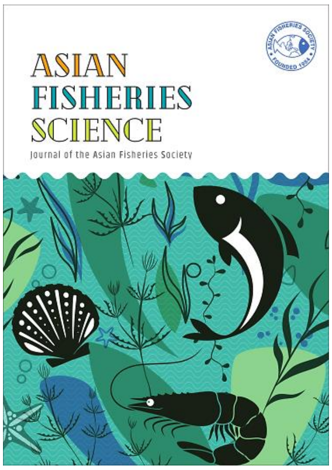


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ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2021.34.1.006

# Generation of Transgenic Medaka *Oryzias curvinotus* (Nichols & Pope, 1927) Carrying a Cyan Fluorescent Protein Gene Driven by Alpha Actin Promoter

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## Abstract

The study aimed to produce fluorescent protein transgenic medaka *Oryzias curvinotus* (Nichols & Pope, 1927) as a novel strain of ornamental fish. These fish were produced by transferring a plasmid consisting of a fluorescent reporter gene and a strong promoter into one-cell stage embryos. For this purpose, myosin light chain 2, but not other promoters, was mainly used. The study also evaluated the stability of the transgenic medaka germline acquiring vivid fluorescent phenotypes via the transgenesis of the cyan fluorescent protein (CFP) gene under the control of *O. curvinotus* skeletal alpha-actin (OCacta) promoter. The pOCacta-CFP plasmid, containing a OCacta promoter and CFP reporter gene, was transferred into the one-cell stage of *O. curvinotus* embryos by a microinjection technique. As a result, 36 of 1386 microinjected *O. curvinotus* embryos exhibited CFP signals in their trunks. The expressed CFP signals in *O. curvinotus* embryos and adults were detected under a microscope using a green fluorescent protein (GFP) filter (450–490 nm wavelength), and blue LED light (400–450 nm wavelength). Five *O. curvinotus* founders showing clear CFP signals were selected and crossed with non-transgenic counterparts to produce subsequent generations. Among strains, the frequency of germline transmission from founder to F1 was highly variable. Only two of the five founders successfully pass the transgene to the F1 generation. At present, the progeny of subsequent generations is being produced and tested for the expression of CFP signals, and therefore, stable lines are ongoing.

**Keywords:** medaka, CFP, LED, *Oryzias curvinotus*, alpha-actin

## Introduction

Transgenesis is described as a transmission process of foreign DNA into the genome of organisms. In 1980, the first genetically modified organism was created by injecting DNA into the nucleus of an egg of the one-cell stage mouse embryo, which later led to extensive genetic manipulation studies (Gordon et al., 1980). Numerous transgenic fish studies have been conducted in the mid-1980s (Maclean and Talwar, 1984; Zhu et al., 1985).

The typical transgenic structure includes three key elements of DNA: a promoter, an indicator gene, and a transcription terminator. The most studied reporter genes are chloramphenicol acetyltransferase (CAT), β-galactosidase (β-gal), luciferase, and green fluorescent protein (GFP) controlled by a functional promoter structure. Zebrafish *Danio rerio* (Hamilton, 1822) was the first fish model to receive the GFP gene and reported as the first transgenic fluorescent fish in 1995 (Amsterdam et al., 1995; Peters et al., 1995). In 2003, Yorktown Technology marketed red fluorescent transgenic zebrafish strains under the name "GloFish." In 2020, they also had fluorescent tetra *Gymnocorymbus ternetzi* (Boulenger, 1895) in six colours (red, green, orange, purple, pink, and blue) and tiger barbs *Puntius tetrazona* (Bleeker, 1855) in red and green, as well as rainbow shark *Epalzeorhynchos frenatus* (Fowler, 1934) in green, orange, blue, and purple (Debode et al., 2020; www.glofish.com).

