The MC1R gene in the guppy (Poecilia reticulata): Genotypic and phenotypic polymorphisms

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Abstract

Background: The guppy (Poecilia reticulata) is an important model organism for studying sexual selection; male guppies have complex and conspicuous pigmentation, and female guppies exhibit preferences for males with specific color spots. Understanding the genetic basis underlying pigmentation variation in the guppy is important for exploring the factors causing the maintenance of color polymorphism in wild populations.

Findings: We focused on the melanic black pigmentation of guppies, and examined genetic variations in the melanocortin 1 receptor (MC1R) gene because variation in this gene is known to contribute to polymorphism of melanin pigmentation in several animal species. The complete coding sequence of the guppy MC1R gene was determined, and two different MC1R alleles (963 and 969 bp) were found in wild populations. Ornamental strain guppies with a 963-bp MC1R tended to show less black pigmentation than those with a 969-bp MC1R, although the association between MC1R genotype and black pigmentation disappeared in the F2 offspring.

Conclusions: The guppy MC1R gene showed variation in the five wild Trinidadian populations we examined, and these populations also differed in terms of allele frequencies. We identified a significant association between black pigmentation and MC1R genotype in fish obtained from aquarium shops. However, the results from F2 families suggest that there are other genes that modify the effects of the MC1R gene.

Background

Pigmentation plays important roles in various aspects of several animal species, including camouflage, warning, thermoregulation, protection from ultraviolet radiation, and courtship display [1]. Therefore, individual variation in pigmentation can be a target of both natural and sexual selection, and thus mutations that change pigmentation influence adaptive evolution. Recently, identification of the genes responsible for pigmentation polymorphism has gained considerable attention in evolutionary biology [2].

The guppy (Poecilia reticulata) exhibits extreme pigmentation polymorphism in the secondary sexual traits of males, which is complex, conspicuous, and manifested as spots, speckles, and lines of various pigmented colors, including black, white, red-orange, yellow and green [3]. These fish also display iridescent structural color [3]. Female guppies show preferences for a specific type of male pigmentation, including orange and black, and various color spots [3]. Although directional selection such as specific female preference is expected to reduce variation in male traits [4], various color spots are maintained as polymorphic traits within populations [3]. Many studies have attempted to explain the maintenance of body color variation in the guppy and have suggested that maintenance of male pigmentation polymorphism is caused by negative frequency-dependent selection [5,6], selection maintaining multiple traits within populations [7], and/or gene flow with divergent selection [8]. Although several evolutionary mechanisms have been proposed, none has been conclusively demonstrated. One of the obstacles to elucidating the evolutionary mechanisms is the lack of information on the genetic basis of pigmentation, since the evolutionary responses to selection for the pigmentation depend largely on how the phenotypes are controlled by genes. For example, the selection force for coloration will be influenced according to whether the genes involved in melanogenesis show multiple pleiotropic effects [9].
Male guppies display many types of color pigment on their bodies. In this study, we particularly focused on a candidate gene that contributes to the polymorphism of black pigmentation. Black pigmentation in guppies plays two distinct roles, namely, in nuptial display and in camouflage. Females from populations with a higher proportion of orange coloration tend to have a stronger preference for orange and black [10]. In contrast, natural selection is predicted to operate against conspicuous black spots in habitats with visually hunting predatory fish (i.e., the so-called “high-predation sites”) [11].

Black pigmentation is produced by melanin synthesis, which involves many genes. The coding sequence of one of these genes, melanocortin 1 receptor (MC1R), is reported to contain variations that are associated with melanin pigmentation polymorphism in natural populations in many animals [Reviewed in 2]. Among the pigmentation genes, MC1R plays a crucial role in controlling melanin synthesis [12]. In mammals and birds, high activity of MC1R leads to the synthesis of black eumelanin, whereas low activity leads to reddish pheomelanin [13,14]. In contrast, fish melanophores contain only eumelanin, or an absence of melanin synthesis [13,14]. Thus, in guppies, like other fishes, lower activity of MC1R may lead to an absence of black pigmentation.

In this study, we focused on MC1R as a candidate gene that contributes to the polymorphism of black pigmentation in guppies. The purpose of this study was to determine the complete coding sequence of guppy MC1R, and to examine whether MC1R polymorphisms are present in wild guppy populations in contrasting habitats. We also examined whether sequence variation in MC1R affects black pigmentation by comparing MC1R genotypes and body color phenotypes.

