014, χ^2 test). The reason is unclear, but one or a combination of the STIII mutations may slightly inhibit normal development of embryos, which could statistically be detected only when thousands are examined (note that such an effect was not detected in Figure 5A, where only 773 embryos were examined). We selectively raised these triple recessive F2 larvae, obtained 34 adult fish, and found only two males and two females with the STIII (i-3/if-gu-il-1 quadruple recessive) phenotype. This count (i.e., four) was also significantly lower than the expected value (34 × 1/4 = 8.5; P = 0.017, χ^2 test), which most likely reflects the viability-reducing effect of the il-1 mutation (described later; see Figure 5). This low count of the quadruple recessive F2s also indicates that the il-1 locus is not closely linked to the i-3, il, or gu loci.

Unfortunately, we could not obtain fertilized eggs from the quadruple recessive F2 males and females, and we could not establish the STIII-HH strain. Therefore, we intercrossed their triple recessive (i-3/if-gu) siblings and maintained the strain as a closed colony. This strain seemed to retain the il-1 mutation in the population, but the majority (e.g., 12/13) exhibited a triple recessive phenotype known as see-through medaka no. 2 [STII; (Wakamatsu et al. 2001)], and we termed the strain STII-HH (Figure 2).

Genome-wide genotyping of the see-through-HH strains

We then investigated whether the genomes of SK2-HH and STII-HH actually consist of half northern and half southern alleles. For this purpose, we used the M-marker 2009, which is a set of 48 sequence-tagged sites (STSs) that were designed at all of the 24 medaka chromosomes (two markers on each chromosome). Polymorphisms in these STSs can be detected as insertions or deletions by genomic poly-merase chain reaction and capillary electrophoresis (Kimura and Naruse 2010).

When we analyzed the original SK2 and STIII strains (three males and three females for each strain; n = 6 each), we found that their genomes did not have a northern (HNI) allele at any loci (Table 2). By contrast, genomes of SK2-HH (six males and eight females; n = 14) and STII-HH (seven males and five females; n = 12) did contain the northern alleles (Table 2). Among the 96 alleles amplified by the M-marker 2009 in each fish, 49.8 ± 2.3% and 41.6 ± 1.4% (mean ± SEM) were northern in SK2-HH and STII-HH, respectively. Thus, the outcrosses successfully introduced the northern alleles into the see-through-HH strains and their genomes literally consisted of half northern and half southern alleles.

Given the allele frequencies in the see-through-HH strains noted above, the genotype frequency in each marker could be expected to be northern-homozygous (N/N):heterozygous (N/S):southern-homozygous (S/S) = 1:3:1 on the assumption of the Hardy–Weinberg equilibrium. However, there are several DNA markers in Table 2 that do not fit with this expectation (P < 0.01, χ^2 test without correction). Nevertheless, we could consider plausible reasons for most of these biased genotype frequencies.

For example, only the S/S genotype was detected in MID0117 on LG01 in both SK2-HH and STII-HH. This is most likely because the If locus is located on LG01 (see Table 1). That is, the mutated If allele is of southern origin, and the i-3/il genotype was selected to establish SK2-HH and STII-HH, which should fix the southern alleles of STSs/genes.
flanking to the *If* locus (including MID0117) in these strains. The same should be the case in MID0517 on LG05, where the *gu* locus is located.

The biased genotype frequency was detected in either (not both) of SK2-HH or STII-HH in terms of MID0424 on LG04 and MID1213/1221 on LG12. This is because the *i-3* mutation on LG04 was fixed only in STII-HH (but not in SK2-HH) and the *be8* mutation on LG12 was fixed only in SK2-HH (but not in STII-HH). From this point of view, the genotype frequency of MID1614 on LG16, which is strongly biased in only STII-HH, but not SK2-HH, may indicate that the *il-i* locus is located on this linkage group (though the mutation has not been completely fixed in the strain).

The genotype frequency of MID1807 on LG18 is differently biased from the cases described previously in that its northern (instead of southern) allele was more frequently detected in both SK2-HH and STII-HH. The reason remains unknown, but northern alleles of the chromosomal region around MID1807 may be more advantageous for survival and/or reproduction than southern alleles, which therefore were selected in both strains.

Another interesting genotype frequency is found in MID2113 on LG21, where the heterozygous *N/S* never appeared in either SK2-HH or STII-HH. Considering that we used adult fish (instead of embryos) for genotyping, *N/S* heterozygotes of this chromosomal region may not be able to survive or grow efficiently because of incompatibility between northern and southern alleles (outbreeding depression).

