pnp4a Is the Causal Gene of the Medaka Iridophore Mutant guanineless

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ABSTRACT See-through medaka lines are suitable for observing internal organs throughout life. They were bred by crossing multiple color mutants. However, some of the causal genes for these mutants have not been identified. The medaka has four pigment cell types: black melanophores, yellow xanthophores, white leucophores, and silvery iridophores. The causal genes of melanophore, xanthophore, and leucophore mutants have been elucidated, but the causal gene for the iridophore mutant remains unknown. Here, we describe the iridophore mutant, guanineless (gu), which exhibits a strong reduction in visible iridophores throughout its larval to adult stages. The gu locus was previously mapped to chromosome 5, but was located near the telomeric region, making it difficult to integrate into the chromosome. We sought the causal gene of gu using synteny analysis with the zebrafish genome and found a strong candidate, purine nucleoside phosphorylase 4a (pnp4a). Gene targeting and complementation testing showed that pnp4a is the causal gene of gu. This result will allow the establishment of inbred medaka strains or other useful strains with see-through phenotypes without major disruption in the genetic background of each strain.

Medaka embryos are transparent and develop externally, making them suitable for the observation of internal organ developmental processes. However, because of the gradual appearance of pigment cells, observation of internal organs from the early juvenile to adult stages becomes difficult. To solve this problem, the see-through strains ST-II, ST-V, and SK2 were bred by crossing multiple medaka color mutants (Wakamatsu et al. 2001; Ohshima et al. 2013). In zebrafish, the casper fish were made for this purpose. The casper fish are doubly mutant for nacre (microphthalmia-associated transcription factor a) mutant and roy oribison. They show a complete lack of melanophores and iridophores and have an almost transparent body from the early juvenile to adult stage (White et al. 2008). These see-through strains allow the observation of internal organs throughout life.

To breed transparent medaka strains, multiple crosses among color mutants were necessary. Thus, the original genetic background was disrupted by the crosses. This is undesirable, because uniform and pure genetic backgrounds of inbred lines are one of their advantageous features. Inbred lines are useful for genetic analysis because each inbred line has a unique phenotype (Kimura et al. 2007, 2012; Shinya 2011; Tsuboko et al. 2014). Thus, to retain the genetic background of inbred lines, another method for creating transparent medaka is required. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system-based RNA-guided endonuclease has emerged as a simple and efficient tool for targeted genome editing in medaka (Ansai and Kinoshita 2014). To apply genome editing for the modification of body color, knowledge of the causal gene for the color mutation is indispensable.

The causal gene of the iridophore-less mutant is the last piece of the jigsaw for making see-through medaka by genome editing. The medaka has four different pigment cells: black melanophores, yellow xanthophores, white leucophores, and silver iridophores. To date, the causal genes of melanophore, xanthophore, and leucophore mutants have been identified (Koga et al. 1995; Fukamachi et al. 2001, 2004; Kimura et al. 2014; Nagao et al. 2014), but the causal gene for the iridophore-less mutant remains unknown. Additionally, the causal
The guaninless gene of *ray orbison*, which is responsible for the iridophore-less phenotype in the casper fish, is currently unknown. Therefore, our goal was to identify the causal gene for the iridophore-less mutant *guanineless* (gu).

The *gu* mutant shows a marked reduction in guanine deposition in iridophores throughout life (Tomita 1992; Kelsh et al. 2004) and is a key mutation for the establishment of transparent medaka strains (Wakamatsu et al. 2001; Oshihama et al. 2013). The *gu* locus has been previously mapped to chromosome 5 (Naruse et al. 2000). However, this locus is located near the telomeric region (Naruse et al. 1988) and no genome sequence information from around the *gu* locus is available. Thus, the conventional positional cloning approach was inefficient for the *gu* locus. To circumvent this problem, we used the conserved synteny information with the information from zebrafish for gene targeting using the CRISPR/Cas system to identify the causal gene of the *gu* mutant.

**MATERIALS AND METHODS**

**Medaka strains and rearing conditions**

Medaka were reared at 26.0°C on a 14-hr light/10-hr dark cycle. The T5 strain, which is homozygous for the *gu* mutation, has been described previously (Tomita 1992; Shimada and Shima 2001; Kelsh et al. 2004) and used as the *gu* mutant. The d-rR strain is a closed colony, derived from a southern Japanese population. The d-rR strain was used as wild-type in this study. All the medaka strains were obtained from the National BioResource Project (NBRP), Medaka (www.shigen.nig.ac.jp/medaka/).