Materials and methods

Determination of the complete coding sequences of guppy MC1R

We determined the coding sequences of MC1R using a wild male guppy that was caught from Okinawa Island in Japan and subsequently reared in our laboratory. Total DNA isolation was performed according to the manufacturer's protocol. cDNA was synthesized from the total RNA using a GeneRacer Kit (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. cDNA was synthesized from the total RNA using TRIzol (Invitrogen, Paisley, UK) according to the manufacturer’s protocol.

In order to determine the 5' and 3' regions, 5' and 3' RACE was performed using the primers GeneRacer 5' Primer, GeneRacer 3' Primer, GeneRacer 5' Nested Primer, GeneRacer 3' Nested Primer (Invitrogen), PrMC1R-F1, PrMC1R-F2, PrMC1R-R1, and PrMC1R-R2 (Table 1). Amplification reactions followed PCR protocols in a volume of 50 μl, using 0.25 μl of the cDNA mixture, 5.0 μl 10× rTaq Buffer, 1 μM dNTP mixture, 0.5 μM MgCl₂, 1 unit rTaq (Takara), and each primer. The amplification conditions were as follows: 2 min at 94°C; then 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1.5 min at 72°C; ending with 15 min at 72°C. Semi-nested PCR was then performed with the PCR products, using the primers GeneRacer 5' Nested Primer, GeneRacer 3' Primer, PrMC1R-F1, PrMC1R-F2, PrMC1R-R1 and PrMC1R-R2 (Table 1). The semi-nested PCR products were separated by 1.5% SeaPlaque GTG Agarose (FMC BioProducts) gel electrophoresis and then gel purified.

Semi-nested PCR products were sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Warrington, UK). The sequencing reactions were carried out using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (ABI) under the following conditions: 1 min at 96°C, then 45 cycles of 30 s at 96°C, 15 s at 50°C, and 4 min at 72°C. The primers used for the initial amplification were subsequently used for sequencing: GeneRacer 5' Nested Primer, GeneRacer 3' Primer, GeneRacer 3' Nested Primer, PrMC1R-F1, PrMC1R-F2, PrMC1R-R1, and PrMC1R-R2 (Table 1). Sequencing reaction products were purified and cleaned by ethanol precipitation. The sequences obtained were edited and aligned using Clustal X software [16].

Determination of MC1R sequences in wild Trinidadian populations

To examine whether MC1R polymorphism is present in wild guppy populations, we used tissue samples from 270 guppies that were collected from five wild populations on the island of Trinidad: Pitch Lake, Lower Aripo River, Upper Aripo River, Lower Guanapo River, and Upper Guanapo River (Figure 1). Because all samples were fixed with 99.5% ethanol before this study, we have no information regarding their natural pigmentation.

Total DNA isolation was performed according to the CTAB protocol [17]. On the basis of the cDNA isolation and purification of DNA, the concentration of the DNA was measured with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The DNA samples were then used for PCR amplification and sequencing. The primers used for PCR amplification and sequencing are listed in Table 1. The PCR amplification conditions were as follows: 2 min at 94°C; then 45 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C; ending with 15 min at 72°C. The PCR products were purified and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (ABI) and an ABI 3130 Genetic Analyzer (Applied Biosystems, Warrington, UK). The sequences obtained were edited and aligned using Clustal X software [16].