**Vitality of the see-through-HH strains**

The most critical period when breeding medaka is up to 2 wk after hatching; after surviving this period, most fish can grow into adults (see Figure 3). We could stably maintain both SK2 and SK2-HH in the laboratory (see the section Materials and Methods for our breeding conditions), but we did not have the impression that SK2-HH was particularly easier to handle than SK2; many larvae of both strains died after hatching. Indeed, survival rates (up to 14 wk after hatching) were not significantly different between SK2 and SK2-HH in any of the three independent comparisons (Figure 3A; *P > 0.05*, logrank test with Bonferroni correction). We also measured the body length of all the survivors in the first and second comparisons (in which the observation was continued for 14 weeks) and detected no significant difference between the strains (24.5 ± 0.3 mm [n = 60] in SK2 and 25.4 ± 0.5 mm [n = 48] in SK2-HH; *P = 0.108*, Student's two-tailed *t*-test).

The fact that we twice failed to maintain STIII, but not STII-HH, should indicate that STII-HH is easier to breed than STIII. Indeed, the survival rate of STII-HH was four times greater than that of STIII, at least in one comparison (Figure 3B; *P < 0.001*, log-rank test). However, this improvement seemed to reflect not heterosis but removal of the viability-reducing *il-i* mutation in STII-HH (explained below), and we did not repeat the comparison.

We also compared the fecundity of SK2 and SK2-HH (Table 3). We prepared four pairs of adult fish from each strain that were spawning every morning and collected their eggs for 7 consecutive days. The body lengths of the females were 33.2 ± 0.8 mm and 33.7 ± 1.0 mm in SK2 and SK2-HH, respectively (*n = 4* each), which were not significantly different (*P = 0.691*, Student's two-tailed *t*-test). The total numbers of the collected eggs were 300 for SK2 and 324 for SK2-HH, which is a ratio not significantly different from 1:1 (*P = 0.337*, *χ²* test). The numbers of fertilized eggs were 155 and 135, and the numbers of hatched larvae were 131 and 107 for SK2 and SK2-HH, respectively, neither of which is a ratio significantly different from 1:1 (*P > 0.05*, *χ²* test). In short, only about five fertilized eggs (and four hatched fries) could be obtained per female per day in both SK2 and SK2-HH of this body size.

Taken together, we could not obtain evidence supporting the notion that the hybrid see-through strains were any easier to handle than the original see-through strains (Figure 3 and Table 3) despite the fact that the genomes of the see-through-HH strains actually consisted of a 1:1 mixture of the northern and southern alleles (Table 2). These results would indicate that inbreeding depression (Charlesworth and Willis 2009) seldom occurred in the original SK2 or STIII strains. That is, although all of the alleles that we detected in SK2 and STIII were southern in size (Table 2), their genomes must be sufficiently heterozygotic to avoid the depression. Alternatively, effects of the heterosis might actually exist in the see-through-HH strains but might be masked and overlooked in the present study. Our breeding conditions, in which STIII could not be stably maintained, are not maximally optimized, and we did not test other breeding conditions (e.g., food, fish densities, or water flow; see Hensley and Leung 2010). Therefore, the masked heterosis may be manifested if similar experiments are performed under different breeding conditions.
Effects of the see-through mutations on viability

Given that the see-through-HH strains did not show improved viability or fecundity (at least under our breeding conditions), the decreased vitality of the original and hybrid see-through strains should most likely be the result of the see-through mutations themselves. To assess this hypothesis, we backcrossed the F1 fish (Figure 1) to the original see-through fish, raised the backcrossed siblings to adult stages en masse, and examined the phenotype and body length of all survivors.

From the SK2 backcross, we obtained a total of 1047 hatched larvae, whose embryonic phenotypes had been determined under a binocular microscope at day 5–6 after fertilization. As expected from the independent location of the b, lf, and gu genes (Naruse et al. 2000), the backcrossed larvae exhibited eight kinds of body-color phenotypes (wild-type, b<sup>8</sup>, lf, gu, b<sup>8</sup>-lf, b<sup>8</sup>-gu, lf-gu, and b<sup>8</sup>-lf-gu) in equal numbers (1047 × 1/2<sup>8</sup> = 131; Figure 4A; P = 0.885, χ<sup>2</sup> test test). About 2 mo after their mixed breeding using three large containers (see Materials and Methods), a total of 552 fish had survived (a survival rate of
mutant siblings (and gu according to the P0.051, χ² test). These results demonstrate that the bg8 and gu mutations, but not the if mutation, significantly decrease the probability of larvae growing into adults. Furthermore, considering that siblings with both of the bg8 and gu mutations (i.e., bg8-gu and bg8-if-gu) survived less than those with either of the mutations (i.e., bg8, gu, bg8-if, and if-gu siblings; Figure 4A), the viability-reducing effect of the bg8 and gu mutations seems to function additively.