**Scaffold mapping**

The 95 F2 DNA panels from the cross between the Hd-rR and Kaga strains were used for scaffold 1311 mapping. PCR was performed as described previously (Kimura and Naruse 2010). The PCR products were analyzed using a DNA-500 kit on MCE-202 MultiNA (Shmadzu). Scaffold 1311 was mapped by scoring for recombination with the PCR-length polymorphism marker S1311N02 (Supplemental Material, Tables S1 and S2 in File S2).

**Synteny analysis**

Synteny analysis was performed using the Genomicus synteny browser (http://www.genomicus.biologie.ens.fr) (Louis et al. 2013, 2015).

**RT-PCR analysis**

RNeasy (QIAGEN) and ReverTra Ace (Toyobo) were used to prepare cDNA from embryos. The primers used for amplification are shown in Table S2 in File S2. The PCR products were electrophoresed using a DNA-1000 kit on MCE-202 MultiNA (Shmadzu).

**Genomic PCR analysis**

Genomic DNA was extracted from fin clips fixed in 100% methanol. The samples were suspended in 100 ml lysis buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA; and 1 mg/ml protease K) and incubated at 55°C for 3 hr, followed by incubation at 95°C for 10 min for protease K inactivation. All genomic DNA samples were stored at −20°C until use. The PCR conditions for exons 1 and 2–3 were as follows: one cycle at 95°C for 2 min; 35 cycles at 95°C for 30 sec, 64°C for 30 sec, and 72°C for 90 sec; followed by 72°C for 3 min. The PCR conditions for exons 4–5 and 6–7 were as follows: one cycle at 95°C for 2 min; 35 cycles at 95°C for 30 sec and 68°C for 30 sec; followed by 72°C for 1 min. The products were electrophoresed using a DNA-120000 kit on MCE-202 MultiNA (Shmadzu). The primers used for amplification are shown in Table S1 in File S2.

**Gene targeting and mutation identification**

Gene targeting using the CRISPR/Cas system was performed as described previously (Ansai and Kinoshita 2014). The sgRNA was designed for exon 2 of *pnp4a* (Figure 3 and Table S1 in File S2), and microinjection was performed using the d-rR strain.

Genomic DNA was purified from fin clips. For sequencing, exon 2 of *pnp4a* was amplified with Ex-taq (TaKaRa). The PCR conditions were as follows: one cycle at 95°C for 2 min; 35 cycles at 95°C for 30 sec and 68°C for 30 sec; followed by 72°C for 1 min. The PCR products were treated with ExoSAP-IT (Affymetrix) and sequenced directly. The primers used for amplification and sequencing are shown in Table S1 in File S2.

**Whole-mount in situ hybridization (WISH)**

The EST clone, olec1e13, contained the full-length cDNA of *pnp4a*. The partial sequence of the olec1e13 clone was ligated into TOPO-II (Invitrogen) via PCR. The primers used for amplification are shown in Table S1 in File S2. A digoxigenin (DIG)-labeled RNA probe was generated using the DIG RNA labeling kit (Roche). WISH was performed as described previously (Takashima et al. 2007).

**Phylogenetic analysis**

Phylogenetic analysis was conducted using the neighbor joining method with 2000 bootstrap replicates using MEGA version 7.0.21 (Zuckerkandl and Pauling 1965; Felsenstein 1985; Saitou and Nei 1987; Kumar et al. 2016). Amino acid sequences were obtained from GenBank and Ensembl. We excluded the *pnp5b* of *Gasterosteus aculeatus*, because their estimated amino acid sequences were markedly different from those of others.

**Data availability**

All medaka strains are available from the NBRP, Medaka (www.shigen.nig.ac.jp/medaka/). The amino acid sequences used in phylogenetic analysis are shown in File S1. Sequence data of medaka *Pnp4a, Pnp4b, Pnp5a,* and *Pnp5b* have been deposited at DDBJ/EMBL/GenBank under accession numbers LC177073, LC177074, LC177075, and LC177076, respectively.

