〈Brief Note〉

Development of experimental teaching material for high school students: determination of the sex of medaka (*Oryzias latipes*) using DNA

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Summary In genetic experiments designed for high school students, genetic privacy should be respected and human DNA samples should not be used. We developed an experimental teaching protocol comprising three simple steps for rapid determination (90 min) of the sex of medaka (*Oryzias latipes*). First, the tail fins of medaka were homogenized using a disposable homogenizer. Second, the DM-domain gene on the Y chromosome (*DMY*) and DM-related transcription factor 1 gene (*DMRT1*) were amplified by polymerase chain reaction (PCR). We designed primers to amplify the target genes that resulted in shorter fragments (*DMY*: 61 bp, *DMRT1*: 93 bp) and therefore required shorter PCR conditions (approximately 50 min) than those in a previous study (approximately 2 h). Finally, the PCR products were electrophoresed using polyacrylamide gels (5%–20%) for 7 min at 24 W. We chose polyacrylamide gels over agarose gels, because they have a higher resolving power for small DNA fragments (5–500 bp) than agarose gels (100–600,000 bp). We developed a method for teaching genetic techniques to high school students, preparing for higher pharmaceutical education.

Key words: Medaka (*Oryzias latipes*), Teaching material, *DMY*, *DMRT1*

1. Introduction

There are several experimental teaching protocols for high school students[1-5], some of which were developed by us[6-12]. In genetic experiments designed for high school students, genetic privacy should be respected and human DNA samples

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should not be used. We previously developed protocols to determine the sex of medaka (*Oryzias latipes*)[11,12] and adapted them here for use in high school teaching.

The medaka, a teleost fish, is a popular model in biomedical research, particularly for elucidating sex differentiation and determination mechanisms. Sex in medaka is determined by the DM-domain gene on the Y chromosome (*DMY*)[13-15], which is expressed exclusively in XY (male) embryos. The *DMY* gene encodes a putative protein of 267 amino acids, containing a DNA-binding domain called DM. The DM-related transcription factor 1 gene (*DMRT1*)[14] is involved in a specific type of XY sex reversal. Recent studies suggest that medaka *DMY* was derived from *DMRT1* through gene duplication[15,16].

Polymerase chain reaction (PCR) is a method for amplifying target DNAs. Using PCR, copies of very small amounts of DNA are exponentially amplified in numerous cycles of temperature change. PCR is now an important technique used in medical science and molecular biology.

In this study, we developed an experimental teaching protocol comprising three simple steps for the rapid determination (<90 min) of the sex of medaka for high school students.

2. Materials and Methods

Medaka of the orange-red variety was used in this study. The medaka were anesthetized with FA100 (4-allyl-2-methoexphernol; DS Pharma Animal Health, Osaka, Japan) and their tail fins were dissected on ice. Each tail fin was homogenized in 100 μL of phosphate-buffered saline (pH 7.0) using a Bio-masher III (Nippi, Tokyo, Japan), followed by centrifugation at 6200 ×g for 30 s. The target genes, *DMY* and *DMRT1*, were amplified from the homogenized samples by PCR. The amplification conditions were as follows: an initial denaturation step at 98°C for 2 min, followed by 30 cycles at 98°C for 10 s, 55°C for 10 s, and 68°C for 10 s. Four PCR primers were designed to amplify *DMY* (accession no. AB071534), and *DMRT1* (accession no. AY157712) (F1, 5'-CTGACATGAGCAAGGAGAAGCA-3'; F2, 5'-AAACCTGCTGAGCTC-3'; R1, 5'-CAGCGGGAGCACTTGGGCA-3'; and R2, 5'-AAGCGCTTGTGGCCTTTCAGC-3') (Fig. 1). The reaction mixture contained 9 μL of distilled water, 25 μL of 2× PCR buffer (KOD FX neo; TOYOBO, Osaka, Japan), 10 μL of 2 mM dNTPs, 1.5 μL of 10 μM forward primer (F1 or F2), 1.5 μL of 10 μM reverse primer