Our data also showed that the bg8 and gu mutations suppress growth; siblings without these mutations grew significantly larger than those with the mutations (P < 0.001, Student’s two-tailed t-test; Figure 4C). Again, such an effect was not detected in the if mutation (P = 0.097; Figure 4C).

From the STIII backcross, we obtained a total of 773 hatched larvae, which had been phenotyped for the i-3, if, and gu (but not il-1) loci during embryonic stages. Supporting their independent locations on chromosomes (Table 1), eight phenotypes appeared in equal numbers (773 x 1/23 = 97; P = 0.173, χ² test; Figure 5A) in the backcrossed siblings. After two months of their breeding en masse, we obtained 282 adult fish (a survival rate of 36.5%), phenotyped each of them for the i-3, if, gu, and il-1 loci and classified them into 16 groups (Figure 5A). As for the case of the SK2 backcross, the numbers of survivors apparently differ among the groups (P < 0.001, χ² test); e.g., we obtained only two quadruple-recessive fish, whereas 36 wild-type siblings survived in the same environment.

Classification of the 282 survivors into wild-type and mutant groups according to either of the i-3, if, gu, and il-1 loci revealed that siblings with the i-3, gu, or il-1 phenotypes, but not the if phenotype, survived less than their corresponding wild-type siblings. It is noteworthy that the results for if and gu were consistent between the SK2 and STIII backcrosses (Figures 4B and 5B). The growth-suppressing effect of gu, but not if, detected in the SK2 backcross (Figure 4C) also was reproduced in the STIII backcrosses (Figure 5C), and the i-3 mutation was shown to have the same growth-suppressing effect (Figure 5C). It is intriguing that—unlike bg8, gu, and i-3—the il-1 mutation, which reduced viability (Figure 5B), did not suppress growth (P = 0.936, Student’s t-test; Figure 5C). Therefore, the mechanism by which the il-1 mutation reduces viability should be different from that of the bg8, i-3, and gu mutations (discussed below).

Potential mechanisms by which the see-through mutations reduce viability

It was understandable that the bg8 and i-3 mutations reduced viability because these mutations suppress melanin deposition in the eyes (and skin), causing a typical phenotype known as albino. The Slc45a2 and Oca2 genes, on which the bg8 and i-3 mutations locate in medaka (Table 1), are also found in humans and are mutated in oculocutaneous albinism type 4 and 2 (OCA4 and OCA2) patients, respectively (Suzuki and Tomita 2008). Because OCA patients face several problems in visual acuity because of their ametropic eyes (Grönstroem et al. 2007), we suspect that the bg8 or i-3 fish might have similar optical problems. These albino fish would not be able to find and catch food efficiently in tanks, which would cause malnutrition, suppress growth (Figures 4C and 5C), and reduce viability (Figures 4B and 5B). However, our data do not exclude the possibility that the growth suppression and reduced viability are directly caused by pleiotropic effects of these albino mutations (such as diminishing food appetite, reducing nutrient absorption from guts, or preventing anabolism).

Considering that the gu mutants showed the same defects in growth and viability (Figures 4, B and C, and 5, B and C), we suspect that the gu mutation causes optical problems similar to those of bg8 and i-3. The eyes of wild-type medaka (and many other fish species) are surrounded by a dense distribution of iridophores, which make them iridescent and silver in color when exposed to light. Because the gu mutation removes many of the iridophores, the amount of light...
coming into the eyes or light reflections inside the eyes would not be appropriately controlled. Considering that the gu mutation and the bg8 and i-3 mutations affect different types of chromatophores, the bg8-gu and i-3-gu double mutants should face more crucial optical problems than the bg8, i-3, or gu single mutants, and this appears to be detected as the additive effects of these mutations on the larval viability (Figure 4A and Figure 5A).

It was surprising to us that the il-1 mutation, which only suppresses iridophore distribution on the opercles (Figure 2), apparently reduced viability (Figure 5B). This effect should not occur in the same mechanism as that of bg8, i-3, and gu (i.e., optical problems leading to malnutrition), because the il-1 fish grow as big as their wild-type siblings (Figure SC). The definite mechanism remains unknown, but the iridophores on the opercles may have an indispensable role in protecting the gills (and/or surrounding tissues) from light exposure, and fish may die young without the protection against this phototoxicity. To our knowledge, however, the negative relationship between light exposure on the gills (or other internal organs) and organismal viability has not been elucidated to date. Alternatively, the il-1 gene may have pleiotropic functions other than the iridophore development that are essential for retaining viability. It is also possible that not the il-1 mutation but one or more other mutations closely linked to the il-1 locus might be the actual cause of the reduced viability (genetic hitchhiking). Cloning and characterization of the il-1 gene would open up a way to assess these possibilities.