**RESULTS**

The *gu* mutant exhibited drastically reduced guanine deposition in iridophores throughout all life stages (Figure 1) (Tomita 1992; Kelsh et al. 2004). The *gu* mutant has a small number of pigmented iridophores. Iridophore differentiation could still proceed normally in the...
same cells, at least based on the gross appearance of the remaining intact iridophores, but the overall number of iridophores was decreased.

The gu locus has already been mapped to chromosome 5 (Naruse et al. 2000); however, the gu locus is located in the telomeric region of chromosome 5. Unfortunately, the medaka genome sequence lacks this region. Previously, we constructed a linkage map for medaka (Kimura et al. 2007). The microsatellite marker, MM05D05K, was the most telomeric side on chromosome 5 in our linkage map. Because MM05D05K was mapped at 0 cM apart from the gu locus and is located in nfs1 on ultracontig 95. Synteny analysis suggests that scaffold 1311 is near ultracontig 95. The polymorphic marker, S1311N02, for mapping the scaffold 1311 position was designed into the genome sequence of the scaffold located at the MM05D05K marker, from the region surrounding the gu locus. We compared the region surrounding the nfs1 region in other fish genomes using the Genomicus synteny browser (Louis et al. 2015) and found that pnp4a was located near nfs1 on zebrafish chromosome 11 (Figure 2). In addition, medaka chromosome 5 and zebrafish chromosome 11 shared an ancestral chromosome (Kasahara et al. 2007). The purine nucleotide phosphorylase metabolizes guanosine to guanine. In particular, pnp4a is a marker gene for iridophores in zebrafish (Curran et al. 2010). To confirm the result of synteny analysis, we attempted to map the position of pnp4a. As pnp4a was located on scaffold 1311 in medaka, we created a polymorphic marker named S1311N02 (Figure 2 and Table S1 in File S2) for mapping and determining the position of the scaffold. The mapping panel showed that scaffold 1311 was located on chromosome 5 and was 0 cM apart from the gu locus (Table S2 in File S2). Therefore, we thought that pnp4a was a strong candidate causal gene on the gu locus.

In order to determine whether the gu mutant possesses a mutation in pnp4a, we performed genomic PCR and RT-PCR using genomic DNA as well as total RNA extracted from the wild-type and gu embryos. We could amplify exons 1 and 2–3 of pnp4a, but could not amplify exons 4–5 and 6–7 when using genomic DNA from the gu mutant, whereas all PCR amplifications were positive in the wild-type embryo (Figure 3B). These data suggest that the gu mutant lacked exon 4–7 (Figure 3A and B). We also performed RT-PCR analysis of pnp4a mRNA from the gu mutant and wild-type. RT-PCR experiments with primers for exon 2–3 were positive both in the gu mutant and wild-type, but were negative in the gu mutant with primers for exon 4–7. Thus, we concluded that exons 4–7 of pnp4a were deleted in the gu mutant.

To confirm that pnp4a was the causal gene of the gu mutant, we generated pnp4a knockout fish using the CRISPR/Cas system (Ansai and Kinoshita 2014). We performed microinjection using one-cell-stage embryos of the d-rR strain. After intercrossing these fish, some progeny exhibited greatly decreased pigmentation of iridophores, a

![Image of synteny analysis between medaka and zebrafish](image-url)

**Figure 2** Synteny between medaka and zebrafish suggests that pnp4a is near the gu locus. The genetic marker MM05D05K is located 0 cM apart from the gu locus and is located in nfs1 on ultracontig 95. Synteny analysis suggests that scaffold 1311 is near ultracontig 95. The polymorphic marker, S1311N02, for mapping the scaffold 1311 position was designed into the genome sequence of the scaffold located at the MM05D05K marker, from the region surrounding the gu locus. Previously, we constructed a linkage map for medaka (Kimura et al. 2007). The microsatellite marker, MM05D05K, was the most telomeric side on chromosome 5 in our linkage map. Because MM05D05K was mapped at 0 cM apart from the gu locus and is located in nfs1 on ultracontig 95. Synteny analysis suggests that scaffold 1311 is near ultracontig 95. The polymorphic marker, S1311N02, for mapping the scaffold 1311 position was designed into the genome sequence of the scaffold located at the MM05D05K marker, from the region surrounding the gu locus. We compared the region surrounding the nfs1 region in other fish genomes using the Genomicus synteny browser (Louis et al. 2015) and found that pnp4a was located near nfs1 on zebrafish chromosome 11 (Figure 2). In addition, medaka chromosome 5 and zebrafish chromosome 11 shared an ancestral chromosome (Kasahara et al. 2007). The purine nucleotide phosphorylase metabolizes guanosine to guanine. In particular, pnp4a is a marker gene for iridophores in zebrafish (Curran et al. 2010). To confirm the result of synteny analysis, we attempted to map the position of pnp4a. As pnp4a was located on scaffold 1311 in medaka, we created a polymorphic marker named S1311N02 (Figure 2 and Table S1 in File S2) for mapping and determining the position of the scaffold. The mapping panel showed that scaffold 1311 was located on chromosome 5 and was 0 cM apart from the gu locus (Table S2 in File S2). Therefore, we thought that pnp4a was a strong candidate causal gene on the gu locus.