Actin is a cytoskeletal protein manifested as other isoforms in vertebrates (Vandekerckhove et al., 1986). Since it is an important component of muscle contraction, cell motility, cytoskeletal function, cell division, intracellular transport, and cell differentiation in eukaryotic cells (Herman1993), its characteristics were discovered in embryo stages of zebrafish *D. rerio* (Higashijima et al., 1997; Hsiao et al., 2001; Bertola et al., 2008), medaka *Oryzias latipes* (Temminck & Schlegel, 1846) (Chou et al., 2001), and two rattail fish *Coryphaenoides acrolepis* (Bean, 1884) and *Coryphaenoides cinereus* (Gilbert, 1896) (Morita, 2000) models. The tissue distribution of these actin genes in the late stage of fish embryos, however, has not been investigated.

In this study, transgenic cyan fluorescent *O. curvinotus* were successfully produced and characterised by microinjecting an alpha-actin promoter-driven recombinant vector containing a CFP gene. This transgenic fish exhibited vivid cyan fluorescence until the F2 generation. Because medaka *O. curvinotus* is native to Vietnam, it was chosen as the experimental model. The successful laboratory breeding of *O. curvinotus* eliminates the threat of its extinction due to urbanisation.

# Materials and Methods

## Maintenance and breeding of medaka Oryzias curvinotus

The medaka *O. curvinotus* (2–3 cm length) were collected from watery areas, including streams and paddy fields in Chap Le commune, Vinh Linh District, Quang Tri Province, Vietnam. The fish were effectively domesticated and reproduced in a laboratory at the Biotechnology Centre of Ho Chi Minh City. Five hundred *O. curvinotus* were reared in 70 L (60 cm × 40 cm × 35 cm) glass tanks. Commercial food (NRD2/3, INVE) and nauplius of artemia were fed twice daily at 8 am and 3 pm, respectively. Thirty percent of the water was exchanged every three days. The water temperature in the fish tank was maintained at 25 ± 1 °C.

The male and female could be distinguished after maintaining the fish in the laboratory for a month. The fish were transferred to 15 L (35 cm × 20 cm × 23 cm) glass tanks for breeding. A mesh screen separated ten males and 30 females in the same tank. The spawning tanks were subjected to 14 h of light and 10 h of darkness. Approximately 10 % of the water was changed daily. To stimulate spawning, the light was switched on and the mesh screen separating the male and female was removed.

After spawning, the eggs were collected using a small fish net (10 × 10 cm; mesh size 0.1 cm). Embryonic stages were visualised using Carl Zeiss microscope. Adult *O. curvinotus* were photographed using a Canon IXUS 210 digital camera.

All the fish experiments were conducted under the regulations of "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms" and "Cartagena Protocol on Biosafety". The study was approved in January 2013 with decision No. 02/QĐ-CNSH of the Biotechnology Centre of Ho Chi Minh City. All experiments were also performed according to "the Guide for the Care and Use of Laboratory Animals at the Biotechnology Centre of Ho Chi Minh City".

## DNA sequencing and phylogenetic analysis

The total genomic DNA was extracted from the caudal fin of the *O. curvinotus* using QIAGEN DNeasy ® Blood and Tissue Kits (Qiagen, Germany).

To obtain the *O. curvinotus* 16S rNRA sequence, the polymerase chain reaction (PCR) was applied to the total genomic DNA by using two primers (F-OC16S: 5'-AAAGATCTATGAAGGCCTGTATGAATGG-3' and R-OC16S: 5'-AAAGATCTAGGTCGTAAACCCTCTTGTC-3') designed from the sequences of *O. curvinotus* mitochondrial gene for 16S rRNA (GenBank: AB188720.1; AB511366.1; and AB188719.1). The sequences of the amplified fragments were analysed using an ABI 3130 DNA Sequencer (Applied Biosystems, USA).

To confirm the identification of medaka *O. curvinotus* from Quang Tri Province of Vietnam, phylogenetic analysis (MEGA version 6) using mitochondrial 16S ribosomal RNA (16S rRNA) gene sequences was performed.