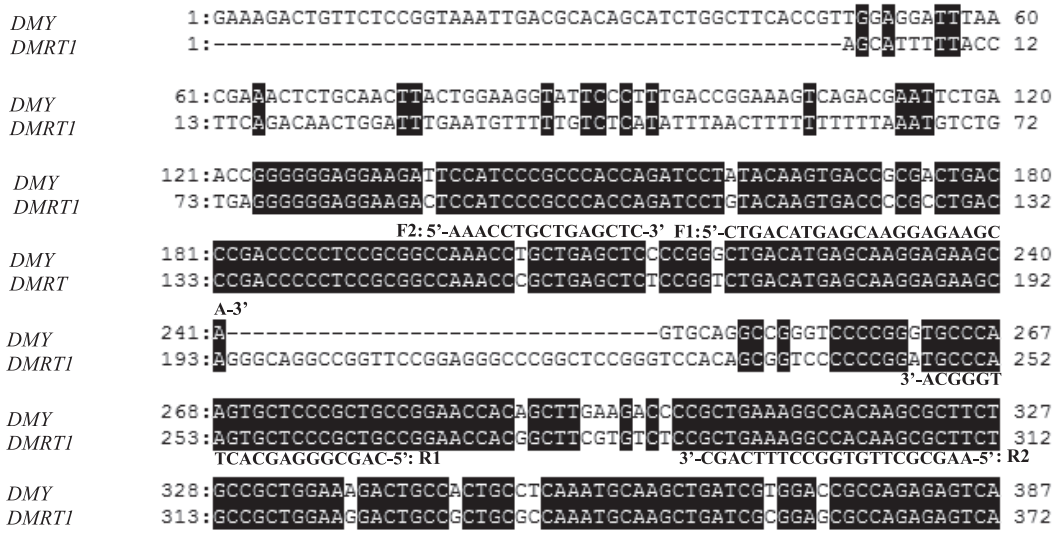

Fig. 1 Alignment of PCR primers with *DMY* and *DMRT1* genes of medaka. Nucleotides that are conserved between the two genes are outlined. Four PCR primers were designed for amplifying the *DMY* and *DMRT1* genes (F1, F2, R1, and R2).

(R1 or R2), 1 μL of KOD FX neo DNA polymerase (1.0 U/μL,) and 2 μL of homogenized sample. The PCR products were electrophoresed using polyacrylamide gels (5%–20% polyacrylamide; C-PAGEL HR, ATTO, Tokyo, Japan) and the Compact PAGE-ACE machine (WSE-1010/25; ATTO, Tokyo, Japan) for 7 min at 24 W, with a 25-bp DNA ladder (FUJIFILM Wako Pure Chemical, Osaka, Japan). The DNA in the gels was stained with SYBR Safe DNA gel stain in 1× TAE (Thermo Fisher Scientific, Tokyo, Japan) for 2 min. Photographs were captured using Printgraph (ATTO, Tokyo, Japan). The PCR products of *DMY* and *DMRT1* were then purified and cloned into the PTZ19R vector (Thermo Fisher Scientific). After the transformation of these recombinants into *Escherichia coli* XLI-blue strain (NIPPON GENE, Tokyo, Japan) using the calcium chloride method, phasmid DNA was recovered and nucleotide sequences were determined using the Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific).

3. Results and Discussion

The medaka has an XX/XY sex-determination system[13-15]. The Y-linked sex-determination gene *DMY* is a duplicate of the autosomal gene *DMRT1*[15,16]. As the nucleotide sequence similarity between *DMY* and *DMRT1* in medaka is very high, several PCR primers can be used to amplify both genes. To distinguish *DMY* and *DMRT1*, we designed primers of different fragment sizes. Male medaka possesses *DMY* and *DMRT1*, while the female medaka only possesses *DMRT1*. By PCR amplification of these genes with the designed primers, male and female medaka can be distinguished.

Primers F1, F2, R1, and R2 were designed to amplify both *DMY* and *DMRT* (Fig. 1). The PCR products resulting from the four possible primer combinations (F1 and R1, F1 and R2, F2 and R1, and F2 and R2) were estimated to be 61, 106, 82, and 127 bp, respectively, for *DMY*. *DMRT1* was estimated to be 93, 138, 114, and 159 bp, respectively. In lanes 1 and 2, containing PCR products obtained using primers (F2 and R2), *DMY* (127 bp) was unclear and *DMRT1* (159 bp) formed a weak band. In lanes 3 and 4 containing PCR products obtained using primers F2 and R1, *DMY* (82 bp) formed a weak band or was unclear, and *DMRT1* (114 bp) formed a weak band; nonspecific gene bands (approximately 140, 150, 160, 240, 270 and 300 bp) were also found. In lanes 5 and 6 containing PCR products obtained using primers F1 and R2, *DMY* (106 bp) and *DMRT1* (138 bp) were clearly identified. In lanes 7 and 8 containing PCR products obtained using primers F1 and R1, *DMY* (61 bp) and *DMRT1* (93 bp) were clearly identified (Fig. 2). Teaching materials for understanding DNA in high school should be simple and rapid. The experiment should preferably be completed within 100 min (a 2-h lesson). Short DNA fragments were amplified more rapidly than long fragments because the annealing time required depends on the size of the amplified DNA, with shorter annealing times for shorter DNA fragments. Therefore, the F1 and R1 primer combination was optimal because of the short size of the fragments produced from *DMY* (61 bp) and *DMRT1* (93 bp). The PCR products were electrophoresed by polyacrylamide gel electrophoresis (PAGE) for 7 min at 24 W. We chose polyacrylamide gels, over agarose gels, because polyacrylamide gels have a higher resolving power for small fragments of DNA (5–500 bp) than agarose gels (100–600,000 bp). The sex of young medaka is not distinguishable based on its appearance. The *DMY* fragment was 61 bp and the *DMRT1* fragment was 93 bp for male medaka (Fig. 3, lanes 1, 3, and 5). The *DMRT1* fragment was 93 bp for females (Fig. 3, lanes 2, 4, and 6). Therefore, the samples in lanes 1, 3, and 5 were estimated to be from young male medaka fish. The samples in lanes 2, 4, and 6 were estimated to be from young female medaka fish. The findings were confirmed by observing these fish in the adult stage. Thus, this method can be used to accurately identify the sex of young medaka.

