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Cystic Fibrosis Transmembrane Conductance Regulator (CFTR): An Apical Marker Protein of Ionocytes for Identifying Hypo-osmoregulation in Gills of the Euryhaline Medaka *Oryzias dancena*

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Chao-Kai Kang, Shu-Chuan Tsai, Shang-Tao Lin, Wan-Yu Lo, Tsung-Han Lee, and Pung-Pung Hwang (2012) Cystic fibrosis transmembrane conductance regulator (CFTR): an apical marker protein of ionocytes for identifying hypo-osmoregulation in gills of the euryhaline medaka *Oryzias dancena*. *Zoological Studies* **51**(8): 1270-1281. This study used a monoclonal antibody against the human cystic fibrosis transmembrane conductance regulator (CFTR) to characterize its expression in gills of the brackish medaka *Oryzias dancena*. Whole-mount double immunofluorescence staining of gills revealed that the CFTR protein was localized in apical membranes of Na^+, K^+-ATPase-immunoreactive ionocytes in gills of seawater (SW)-acclimated medaka. Immunoreactive signals of the CFTR protein were detected by the antibody in gills of SW medaka rather than in those of freshwater (FW) fish. Furthermore, the full-length complementary (c)DNA of *Odcftr* was cloned and identified from gill tissues to evaluate specific affinities of the CFTR antibody. Using the RT-PCR to survey gene expressions in various tissues, *Odcftr* was prominently expressed in gills of the brackish medaka. Higher messenger (m)RNA levels of *Odcftr* were found in gills of medaka acclimated to environments with increasing salinities. The present study validated the salinity-dependent expression of the CFTR in branchial ionocytes of the brackish medaka and illustrated that the CFTR protein is a marker of the hypo-osmoregulatory role of the gill epithelium of this euryhaline teleost. http://zoolstud.sinica.edu.tw/Journals/51.8/1270.pdf

Key words: CFTR, Medaka, Ionocytes, Gills, Hypo-osmoregulation.

The gills of fish are directly exposed to aquatic environments and are the major organ for ion transport. One type of gill epithelial cells, ionocytes, also called chloride cells and mitochondrion-rich cells, are responsible for absorbing ions in fresh water (FW) and secreting ions in sea water (SW; Wood and Marshall 1994, Evans et al. 1999, Marshall 2002, Hirose et al. 2003, Hwang and Lee 2007, Kaneko et al. 2008, Evans 2008, Hwang et al. 2011). Immunohistochemical studies demonstrated that branchial ionocytes contain the most abundant Na^+/K^+-ATPase (NKA) in the basolateral membrane which provides the driving force for secondary ion transporters (Dang et al. 2000, Lee et al. 2000, Sakamoto et al. 2001, Brauer et al. 2005). In numerous studies, gill ionocytes refer to NKA-immunoreactive (IR) cells with basolateral signals (Dang et al. 2000, Lee et al. 2000, Marshall et al. 2002, Lin et al. 2003, Kaneko et al. 2008, Kang

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et al. 2008). Two fluorescent lectin molecules are used as markers to identify the apical region of ionocytes in teleost gills. In gills of tilapia (*Oreochromis mossambicus*) and zebrafish (*Danio rerio*), apical membranes of ionocytes were labeled with concanavalin A (Li et al. 1995, Van der Heijden et al. 1997, Lee et al. 2003, Lin et al. 2006). On the other hand, peanut lectin agglutinin was used to recognize apical membranes of various ionocytes in gills of rainbow trout (*Oncorhynchus mykiss*, Goss et al. 2001). In branchial ionocytes of SW-adapted teleosts, the current model shows that Cl^- secretion is conducted by 3 molecules, NKA which supplies the driving force, the basolateral Na^+, K^+, $2Cl^-$ cotransporter (NKCC), and the apical cystic fibrosis transmembrane conductance regulator (CFTR) which transports Cl^- from the blood through cells to external media for hypo-osmoregulation (Marshall 2002, Hirose et al. 2003, Hwang and Lee 2007, Evans 2008, Hwang et al. 2011). Increased levels of the CFTR gene and protein were correlated with environmental salinities in gills of various teleosts (Singer et al. 1998, Scott et al. 2005, Madsen et al. 2007, Shaw et al. 2008 2010, Ouattara et al. 2009, Bodinier et al. 2009). A monoclonal antibody (mAb) against C-terminus sequences of the human CFTR (24-1, R&D Systems, Boston, MA, USA) was used for immunocytochemical staining to demonstrate that the CFTR protein was localized to apical regions of ionocytes with Cl^- secretion when various teleosts were exposed to high-salinity environments (Marshall et al. 2002, Hirose et al. 2003, Katoh and Kaneko 2003, Tse et al. 2006, Bodinier et al. 2009, Ouattara et al. 2009, Tang et al. 2011). Results implied that the CFTR antibody could be a potential marker for identifying SW ionocytes with the capacity for hypo-osmoregulation in teleosts.