## Experimental Oryzias curvinotus alpha-actin promoter isolation

To identify *O. curvinotus* alpha-actin promoter sequences, two medaka *Oryzias dancena* (Hamilton, 1822) (JQ905608.1) and *O. latipes* (AB015886) alpha-actin promoter sequences were aligned. The coding promoter region of *O. curvinotus* alpha-actin (OCacta) was amplified using PCR with two primers F-OCacta: 5'-GCTAGCCATATGGACTCCTCCCTWCTTTGAG-3' (*Nhe*I site underlined) and R-OCacta: 5'-GGATCCACCGGTAACCAGTGCTGCTGCTGAAC-3' (*Age*I sites underlined). The insert OCacta fragment was digested by *Nhe*I and *Age*I enzyme and subsequently cloned into the *Nhe*I/*Age*I sites of pJET1.2/blunt to generate the pJET1.2-OCacta plasmid. Positive OCacta clones were sequenced with two primers SO501–pJET1.2:5'- (CGACTCACTATAGGGAGAGCGGC)-3', and SO511–pJET1.2: 5'- (AAGAACATCGATTTTCCATGGCAG)-3' to verify the insert.

## Construction of recombinant pOCacta-CFP plasmid

To construct an OCacta expression plasmid driven downstream with CFP, pJET1.2-OCacta and pAmcyan-C1 plasmids were digested with *Nhe*I and *Age*I restriction enzymes. The linearised plasmid pAmcyan-C1 was then ligated to the *Nhe*I/*Age*I OCacta fragment encoding CFP (Amcyan) to generate the pOCacta-CFP expression plasmid.

## *Microinjection and detection of CFP expression*

The pOCacta-CFP plasmid was purified with ISOLATE II Plasmid Mini Kit (Bioline, England). The purified pOCacta-CFP plasmid was adjusted to 50 µg.mL$^{-1}$ in 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and microinjected into one-cell stage *O. curvinotus* embryos by following Vu et al., 2014. The average amount of DNA introduced to each embryo was approximately 50 pg, as suggested by Cho et al. (2011). Microinjected embryos were kept in the incubator at 28 °C. The dead and unfertilised embryos were counted and removed.

CFP expression embryos [from 1- to 14-day post fertilisation (dpf)] were observed using Nikon Eclipse TS100 fluorescence microscope with B-2A filter (excitation filter wavelengths: 450–490 nm; emission filter wavelength: 520 nm). The image of CFP-positive embryos was photographed using Nikon Eclipse TS100 digital camera. The *O. curvinotus* embryos that expressed CFP in the skeletal muscle were selected and maintained for adult growth.

# Results

## *Identification of* Oryzias curvinotus

The mitochondrial 16S rRNA gene is a conserved mitochondrial genome sequence that allows for rapid species identification using PCR technique. As shown in Figure 1A, the 409 bp PCR product of *O. curvinotus* 16S rRNA fragment was amplified. The nucleotide sequence of *O. curvinotus* 16S rRNA gene was deposited in GenBank under accession number KM491555.1.

In the phylogenetic analysis based on 16S rRNA gene sequences, *O. curvinotus* from Hong Kong (GenBank: AB511366.1; AB188719) and Hanoi (GenBank: AB188720.1) formed a single clade along with Quang Tri Province of Vietnam (GenBank: KM491555.1) (Fig. 1B). For this clade, the bootstrap value was 99 %. These results suggest that the fish found at freshwater areas in Quang Tri Province of Vietnam is medaka *O. curvinotus*.

## *Identification and cloning of OCacta promoter*

To isolate OCacta promoter sequences, a 1110 bp DNA fragment (Fig. 2A) was amplified by PCR. The OCacta promoter sequence was deposited in GenBank with the accession number: KF764703. The recombinant plasmid pOCacta-CFP by ligating a 1110 bp *NheI/AgeI* OCacta promoter to a modified CFP (Amcyan1) reporter gene (Fig. 2B).

## *Generation and screening of transgenic* Oryzias curvinotus *founders*

Out of 1386 microinjected embryos, 624 embryos (45 %) hatched, and 63 hatched embryos (10.1 %) had CFP signals under the fluorescence microscope (Table 1; Fig. 3).

*Oryzias curvinotus* pOCacta-CFP microinjected embryos began to express CFP signalling in the tail and head bud region of 4-dpf (Figs. 3Aa, 3Aa1). The expression level of CFP was highest from 8-dpf (Fig. 3Ad1). Figures 3Aa1, 3Ab1, 3Ac1, 3Ad1, 3Ae1, and 3Af1 show some examples of different mosaic distribution of microinjected DNA. Because of the irregular DNA distribution during microinjection, most transgenic fish were mosaic (Figueiredo et al., 2007).