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![Image of PCR results](image-url)

**Figure 3** PCR suggests that exon 4–7 of pnp4a are lost in the guanineless mutant. (A) Scheme of the pnp4a gene. The medaka pnp4a gene comprises seven exons. Vertical bars indicate exons. Red arrows indicate the positions of primers used in RT-PCR. Black arrows indicate the positions of primers used in genomic PCR. (B) Genomic PCR result of WT and gu. Genomic PCR could not detect exon 4–7 in the gu mutant. KO indicates pnp4a KO. (C) pnp4a RT-PCR results of WT, pnp4a KO using CRISPR, and the gu mutant. RT-PCR could not detect exon 4–7 of pnp4a in the gu mutant. The amplicon of exon 2–3 of the pnp4a knockout is smaller than that of WT and the gu mutant because the knockout has a 13-bp deletion in exon 2 induced by CRISPR. β-actin is the positive control. cDNA, complementary DNA; Cas, CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; gu, guanineless; KO, knockout; PCR, polymerase chain reaction; pnp4a, purine nucleoside phosphorylase 4; RT-PCR, reverse transcription PCR; WT, wild-type.
phenotype similar to that of the gu mutant. We outcrossed the gu-like fish with d-rR and obtained F1 fish. As expected, sequence analysis showed that the CRISPR/Cas system induced a 13-bp deletion on exon 2 of pnp4a and generated an early stop in the 71st codon of pnp4a (Figure 3B and Figure 4, A and B). We then created a homozygote for the 13-bp deletion of pnp4a. The pnp4a knockout fish exhibited a guanineless phenotype indistinguishable from that of the gu mutant (Figure 4, C–E). Because the pnp4a knockout fish were viable and fertile, we performed a complementation test to confirm that pnp4a was the causal gene of the gu mutant (Figure 4, C–E). The pnp4a knockout embryos were healthy and fertile, so we performed a complementation test to confirm that pnp4a was the causal gene of the gu mutant. We obtained 48 embryos in total from the cross between the pnp4a knockout fish (13-bp deletion homozygous) and the gu mutant. All embryos showed the same phenotype as that of the gu mutants (Figure 5). Thus, we concluded that pnp4a was the causal gene of the gu mutant.

To determine pnp4a expression, we performed WISH. Guanine pigmentation was observed in the eyes of 3 dpf postfertilization (dpf) embryos, and iridophores emerged on the abdominal region. Guanine pigmentation was observed in the eyes of 3 dpf embryos, and iridophores emerged on the abdominal region. In 4 dpf embryos, the pnp4a signal was observed as pigmented iridophores as well as colorless cells on the abdominal region (Figure 6, C and D), but not in leucophores. These results suggest that pnp4a functions as a cell autonomous protein and is the responsible enzyme for iridophore pigmentation.
Since our WISH probe sequence was complementary to exon 4–7 of the pnp4a sequence, we performed WISH using gu embryos to assess probe specificity. As expected, the 5 dpf embryo of gu, which did not possess exon 4–7 of pnp4a, did not show any signal (Figure S1A in File S2). Additionally, the 5 dpf pnp4a knockout embryo showed signals similar to those in wild-type embryos, consistent with the RT-PCR results (Figure 3C). These results indicate that the probe was highly specific to pnp4a mRNA.