Table 1 Primers used for cDNA synthesis, amplification, and sequencing

| Primer   | Sequence (5'-3') |
|----------|------------------|
| PrMc1r-F1 | CAAGAACAGSAATCTTCATTCRCCCATGTA |
| PrMc1r-F2 | CCATCTTTTACGCRCTSCGGTACCACAG |
| PrMc1r-R1 | CTGYGGTARGCGTADATGAGMGGGTC |
| PrMc1r-R2 | AGRAGAAAVWGGCCCGACCAGAG |
| PrMc1r-Fp1 | TCTCCCGGTGGAATGAGAAACTTA |
| PrMc1r-Fp2 | CAGATCCGCATCGGCAAGAGGTTC |
| PrMc1r-Rp1 | CAGCTGCAGCTTATGGCTCATAAT |
| PrMc1r-Rp2 | GGGCCGGCGGAGGTGCAACAGAGG |
sequences determined in this study, we designed primers from the *MC1R* untranslated regions: PrMC1R-Fp1 and PrMC1R-Rp1 (Table 1). Complete coding sequences of *MC1R* were amplified using these primers. Amplification reactions followed PCR protocols in a volume of 50 μl, using a 2.5 μl of the DNA mixture, 5.0 μl 10× rTaq Buffer, 1 μM dNTP mixture, 0.5 μM MgCl₂, 1 unit rTaq (Takara), and each primer. The amplification conditions were as follows: 2 min at 94°C; then 30 cycles of 30 s at 94°C, 30 s at 50°C, and 1.5 min at 72°C; ending with 15 min at 72°C. The PCR products were purified and cleaned by PEG precipitation. Sequences were determined in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems). The sequencing reactions were carried out using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (ABI). Sequencing was performed under the same conditions as described previously. Sequencing was performed using the PCR primers and additional internal primers: PrMC1R-Fp2 and PrMC1R-Rp2 (Table 1). The sequences obtained were edited and aligned using Clustal X software [16].

**Association between MC1R genotypes and phenotypes**

To confirm the association between phenotypes and *MC1R* genotypes, a total of 67 guppies were obtained from four aquarium shops. By choosing guppies that showed distinctly different pigmentation patterns, we ensured that fish from more than two different families were included in the sample obtained from each shop.

We evaluated black pigmentation using two traits: “body blackness,” which is overall body blackness used in crypsis, and “black traits,” which are conspicuous discrete black spots and lines on several parts of the body that are used as sexual signals. Although both male and female guppies display “body blackness,” only the males express “black traits,” which might be a secondary sexual trait in wild populations [3]. We evaluated the two traits independently because these traits might be controlled by different genes. Male-specific ornaments such as black spots and lines (i.e., black traits) are highly heritable between father and son, and thus certain sex-dependent genes (such as those on the Y chromosome) are assumed to affect black traits [3]. On the other hand, individuals of both sexes show whole-body blackness. Thus, we considered that the combinations of genes that affect black pigmentation in black traits and body blackness are different.

The chromatophores of fish are under neuroendocrine control, such that color and patterns can be changed almost instantaneously [18]. Therefore, the estimation of pigmentation was performed by taking photographs before and after anesthesia (Figure 2). We used phenoxethanol at a 1:1500 dilution as an anesthetic. Anesthetized specimens showed melanin pigmentation that was similar to or darker than that in the un-anesthetized specimens (Figure 2). If an individual has melanin pigments and melanophores in its cells, anesthetic treatment cannot suppress melanin aggregation, and consequently, the body color becomes darker.

For body blackness, each guppy was categorized as either grayish or yellowish: “Grayish” guppies have black pigmentation whereas “Yellowish” guppies lack black pigmentation. The existence of black pigmentation is evident when the guppy is anesthetized. Thus, a guppy could be correctly categorized as yellowish when we failed to observe black pigmentation even under anesthetized conditions. If a guppy has melanin pigments, its body color becomes darker under anesthetized conditions. For black traits, each guppy was categorized as either black or none. “Black” guppies had more than one black spot and/or line. “None” guppies had no black spots or lines under both anesthetized and un-anesthetized conditions. Although the categorizations were performed by eye, we were confident that the guppies were correctly assigned since four persons independently scored the guppies and each of these individuals gave the same assessment.

After taking photographs, a part of the caudal fin was clipped from each fish for DNA isolation. All tissues were fixed with 99.5% ethanol and stored at -20°C until dissected. Genotyping was conducted using previously described protocols.

**Association between MC1R genotypes and phenotypes in F₁ and F₂ individuals**

We found two different alleles of the *MC1R* gene (Del and +, see Results). F₁ families were established by crossing individuals homozygous for the two different
MC1R alleles: a Del/Del male obtained from an aquarium shop and a +/+ female derived from a wild population in Okinawa Island. We successfully obtained F1 offspring from two pairs. We raised the two families, and selected four pairs of F1 individuals (i.e., a total of eight F1 individuals; one pair in one family and three pairs in the other family). A total of 39 F2 individuals were obtained by two crosses of the F1 pairs. All F2 individuals were raised in groups of three to five fish per tank until 3 months of age. All the F1 and F2 fishes were maintained at 25°C with a constant photoperiod of 12 h light and 12 h dark, and were fed with Tetramin fish food. Genotyping and an estimation of phenotypes were performed using previously described protocols.