## *Generation of fluorescent* Oryzias curvinotus *transgenic germline*

Only five founders with clear CFP signals were further crossbred with wild-type fish to analyse the germline transmission. Of the five transgenic founders identified, two founders transmitted the transgene to the F1 generation. The first founder transmitted 51 % (153 per 300 embryos had CFP signals), while the second founder transmitted 8 % (12 per 150 embryos had CFP signals). The transmission rate was less than 50 % due to the mosaic distribution of the transgene (Nam et al., 1999; Cho et al., 2011; Lee et al., 2013).

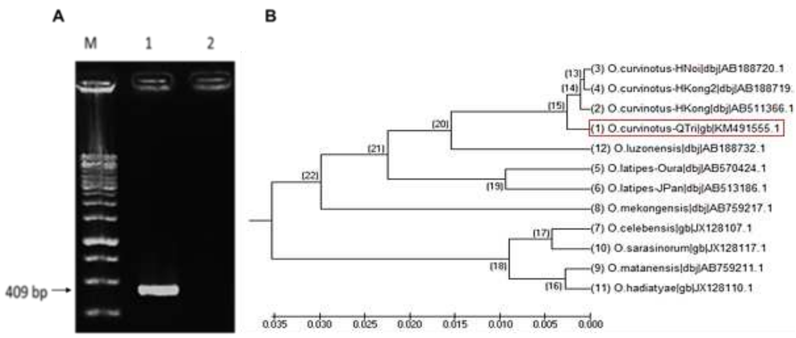


Fig. 1. Medaka *Oryzias spp.* phylogenetic relationship based on 16S rRNA gene sequence. A: PCR product of 16S 16S rRNA gene. B: The phylogenetic tree was produced using MEGA version 6.

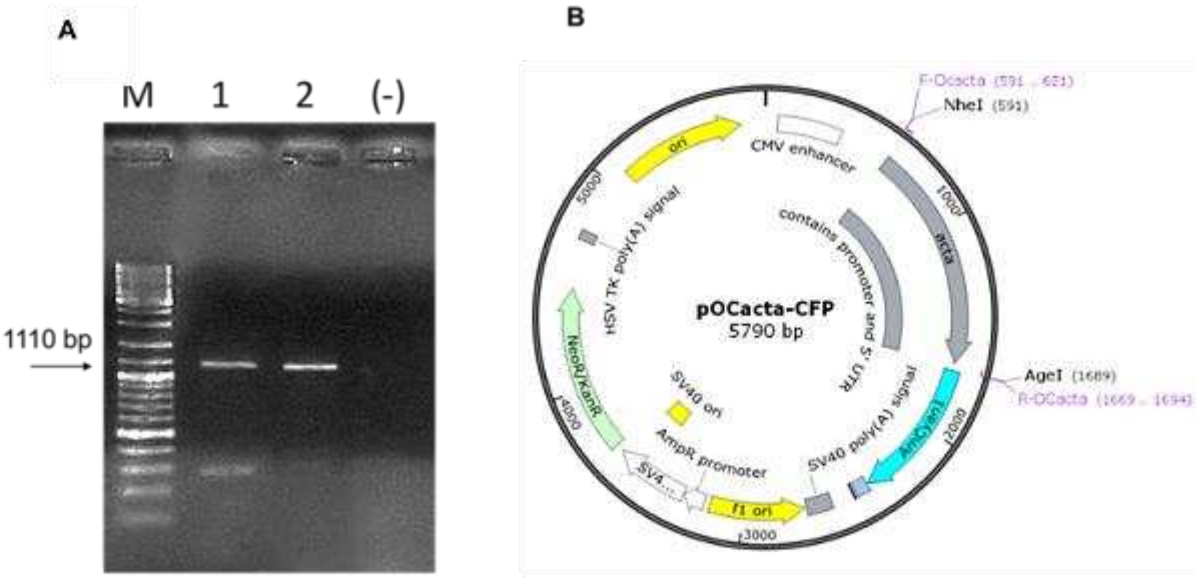


Fig. 2. Generation of recombinant pOCacta-CFP plasmid. A: PCR products of OCacta promoter. M: DNA ladder (10 kb). 1; 2: PCR products of OCacta promoter (1110 bp); (-): negative. B: pOCacta-CFP recombinant plasmid was generated by the ligation of OCacta promoter to the pAmCyan1-C1 vector with the *NheI* and *AgeI* restriction enzyme.