Since pnp4a was the causal gene of the gu phenotype, we investigated whether pnp4 has a conserved role in iridophore pigmentation of vertebrates by generating a phylogenetic tree of the pnp gene family. The pnp gene family of vertebrates is divided into at least three clades, PNP4, PNP5, and PNP6 (Figure 7). Iridophores are conserved among lamprey, teleost, amphibians, and reptiles. On the other hand, pnp4 genes are conserved in teleost, latimeria, and anole lizards. Although pnp4a is conserved in teleosts, pnp4 gene conservation is not consistent with that of iridophores.

**DISCUSSION**

We showed that pnp4a was the causal gene of the gu mutant and found that pnp4a, involving guanine synthesis, was located on the gu locus. The pnp4a gene was expressed in iridophores in concordance with the gu phenotype, which exhibited diminished pigmentation of iridophores. The pnp4a knockout experiment showed that pnp4a disruption caused a decrease in the pigmentation of iridophores, similar to that in the gu mutant. The cross between gu and pnp4a knockout fish demonstrated that pnp4a was the causal gene of the gu mutant.

The genomic PCR, RT-PCR, and WISH results indicated that the causal mutation of gu was the deletion of exon 4–7 of pnp4a (Figure 3, A and B and Figure S1 in File S2). Thus, the gu mutation is a null allele of pnp4a. The 13-bp deletion in exon 2 of pnp4a was also null because the homozygous phenotype was indistinguishable from that of the gu mutant (Figure 4). Additionally, pnp4a knockout fish expressed mRNA that had the 13-bp deletion, which caused a frame-shift and an early stop codon (Figure 3C).

Although the gu mutant has no functional pnp4a gene, the gu iridophores became slightly pigmented. It is probable that paralogs compensate for pnp4a function as another medaka iridophore mutant, iridophoreless-1 (il-1), is reported (Ohshima et al. 2013). In the double mutant gu and il-1, iridophore pigmentation is more repressed than in the single gu mutant (Wakamatsu et al. 2001; Ohshima et al. 2013). This suggests that pnp4a is not the only purine nucleoside phosphorylase present in iridophores. In zebrafish, all pnp paralogs are expressed in iridophores (Higdon et al. 2013). Thus, it is plausible that the causal gene of il-1 is a purine nucleoside phosphorylase.

Similar to tyrosinase in melanophores, is pnp4 a common guanine synthetic enzyme in iridophores in vertebrates? Our phylogenetic analysis suggests that it is not (Figure 7). The distribution of pnp4 is inconsistent with iridophore distribution. Specifically, both Xenopus species have iridophores, but they do not have the pnp4 gene. It is possible that the pnp4 gene has not yet been discovered in the Xenopus genome because Anolis carolinensis has PNP4. However, birds (Gallus gallus, Meleagris gallopavo, and Taeniopygia guttata) only have the PNP6 gene, suggesting that the subfunctionalization process of pnp genes is complicated and that pnp4 is not a common guanine synthetic gene in vertebrate iridophores. On the other hand, humans and mice with the absence of iridophores only have PNP5, whereas A. carolinensis with iridophores has PNP4 and PNP5. This suggests that pnp4 has an important role in guanine synthesis in iridophores. Although further analysis is required to elucidate the relationship between pnp genes and iridophore pigmentation, we conclude that pnp4a plays a major role in iridophore pigmentation in teleosts.

In this paper, we showed that the pnp4a knockout demonstrated an iridophore-less phenotype, and is the first choice to generate an iridophore-less phenotype in fish. Because several causal genes of melanophore mutants are known (Koga et al. 1995; Fukamachi et al. 2001, 2004) and the pax7a mutant has no xanthophores and leucophores (Kimura et al. 2014), we expect that our results pave the way for the production of transparent medaka using the CRISPR/Cas system without outcrossing. Since the causal gene of the iridophore-less zebrafish mutant roy is not identified, we also hope that our results will be helpful for the establishment see-through strains in zebrafish or in other fish species.

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Phylogenetic tree of the pnp amino acid sequences. The phylogenetic tree was constructed by Neighbor joining method using MEGA7. Numbers indicate the percentage of replicate trees in which the associated clade clustered together in the bootstrap test (2000 replicates). pnp, purine nucleoside phosphorylase.
LITERATURE CITED

Ansai, S., and M. Kinoshita, 2014 Targeted mutagenesis using CRISPR/Cas system in medaka. Biol. Open 3: 362–371.