Statistical analysis
Differences in frequencies of the different MC1R alleles among Trinidadian populations were analyzed using a G-test, and multiple comparisons of the allele frequencies of the populations were conducted using a simultaneous test procedure [19]. Deviation from Hardy-Weinberg equilibrium in the genotype frequencies was tested using a randomization test. Differences in frequencies of the different MC1R genotypes between Grayish and Yellowish body blackness and between Black and None black traits were tested using Fisher’s exact test. The excess of heterozygous genotypes in F1 families was also tested using Fisher’s exact test. The significant values were adjusted using sequential Bonferroni procedures. These statistical analyses were performed using the R statistical computing environment [20].

Results
DNA sequencing of guppy MC1R and MC1R polymorphism
We determined the complete coding sequence and partial untranslated regions of the guppy MC1R gene. The complete coding sequence of the guppy MC1R gene contains 969 bp (+), which is predicted to encode a protein of 322 amino acids. The 969-bp guppy MC1R gene has the same length as the MC1R gene of a related species, the platyfish Xiphophorus maculatus (GenBank accession number DQ866828) [21]. To examine whether MC1R polymorphism is present in wild populations, we genotyped 270 individuals sampled from five wild populations in Trinidad. MC1R polymorphism was observed in two of these wild populations: Pitch Lake and Lower Guanapo River (Table 2). We identified two alleles of the guppy MC1R gene differing in length by 6 bp: one allele of 969 bp (+) (GenBank number AB563501); the other of 963 bp (Del) (GenBank number AB563502),
which lacks two amino acids (Ser35 and Ser36) in the extracellular region. We determined the sequences using four indel-flanking primers. When we compared the sequences of homo- and heterozygous individuals, we found those from the heterozygotes to be unreadable. We were unable to detect any further alleles in any of the homozygotes examined in this study. Figure 3 shows an amino acid alignment of the extracellular region and the transmembrane region of \textit{MC1R} that includes the deleted sites. We designated the homozygous genotype of the 963-bp (Del) length morph "Del/Del" and that of the 969-bp (+) length morph "+/+." In the Pitch Lake population, genotype frequencies were in approximate Hardy-Weinberg equilibrium (Table 2). A small non-significant deviation in the \(+/\text{Del}\) genotype was observed in the Lower Guanapo population (Ho = 0.040 and He = 0.113) (randomization test: $p = 0.163$).

Individuals with the 963-bp (Del) allele were mostly found in Pitch Lake. There was a significant difference in the frequency of the 963-bp (Del) allele among the five wild populations sampled ($G = 48.243$, df = 4, $P < 0.0001$). The frequencies of the 963-bp (Del) allele in Pitch Lake (PL) and the Lower Guanapo River (LG) were significantly higher than those in the other three populations (a simultaneous test procedure using the G-test: PL vs. LG, $G = 4.822$, df = 4, $P = 0.306$; PL and LG vs. Upper Aripo River, $G = 20.1681$, df = 4, $P < 0.0004$).

### Table 2

| Populations     | MC1R genotype | Allele frequencies |
|-----------------|---------------|-------------------|
|                 | +/+           | +/Del             | Del/Del           |
| Pitch Lake      | 68            | 20                | 3                 |
| Lower Aripo     | 45            | 0                 | 0                 |
| Upper Aripo     | 36            | 0                 | 0                 |
| Lower Guanapo   | 46            | 2                 | 2                 |
| Upper Guanapo   | 48            | 0                 | 0                 |

The number of individuals shown is based on MC1R genotypes in each population.