Table 1: Results of microinjection of plasmid pOCacta-CFP into one-cell stage embryos.

| Recombinant plasmid | Number of microinjected embryos | Number of hatched embryos | Number of expressed CFP embryos | Number of expressed CFP fish at 2 months after fertilisation (Founders) |
|---|---|---|---|---|
| pOCacta-CFP | 1386 | 624 (45 %) | 63 (10.1 %) | 36 |

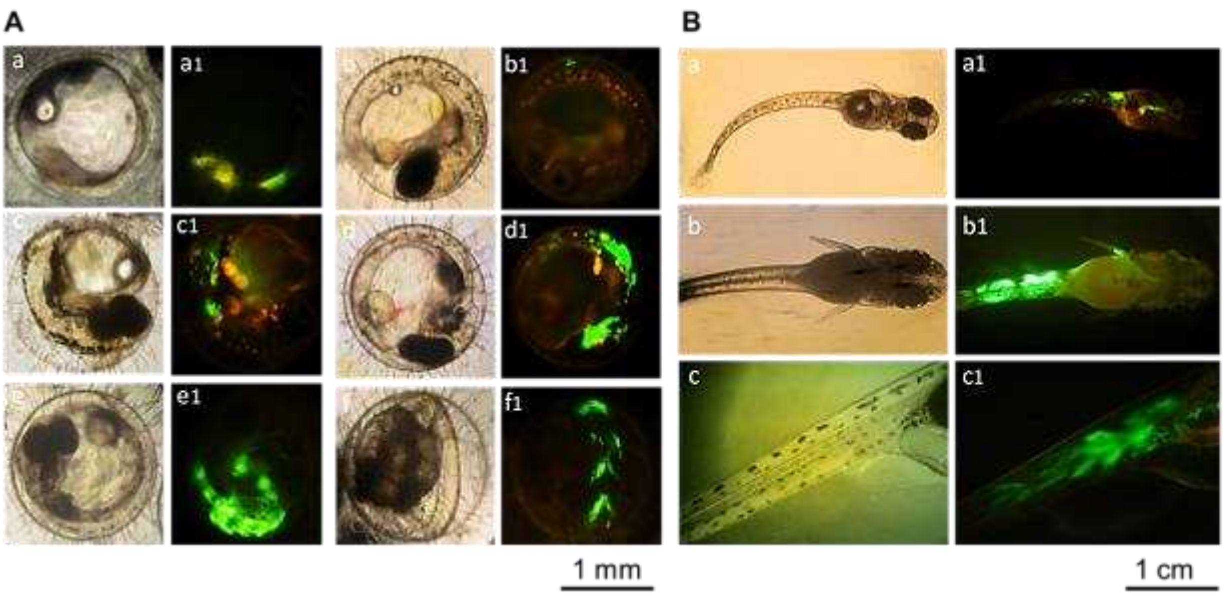


Fig. 3. Expression of founder (F0) *Oryzias curvinotus* embryos after microinjected pOCacta-CFP. A: CFP expression in *O. curvinotus* embryos at 4-dpf (Aa; Aa1), 8-dpf (Ab, Ab1; Ac, Ac1; Ad, Ad1), 10 dpf (Ae, Ae1; Af1). Aa, Ab, Ac, Ad, Ae, and Af: medaka embryos were observed under normal light, while Aa1, Ab1, Ac1, Ad1, Ae1, and Af1 observed under the B-2A green filter (450–490 nm excitation filter: 520 nm filter). Aa1: showed the first CFP expression after 4-dpf microinjection. Ab1; Ac1; Ad1; Ae1; and Af1 showed different embryonic phenotypes. Scale bar: 1 mm. B: *O. curvinotus* larvae CFP expression. Ba; Bb; Bc showed 1-day post-hatching (dph) transgenic fish with normal light. Ba1; Bb1; and Bc1 observed under green filter B-2A. Scale bar: 1 cm.