**Future establishment of an easy-to-breed see-through strain**

Since the establishment of SK2 (Fukamachi et al. 2008), we have had an impression that it is much easier to breed than STIII. We believe that this is because the bg8 mutation suppresses melanin deposition in the eyes less severely than the i-3 mutation (Figure 2; Fukamachi et al. 2004). Indeed, SK2, but not STIII larvae, could be raised outdoors (K. Naruse and S. Fukamachi, unpublished data), indicating that STIII with their less-pigmented eyes face more optical troubles than SK2 under strong light conditions. Under the present indoor conditions, however, the bg8 and i-3 mutations seemed to reduce viability to a similar degree, because the wild-type-to-mutant ratios of the backcrossed survivors (Figures 4B and 5B) were 71:29 and 73:27 when the fish were grouped based on the bg8 and i-3 phenotypes, respectively. Therefore, the aforementioned scenario would most likely reflect the fact that SK2 has only two viability-reducing mutations (bg8 and gu), whereas STIII has three (i-3, gu, and il-1).

Given that the outcrosses did not increase the vitality of the see-through strains and that four of the five see-through mutations reduced viability (Figures 3–5, Table 3), an easy-to-breed see-through strain will only be established by adopting more complex methods. One such method could include screening of body-color mutations that do not decrease vitality. This study has already revealed that one of the four types of chromatophores in medaka, leucophores, can be perfectly removed by the lf mutation without reducing viability or suppressing growth (Figures 4, B and C, and 5, B and C). However, to eliminate the other three types (melanophores, iridophores, and xanthophores), screening of viable mutations other than bg8, i-3, gu, and il-1 is necessary. Such mutations may already exist in mutant stock, such as the Tomita collection (Kelsh et al. 2004) or commercially available strains.

Genetic engineering is another possible method. Transgenesis and gene targeting are applicable in medaka (Ishikawa et al. 2010; Yan et al. 2013). Therefore, identification/utilization of cis-regulatory elements to express the b, i-3, and gu gene specifically in the eyes may

### Table 3 Fecundity of SK2 and SK2-HH

| Strain | Body Length of Females (n = 4), mm | Total no. of Eggs Collected in 7 Contiguous Days | No. Fertilized Eggs | Fertility Rate, % | No. Hatched Larvae | Hatching Rate, % |
|--------|-----------------------------------|-----------------------------------------------|---------------------|------------------|------------------|-----------------|
| SK2    | 33.2 ± 0.8                        | 300                                           | 155                 | 51.7%            | 131              | 84.5            |
| SK2-HH | 33.7 ± 1.0                        | 324                                           | 135                 | 41.7%            | 107              | 79.3            |

---

![Image](image-url)
solves optical problems and enhances growth and viability of the see-through strain.

The il-1 phenotype was originally described as having an absence of iridophores in the opercles. Against the gu background, however, it becomes apparent that the il-1 mutation also removes iridophores in the anterior part of the abdomen (Figure 2), and therefore it should be better when introduced into see-through strains to increase transparency. However, the mutation (or a hitchhiking mutation/s) reduces viability by an unpredictable mechanism (Figure 5B). If it is a hitchhiking mutation that reduces viability or if the il-1 gene has other pleiotropic functions that directly affect viability, genetic manipulation would enable removal of the iridophores without reducing viability. However, if the phototoxicity in the gill or surrounding tissues/organs is the cause of the reduced viability, a reduction in viability (Figure 5B) seems to be inevitable. Breeding under dim light (or in darkness) may be a solution, but such a strain will not be easy to breed in laboratories with ordinary breeding apparatuses. Establishment of optimized and simplified protocols would be another effective approach for breeding see-through strains (Hensley and Leung 2010).

In summary, considering that the genomic background (and consequently the phenotype) of the see-through-HH strains is less uniform than that of the original strains (Table 2), we propose that the original SK is the most recommended easy-to-breed see-through strain at this time. This fish is now available at the National Biore-source Project (NBRP) Medaka upon request, and we hope that this availability expands opportunities for investigators.

ACKNOWLEDGMENTS

We thank the NBRP Medaka for providing the STIII strain. This study was supported by a Grant-in-Aid for Young Scientists A (#23687011) from the Japan Society for the Promotion of Science, individual collaborative research funds from the National Institute for Basic Biology (#10-349 and #11-317), and research funds from Japan Women’s University to S.F.