Curran, K., J. A. Lister, G. R. Kunkel, A. Prendergast, D. M. Parichy et al., 2010 Interplay between Foxd3 and Mitf regulates cell fate plasticity in the zebrafish neural crest. Dev. Biol. 344: 107–118.

Felsenstein, J., 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

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.

Higdon, C. W., R. D. Mitra, and S. L. Johnson, 2013 Gene expression analysis of zebrafish melanocytes, iridophores, and retinal pigmented epithelium reveals indicators of biological function and developmental origin. PLoS One 8: e67801.

Kasahara, M., K. Naruse, S. Sasaki, Y. Nakatani, W. Qu et al., 2007 The medaka draft genome and insights into vertebrate genome evolution. Nature 447: 714–719.

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., A. Shimada, N. Sakai, H. Mitani, M. Kondo, T. Matsuoka et al., 2007 The see-through medaka: a model that is transparent throughout life. Proc. Natl. Acad. Sci. USA 111: 7343–7348.

Koga, A., H. Inagaki, Y. Bessho, and H. Hori, 1995 Insertion of a novel transposable element in the tyrosinase gene is responsible for an albino mutation in the medaka fish, Oryzias latipes. Mol. Gen. Genet. 249: 400–405.

Kumar, S., G. Stecher, and K. Tamura, 2016 MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33: 1870–1874.

Louis, A., M. Muffato, and H. Roest Crollius, 2013 Genomics: five genome browsers for comparative genomics in eukaryota. Nucleic Acids Res. 41 (Database issue): D700–D705. Louis, A., N. T. Nguyen, M. Muffato, and H. Roest Crollius, 2015 Genomicus update 2015: KaryoView and MatrixView provide a genome-wide perspective to multispecies comparative genomics. Nucleic Acids Res. 43 (Database issue): D682–D689.

Nagao, Y., T. Suzuki, A. Shimizu, T. Kimura, R. Seki et al., 2014 Sox5 functions as a fate switch in medaka pigment cell development. PLoS Genet. 10: e1004246.

Naruse, K., A. Shimada, and A. Shima, 1988 Gene-centromere mapping for 5 visible mutant loci in multiple recessive tester stock of the medaka (Oryzias latipes). Zoolog. Sci. 5: 489–492.

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.

Ohshima, A., N. Morimura, C. Matsumoto, A. Hiraga, R. Komine et al., 2013 Effects of body-color mutations on vitality: an attempt to establish easy-to-breed see-through medaka strains by outcrossing. G3 3: 1577–1585.

Saitou, N., and M. Nei, 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425.

Shimada, A., and A. Shima, 2001 High incidence of mosaic mutations induced by irradiating paternal germ cells of the medaka fish, Oryzias latipes. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 495: 33–42.

Shinya, M., 2011 Craniofacial traits, pp. 185–199 in Medaka, A Model for Organogenesis, Human Disease and Evolution, edited by Naruse, K., M. Tanaka, and H. Takeda. Springer, Tokyo.

Takashima, S., A. Shimada, D. Kobayashi, H. Yokoi, T. Narita et al., 2007 Phenotypic analysis of a novel chordin mutant in medaka. Dev. Dyn. 236: 2298–2310.

Tomita, H., 1992 The lists of the mutants and strains of the medaka, common gambusia, silver crucian carp, goldfish, and golden venus fish maintained in the Laboratory of Freshwater Fish Stocks, Nagoya University. Fish Biol. J. Medaka. 4: 45–47.

Tsuboko, S., T. Kimura, M. Shinya, Y. Suehiro, T. Okuyama et al., 2014 Genetic control of startle behavior in medaka fish. PLoS One 9: e112527.

Wakamatsu, Y., S. Pristazyhnyuk, M. Kinoshita, 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.

White, R. M., A. Sessa, C. Burke, T. Bowman, J. LeBlanc et al., 2008 Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell 2: 183–189.

Zuckerkandl, E., and L. Pauling, 1965 Evolutionary divergence and convergence in proteins, pp. 97–166 in Evolving Genes and Proteins, edited by Bryson, V., and H. J. Vogel. Academic Press, New York.

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