Association between MC1R genotypes and black pigmentation

We examined the association between the genotypes of MC1R (+/+, +/Del, and Del/Del) and black pigmentation using the specimens obtained from aquarium shops. The typical black pigmentation of each MC1R genotype is shown in Figure 2. In males, +/+ individuals tended to exhibit a grayish body color and have black traits, whereas the +/Del and Del/Del individuals tended to exhibit a yellowish body color and have no black traits, which suggests that the yellowish pigmentation was highly visible in these individuals because black pigmentation was absent. In females, the +/+ individuals tended to exhibit a grayish body color, whereas the Del/Del individuals tended to exhibit a yellowish color. The +/Del females had phenotypes intermediate between those of +/+ and Del/Del. We found statistically significant differences in the frequencies of different genotypes (+/+:Del/Del, +/+:+/Del, and +/+:+/Del:Del/Del) between different blackness (Grayish vs. Yellowish) and also in the frequencies of different genotypes (+/+:Del/Del, and +/+:+/Del:Del/Del) between different black

| amino-acid sequence | extracellular region | trans membrane region |
|---------------------|----------------------|-----------------------|
| Guppy (969-bp)      | T N S T L G E R S S | P S C V Q I R I P Q E L F |
| Guppy (963-bp)      | - - - - - - - - - - | - - - - - - - - - - - - |
| Platypus            | - - - - - - - - - - | - - - - - - - - - - - - |
| Tilapia             | - - - - - A - - - - | - N W L N - - - - - - |
| Stickleback         | - - - - - A - - - - | - N G - - - - - - - - |
| Tetraodon           | - - I - N - D Q N T L G | - - - - - - - - - - - - |
| Medaka              | - - - - - A - - - - | - N L L G - F - - - - - - |
| Zebrafish           | S - - - - A S D I N V T G I A - - M - - - - - - |
| Chicken             | S N A - A - A G G A W - Q G L D - - N - - - - - - |
| Human               | Q L G L A N Q T G A R - L E V S - S D G - - - - - - |
| Mouse               | H L G L A T N Q S E P W - L Y V S - D G - - - - - - |

Figure 3 Amino acid alignment in the extracellular region and the transmembrane region surrounding the deletion sites in MC1R. The sites of the deletion mutation in guppy MC1R are highlighted. Dashes and cross marks indicate sequence identities and insertion/deletion differences, respectively. These sequences are as follows: Platypus \textit{X. maculatus} (DQ866682), Tilapia \textit{Oreochromis mossambicus} (AJ871147), Stickleback \textit{Gasterosteus aculeatus} (group II contig 348), Tetraodon \textit{Tetraodon nigroviridis} (AY332338), Medaka \textit{Oryzias latipes} (scaffold 45), Zebrafish \textit{Danio rerio} (NM_180970), Chicken Gallus gallus (NM_001031462), Human \textit{Homo sapiens} (AF326275), and Mouse \textit{Mus musculus} (NM_008559).
traits (Black vs. None) (Table 3) (sequential Bonferroni corrections after Fisher’s exact test: P < 0.05).

**Association between MC1R genotypes and black pigmentation in F1 and F2**

We obtained two F2 families (N = 39) from eight F1 individuals established by crossing two different homozygotes. The composition ratio of genotypes in the F2 individuals deviated from 1:2:1, with the +/Del genotype occurring at a frequency slightly greater than expected (overall ratio: 8:24:7). However, this bias was statistically non-significant (Fisher’s exact test: P = 0.2).

All of the F1 individuals had grayish body color and black traits. The association between genotype and black pigmentation in the F2 individuals is shown in Table 4. There was no significant association between MC1R genotype and black trait (Black vs. None) (Fisher’s exact test: P > 0.05) (Table 4).

**Discussion**

This study showed that there was MC1R polymorphism both within and among wild guppy populations. We identified two alleles of the MC1R gene that differed in length by 6 bp: 969 bp (+) and 963 bp (Del). The 969-bp (+) guppy MC1R gene is the same length as the MC1R gene of the platyfish, which is a related species. Thus, the 963-bp (Del) guppy MC1R allele may have lost a 6-bp sequence after divergence between the guppy and the platyfish. Although several studies have reported that polymorphism of color-related genes is maintained in wild fish populations [22-24], this is the first study to detect polymorphism in the color-related genes of guppies in the wild.

### Table 3 Association between MC1R genotype and black pigmentation in guppies obtained from aquarium shops

| MCHR | No. of males | No. of females |
|------|--------------|----------------|
|      | Grayish      | Yellowish      |
|      | Grayish      | Yellowish      |
| +/+  | 8            | 3              | 11            | 3              |
| +/Del| 3            | 14             | 4              | 7              |
| Del/Del | 1     | 7              | 1              | 5              |

(1) For body blackness, “Grayish” guppies have black pigmentation and “Yellowish” guppies have no black pigmentation. (2) For black traits, “Black” and “None” indicate the presence and absence of black spots and lines, respectively.