LITERATURE CITED

Aljanabi, S. M., and I. Martinez, 1997 Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. 25: 4692–4693.

Asai, T. S., H. Senou, and K. Hosoya, 2011 Oryzias sakaizumi, a new ricefish from northern Japan (Teleostei: Adrianichthyidae). Ichthyol. Explor. Freshwat. 22: 289–299.

Charlesworth, D., and J. H. Willis, 2009 The genetics of inbreeding depression. Nat. Rev. Genet. 10: 783–796.

Deguchi, T., K. E. Fujimori, T. Kawasaki, K. Maruyama, and S. Yuba, 2012 In vivo visualization of the lymphatic vessels in pFLT4-EGFP transgenic medaka. Genesis 50: 625–634.

Fujii, R., 2000 The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13: 300–319.

Fukamachi, S., 2011 Medaka spontaneous mutants for body coloration, pp. 173–184 in Medaka: Model for Organogenesis, Human Disease and Evolution, edited by M. Tanaka, K. Naruse, and H. Takeda. Springer Japan, Tokyo.

Fukamachi, S., A. Shimada, and A. Shima, 2001 Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. Nat. Genet. 28: 381–385.

Fukamachi, S., S. Asakawa, Y. Wakamatsu, N. Shimizu, H. Mitani et al., 2004 Conserved function of medaka pink-eyed dilution in melanin synthesis and its divergent transcriptional regulation in gonads among vertebrates. Genetics 168: 1519–1527.

Fukamachi, S., Y. Wakamatsu, and H. Mitani, 2006 Medaka double mutants for color interfere and leucophore free: characterization of the xanthophore-somatolactin relationship using the leucophore free gene. Dev. Genes Evol. 216: 152–157.

Fukamachi, S., M. Kinoshita, T. Tsujimura, A. Shimada, S. Oda et al., 2008 Rescue from oculocutaneous albinism type 4 using medaka slc45a2 cDNA driven by its own promoter. Genetics 178: 761–769.

Gronskov, K., J. Ek, and K. Brondum-Nielsen, 2007 Oculocutaneous albinism. Orphanet J. Rare Dis. 2: 43.

Hensley, M. R., and Y. F. Leung, 2010 A convenient dry feed for raising zebrafish larvae. Zebrafish 7: 219–231.

Hirobe, T., 2011 How are proliferation and differentiation of melanocytes regulated? Pigment Cell Melanoma Res 24: 462–478.

Ishikawa, T., Y. Kamei, S. Otozai, J. Kim, A. Sato et al., 2010 High-resolution melting curve analysis for rapid detection of mutations in a Medaka TILLING library. BMC Mol. Biol. 11: 70.

Iwamatsu, T., H. Nakamura, K. Ozato, and Y. Wakamatsu, 2003 Normal growth of the “see-through” medaka. Zool. Sci. 20: 607–615.
Kashiwada, S., 2006 Distribution of nanoparticles in the see-through medaka (Oryzias latipes). Environ. Health Perspect. 114: 1697–1702.

Kelsh, R. N., C. Inoue, A. Momoi, H. Kondoh, M. Furutani-Seiki et al., 2004 The Tomita collection of medaka pigmentation mutants as a resource for understanding neural crest cell development. Mech. Dev. 121: 841–859.

Kimura, T., and K. Naruse, 2010 M-marker 2009, a marker set for mapping medaka mutants using PCR length polymorphisms with an automated microchip gel electrophoresis system. Biotechniques 49: 582–583.

Naruse, K., S. Fukamachi, H. Mitani, M. Kondo, T. Matsuoka et al., 2000 A detailed linkage map of medaka, Oryzias latipes: comparative genomics and genome evolution. Genetics 154: 1773–1784.

Suzuki, T., and Y. Tomita, 2008 Recent advances in genetic analyses of oculocutaneous albinism types 2 and 4. J. Dermatol. Sci. 51: 1–9.

Wada, H., A. Shimada, S. Fukamachi, K. Naruse, and A. Shima, 1998 Sex-linked inheritance of the if locus in the medaka fish (Oryzias latipes). Zoolog. Sci. 15: 123–126.

Wakamatsu, Y., S. Pristyazhnyuk, M. Kinosita, M. Tanaka, and K. Ozato, 2001 The see-through medaka: a fish model that is transparent throughout life. Proc. Natl. Acad. Sci. USA 98: 10046–10050.

Yan, Y., N. Hong, T. Chen, M. Li, T. Wang et al., 2013 p53 gene targeting by homologous recombination in fish ES cells. PLoS ONE 8: e59400.

Communicating editor: B. J. Andrews