The F1 generation with clear CFP expression (Fig. 4Bc) was selected under the blue LED light and further crossbred with wild-type fish to produce stable transgenic F2 germline. As a result, more than 1000 F2 germline were produced. Each individual had a uniform fluorescence colour across the body that glowed brightly under the blue LED light (Fig. 5).

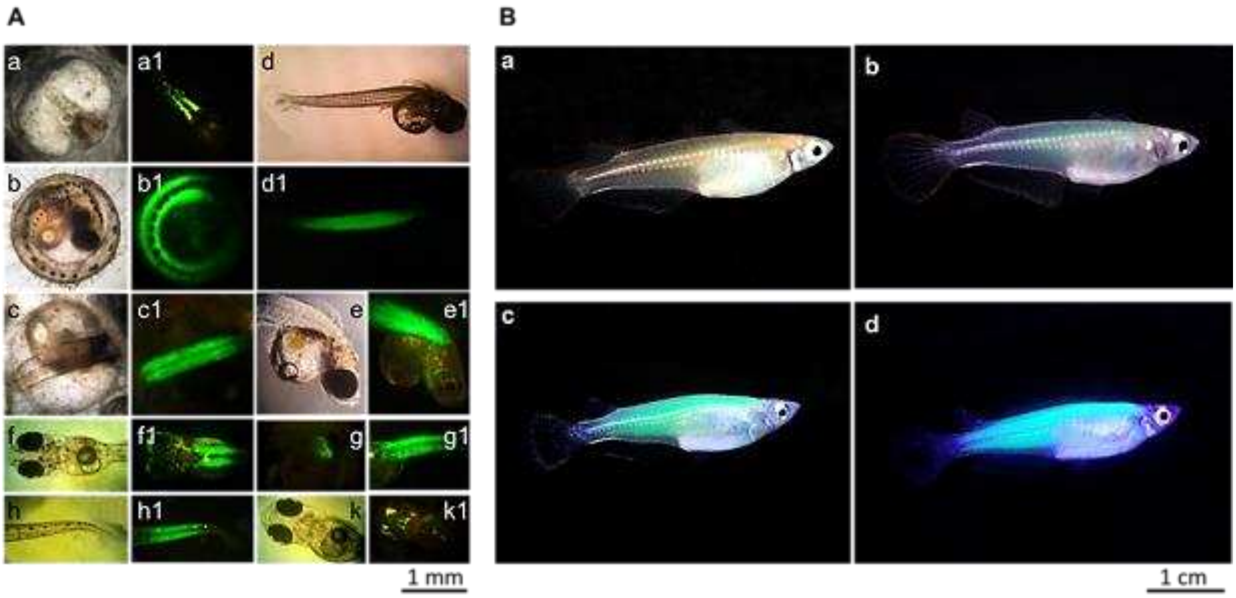


Fig. 4. The fluorescent expression of genetically modified *Oryzias curvinotus* in F1 generation. Aa1: expression of CFP in 4-dpf embryos. Ab1 and Ac1: horizontal or vertical view of 8-day medaka embryos. Ad1: CFP expression fry 1-day after hatching. Ae1, Af1, Ag1, Ah1, and Ak1 CFP-appearance of Ad1-fish viewed horizontally and vertically at the head; the torso, the tail viewed vertically from the top down, and the head area of the lower abdomen, respectively. Ba: An F1-non-transgenic *O. curvinotus*. Bb; Bc; and Bd showed fluorescent expression of F1 transgenic *O. curvinotus* under normal light, normal light and blue LED, and blue LED.

Fig. 5. Aquarium with cyan and red fluorescent *Oryzias curvinotus* transgenic fish (F2 generation) under blue LED.

## Discussion

In the present study, the transgenic medaka *O. curvinotus* containing a CFP reporter gene driven by an alpha-actin promoter was first produced and characterised in Vietnam. The aquarium industry has focused on artificial breeding of high-quality species and the reproduction of rare fish in danger of extinction. Technological advances, including transgenic methods, have been used to generate different fish species for customers.