In genetic experiments designed for high school students, genetic privacy should be respected and human DNA should not be used. We developed a protocol to rapidly determine the sex of medaka (*O.*

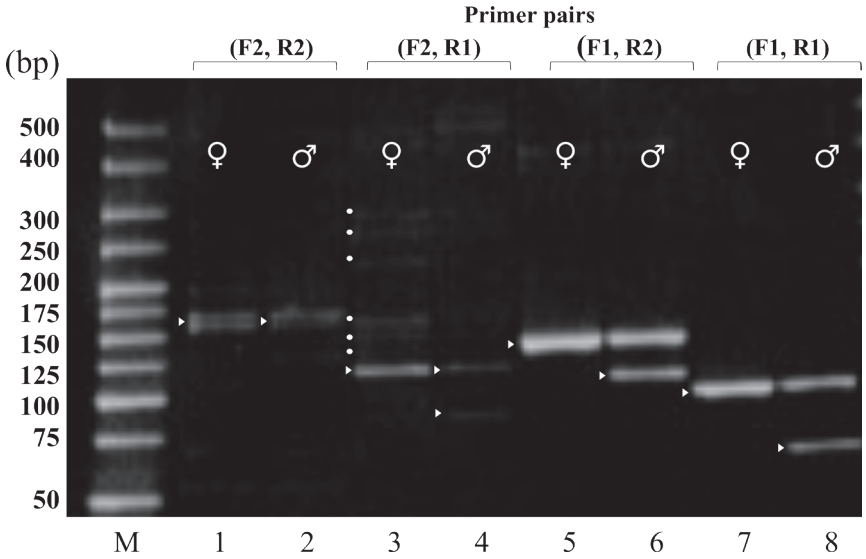

Fig. 2 Polyacrylamide gel electrophoresis patterns produced using different primer pairs to amplify *DMY* and *DMRT1* in medaka. The arrows(▷) indicate PCR products of target genes. The circles (○)indicate those of nonspecific genes.

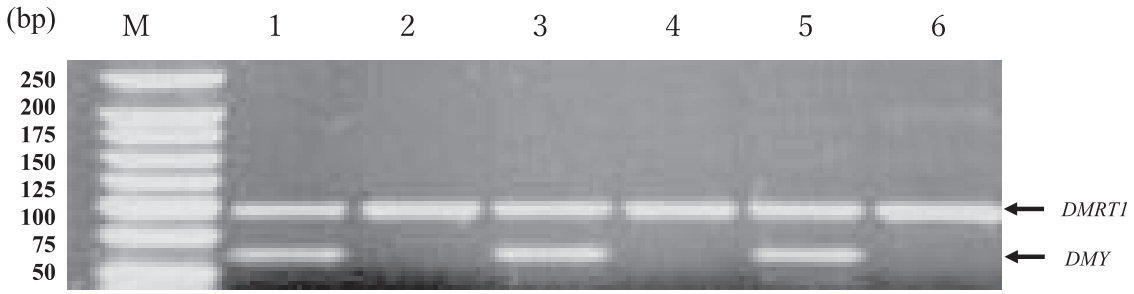

Fig. 3 Identification of genetic sex of young medaka using the F1 and R1 primers to amplify *DMY* and *DMRT1*. Only males carry *DMY*. Lane M contains a 25-bp DNA ladder, lanes 1, 3, and 5 contain samples from male medaka, and lanes 2, 4, and 6 contain samples from female medaka.

latipes). The primers (F1 and R1) were designed to amplify fragments in a shorter time (approximately 50 min) than previous methods (approximately 2 h)[12-13]. This method is simple and rapid and can be completed within 90 min because the amplified PCR products are shorter.