### Table 4 Association between MC1R genotype and black pigmentation in an F2 population

| MCHR | No. of males | No. of females |
|------|--------------|----------------|
|      | Black        | None           |
|      | Black        | None           |
| +/+  | 8            | 3              | 8              | 5              |
| +/Del| 4            | 13             | 4              | 7              |
| Del/Del | 2     | 6              | 1              | 5              |

(1) For body blackness, “Grayish” guppies have black pigmentation and “Yellowish” guppies have no black pigmentation. (2) For black traits, “Black” and “None” indicate the presence and absence of black spots and lines, respectively.

The present study showed that the frequency of the 963-bp (Del) allele of MC1R was higher in the Pitch Lake population than in the other sampled populations. Unlike guppies in the other populations, those in Pitch Lake are exposed to direct sunlight and high water temperatures, and the background color of the sediment in this habitat is black [25]. Similarly, a uniquely colored killifish (Rivulus hartii) with pink and white hue and two mottled darker patches has been reported in the Pitch Lake [26]. The relatively abundant lighter colored fish with +/Del and Del/Del genotypes may therefore be particularly conspicuous in the Pitch Lake environment. MC1R polymorphisms are associated with coloration in approximately half of the investigated species [9,27-35]. These authors mostly suggest that MC1R polymorphism contributes to the adaptation to divergent background colorations for crypsis. For example, in pocket mice (Chaetodipus intermedius), melanic MC1R mutants are found on dark lava fields, whereas light-colored mutants are found on light-colored rocks, suggesting that these variations in MC1R contribute to adaptive camouflage for predator avoidance on different backgrounds [28-30]. On the other hand, in several bird species, it has been reported that MC1R polymorphism is selected by sexual selection [31]. For example, in arctic skus, females prefer dark males, and also those males having the same color as that of their parents, which contributes to positive assortative mating [32]. At this stage of our study, it is unclear whether the differences in allele frequencies across populations have been caused by random genetic drift and natural selection. To test the hypothesis that the differences are a result of natural or sexual selection, we ideally need to use statistical methods for detecting natural selection.
The present results suggest that there is a significant association between MC1R genotype and black pigmentation in the guppies obtained from aquarium shops (Table 3). These results indicate that the deletion of two amino acids in MC1R disturbed both overall grayish body blackness and black traits. MC1R plays crucial roles in controlling melanin synthesis [12]; therefore, by mutating MC1R, it is possible to increase or decrease melanin synthesis. Thus, deletion of two amino acids in the extracellular regions (Figure 3) might affect the affinity of MC1R for its ligands, i.e., MSH and β-defensin. If the affinity for these ligands is decreased, melanin synthesis is disturbed. In this study, most of the Del/Del individuals exhibited less distinct black pigmentation than +/+ individuals, and thus the deletion of two amino acids in MC1R might inhibit black pigment production. However, three of 14 Del/Del individuals had black pigmentation (Table 3), suggesting that other factors are involved in pigmentation. Furthermore, no association between MC1R genotype and black pigmentation could be found in the investigation using F2 individuals (Table 4). However, in this case, it is possible that, owing to an excess of heterozygous (+/Del) individuals, there was an insufficient number of homozygous F2 offspring to enable detection of an association.

There are two possible explanations to account for the lack of association between genotypes and phenotypes in the F2 population. First, the variously pigmented guppy populations examined. Guppies with a 963-bp (Del) MC1R tend to exhibit less black pigmentation than those with a 969-bp (+) MC1R. Although the results of this study indicate the possibility of an association between MC1R genotype and black pigmentation, results from the study of F2 individuals did not allow us to confirm the link between MC1R genotype and black pigmentation. This suggests that there are other genes that modify the effects of the MC1R gene.

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Authors’ contributions
AT carried out the molecular genetic studies and the laboratory experiments. HY and JY advised on the molecular genetic studies. CvO provided the samples of Trinidad population and advice on. MK supervised the research. AT and MK wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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