The pAmcyan1-C1 vector, which encodes an optimised cyan fluorescent protein variant from wild *Anemonia majano* (Carlgren, 1900), was used in this study. The coding sequence of Amcyan1 contains a series of silent nucleotide pairs that correspond to the best human codon adaptation and expression in mammalian cells. To improve Amcyan emission properties (maximum excitation at 458 nm; maximum emission at 489 nm), two amino acid changes were made (replacing Asn-34 with Ser, and Lys-68 with Met) (pAmCyan1-C1 Vector Information, Clontech).

Many promoters have been used in genetically modified fish studies. The Krt8 promoter (cytokeratin gene) was highly expressed in epithelial cells and remained stable from the embryonic to the adult stage (Gong et al., 2002). Meanwhile, the CMV promoter exerted a strong influence on early embryonic tissue, but it vanished after 3 days of development (Chou et al., 2001). Under the β-actin promoter, transgenic medaka expressed GFP signals throughout the whole body. This transgene was transmitted to at least three generations (Chou et al., 2001; Cho et al., 2011). Zeng et al. (2005) demonstrated that the zebrafish *D. rerio* mylz2 (Myosin light chain 2 polypeptide) promoter could be used to generate the fluorescent medaka *O. latipes* embryo. In vice versa, medaka *O. latipes* mylz2 promoter resulted in vivid expression of GFP in zebrafish *D. rerio* embryos. The findings of this study are consistent with those of Chou et al. (2001), who found that fluorescent expression induced by the alpha-actin promoter was abundant in muscle tissue (Figs. 4 and 5), and that the intensity of expression depended on the embryonic stage.

The assumed transgene transmission rate for *O. curvinotus* founders (Table 1) was similar to that for *O. latipes* (Tsai, 2003) and *O. dancena* (Vu et al., 2014). Noticeably, the effect of the transgene depends on the amount of DNA, organisms, the size of embryos, microinjected buffers, the concentration of DNA (Kinoshita, 1995) and type (circular or linear DNA) of the plasmid (Chourrout et al., 1986; Marini and Benbow, 1991). In addition, the survival rate of microinjected embryos varies depending on the species (For example: 16 % in zebrafish (Stuart et al., 1988) and 85 % in salmon (*Oncorhynchus kisutch* (Walbaum, 1792)) (Devlin et al., 1995) as well as the skill of the microinjection technique (Kinoshita, 1995).

Medaka *O. curvinotus* is a small egg-laying teleost, (3–4 cm total length), found in freshwater rice fields and small canals in Quang Tri Province of Vietnam. However, due to urbanisation and its diminishing habitat the fish is endangered (Kottelat, 2001; Parenti, 2012). In this study, successful laboratory breeding of medaka *O. curvinotus* resulted in the production of new fluorescent model fish species.

## Conclusion

The study confirmed the presence of medaka *Oryzias curvinotus* in Quang Tri Province of Vietnam. This fish is considered as an endangered species due to the loss of its habitat mainly due to urbanisation. The present study produced the fluorescent protein transgenic medaka strains as an ornamental fish. These fish were produced by transferring a plasmid consisting of a fluorescent reporter gene and a strong promoter into one-cell stage embryos. The transgenic lines of medaka *O. curvinotus* expressing cyan fluorescent protein driven by the alpha-actin promoter were produced and characterised.


## Acknowledgements

Vy Nguyen Hoang Thuy and Trung Mai Nguyen Thanh contributed equally to this work. We would like to thank Professor Zhiyuan Gong at Singapore National University for helping with the pAmcyan 1-C1 vector. Mr Le Van Hau, Mr Tran Pham Vu Linh, and Miss Nguyen Thi Diem Phuong helped maintain transgenic fish. Dr Ngo Huynh Phuong Thao, and Mrs Vu Thi Thanh Huong commented on the manuscript. This study was funded by Biotechnology Centre of Ho Chi Minh City under grant number TS01/13-15 and the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 108.06-2020.19.


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