In conclusion, we have developed an experimental teaching material for high school students. This method is easy, simple, and rapid (<90 min). This method can be applied for effective teaching in high school lessons because the chapters about DNA and RNA are included in the model core curriculum for pharmacy education[17]—chapters C6(2): fundamentals of biomolecules and C6(4): fundamentals of genetics. The model core curriculum for pharmacy education plays a major role in ensuring that pools of pharmacists and pharmaceutical researchers are available by improving the quality of pharmacy education offered and meeting social responsibilities to uphold the highest standards for their qualification. This developed method is an important analytical technique for pharmacists because they need to correctly understand case report forms containing the treatment of DNA and RNA. The principle of this study relates to the chapters, *naturally occurring polymers*, in chemistry and *expression of genetic information*, in biology for high school.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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Statement on the use of animals

The use of animals in this study complied with the animal ethics guidelines produced by Josai International University.

References

1. Asai R, Nakasone A, and Kayama A: Development of the teaching materials of “child care” for senior high school student (I) [Jpn]. Journal of Ryukyus University of Education, 7: 93-102, 1999.
2. Sasaki Y and Nagumo S: A study of plant DNA polymorphism experiments for students [Jpn]. Yakugaku Zasshi, 125: 205-211, 2005.
3. Hayashida M, Iwao-Koizumi K, Murata S, and Kinoshita K: Single-tube genotyping from a human hair root by direct PCR. Anal Sci, 25: 1487-1489, 2009.
4. Hayashida M, Iwao-Koizumi K, Murata S, Yokoyama A, and Kinoshita K: Genotyping of polymorphisms in alcohol and aldehyde dehydrogenase genes by direct application of PCR-RFLP on dried blood without DNA extraction. Anal Sci, 26: 503-505, 2010.
5. Komatsu E, Komatsu J, and Morita M: An analysis of high school students’ consciousness of practical learning in home economics for development of dyeing teaching material [Jpn]. J Hokakaido Univ of Educ, 2: 225-231, 2014.
6. Okamoto M and Nishiguchi Y: Rapid measurement of caffeine in green tea with a UV/vis spectrometer. Med Biol, 156: 326-3, 2011.
7. Nishiguchi Y, Kitamura K, Goromaru-Shinkai M et al.: Improvement of cell fusion from two types of protoplast, the leaf from petunia (*Petunia hybrida*) and petal from surfinia (*Petunia hybrida*) as teaching material in science education. Med Biol, 157: 22-26, 2013.
8. Goromaru-Shinkai M, Nakazawa K, Shimizu M Kuroda J, Nishiguchi Y, Nakahama T, and Akimoto Y: A simple experimental teaching material for radiation with educative effect [Jpn]. Toho Liberal Arts Review, 47: 1-9, 2015.
9. Nishiguchi Y, Goromaru-Shinkai M, Kuroda J, Kikuchi S, and Ihara H: Estimation of protein, total polyphenol, chlorogenic acid, caffeine, and caffeic acid contents in Indonesian palm civet coffee (*Kopi Luwak*). Int J Anal Bio-Sci, 5: 53-56, 2017.
10. Goromaru-Shinkai M, Kuroda J, Nakzawa K, Narusuye K, Nishiguchi Y, and Ichiva H: Effect of cigarette extract against vitamin C contents of broccoli sprout. Int J Anal Bio-Sci 6: 1-6, 2018.
11. Nishiguchi Y and Goromaru-Shinkai M: Identification of genetic sex in young Japanese medaka, *Oryzias latipes*: development of teaching materials for understanding DNA determination and human DNA polymorphisms [Jpn]. Med Biol, 153: 493-498, 2009.
12. Nishiguchi Y and Goromaru-Shinkai M: Simple, rapid identification genetic sex in young Japanese medaka, *Oryzias latipes* [Jpn]. Med Biol, 155: 78-82, 2011.
13. Kobayashi T, Matsuda M, Kajiura-Kobayashi H et al.: Two DM domain genes, *DMY* and *DMRT1*, involved in testicular differentiation and development in the medaka, *Oryzias latipes*. Dev Dyn, 231: 518-526, 2004.
14. Matsuda M, Shinomiya A, Kinoshita M et al.: *DMY* gene induces male development in genetically female (XX) medaka fish. Proc Natl Acad Sci USA, 104: 3865-3870, 2007.
15. Nanda I, Kondo M, Hornung U et al.: A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. Proc Natl Acad Sci USA, 99: 11778-11783, 2002.
16. Matsuda M, Nagahama Y, and Shinomiya A: *DMY* is a Y-specific DM domain gene required for male development in the medaka fish. Nature, 417: 559-563, 2002.
17. Ozawa K, Ichikawa A, Furusawa Y et al.: Model core curriculum for pharmacy education, 2015 version, 24: 14-15, 2018.