Euryhaline teleosts are able to tolerate a broad range of environmental salinities by utilizing efficient osmoregulatory mechanisms to maintain homeostasis. The brackish medaka *Oryzias dancena* primarily inhabits river mouths and estuaries of eastern India, Bangladesh, and Myanmar (Roberts 1998), and is closely related to the Japanese medaka *O. latipes*. There is a genomic database for this species. This euryhaline species was reported to exhibit better salinity tolerance than the Japanese medaka according to survival rates of adult fish and hatching rates of embryos (Inoue and Takei 2002 2003). With the Japanese medaka genetic database, it is convenient to identify sequence information of target genes of this brackish-water fish. Previous studies examined expressions of 2 crucial ion transporters in the ion secretory model of ionocytes, the NKA and NKCC1a, in gills of the brackish medaka acclimated to environments with different salinities (Kang et al. 2008 2010). The CFTR, another important ion transporter in the model; however, has not yet been validated in the brackish medaka.

The aims of the present study were to examine expression of the CFTR in gills of the brackish medaka to validate the SW model, and we propose that the CFTR antibody could be used as a marker for identifying SW ionocytes with the capacity for hypo-osmoregulation in teleosts acclimated to hyperosmotic environments. Distributions of the CFTR protein in NKA-immunoreactive (IR) cells (ionocytes) of SW and FW fish were compared to verify the Cl^- excretion model in gills of medaka acclimated to SW and FW by whole-mount double immunofluorescence staining. A full-length sequence of *Odcftr* was cloned and analyzed with other homologous CFTRs from other vertebrates to examine the specific affinity of the CFTR antibody. *Odcftr* messenger (m)RNA was surveyed by a reverse-transcription polymerase chain reaction (RT-PCR) in various tissues. Abundances of *Odcftr* mRNA in gills of SW-, 50% SW-, and FW-acclimated fish were also determined to support the protein profiles of immunofluorescence staining.

MATERIALS AND METHODS

Fish and experimental conditions

Adult brackish medaka *Oryzias dancena* obtained from a local aquarium averaged 2.5 ± 0.3 cm long. SW (35‰) and 50% SW (15‰) were prepared from local fresh tap water (FW) with proper amounts of RealOcean™ Synthetic Sea Salt (TAAM, Camarillo, CA, USA) for raising fish. The medaka were acclimated to FW, 50% SW, or SW for at least 3 wk. The water was continuously circulated through fabric-floss filters and partially refreshed every 2 wk. The water temperature was maintained at 28 ± 1°C. The photoperiod cycle was 14 h of light: 10 h of dark. Fish were fed a daily diet of commercial pellets. Following the experiment, fish were not fed and were anesthetized with MS-222 (100-200 mg/L) before sampling. The facilities and animal use protocols of the experimental fish were reviewed and approved by the Institutional Animal Care and

Use Committee of the National Chung Hsing Univ. (IACUC approval no. 96-48).

Preparation of whole-mount samples

Gills of medaka were fixed in neutral formalin (pH 7.2) at 4°C for 3 h. After washing in phosphate-buffered saline (PBS), samples were permeated with methanol for 30 min at -20°C. Samples were then stored in the methanol at -20°C before performing the following experiments.

Double immunofluorescence staining

Whole-mount samples were rehydrated by rinsing with PBS 3 times. Subsequently, samples were incubated in 5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) at room temperature for 30 min. Samples were then incubated with a mouse mAb (MAB25031, 24-1, R&D Systems) directed against 104 amino acids at the C-terminus of the human CFTR (diluted 1: 100 with 1% BSA) at room temperature for 2 h. Samples were then washed 3 times with PBS, incubated with Dylight-549 goat anti-mouse immunoglobulin G (IgG, Jackson Immunoresearch, West Baltimore Pike, PA, USA; diluted 1: 500 with 1% BSA) at room temperature for 1 h, and washed several times with PBS. After the 1st staining, samples were incubated with a rabbit mAb (EP1845Y; Abcam, Cambridge, UK) raised against the N-terminus of the human NKA α-subunit (diluted 1: 250 with 1% BSA) for 2 h at room temperature. Samples were then washed several times with PBS and incubated with Dylight-488 goat anti-rabbit IgG (Jackson Immunoresearch, diluted 1: 200 with 1% BSA) at room temperature for 1 h followed by several PBS washes. Samples were then mounted in 50% glycerol with PBS and examined using a laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan).

Total RNA extraction

Total RNA samples from various tissues were extracted using RNA-Bee™ (Tel-Test, Friendwood, TX, USA) following the manufacturer's instructions. Genomic DNA was eliminated using the RNA clean-up protocol of the RNAspin Mini RNA isolation kit (GE Health Care, Piscataway, NJ, USA).

RT-PCR analysis of the CFTR in different tissues

Expression of the *Odcftr* gene in various organs of brackish medaka was examined with an RT-PCR. Primers were designed to identify specific regions of the CFTR sequence according to the *cftr* gene of Japanese medaka in the medaka database (Table 1). Total RNA was extracted from the brain, gills, eyes, heart, intestines, kidneys, liver, muscles, fins, ovaries, and testes from 10 SW-acclimated fish. RNA samples were stored at -80°C after isolation. First-strand complementary (c)DNA was synthesized by reverse-transcribing 4 μg of total RNA using 1 μl of the Oligo-dT (0.5 μg/μl) primer and 1 μl PowerScript™ Reverse Transcriptase (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. For PCR amplification (28 cycles), 1 μl of cDNA was used as a template in a 25-μl final reaction volume containing 0.25 μM dNTPs, 1.25 units Hot start EX-Taq polymerase (Takara, Shiga, Japan), and 0.2 μM of each primer. PCR products were

Table 1. Primer sequences used for cDNA cloning, RACE, and a quantitative real-time PCR of *Odcftr* genes of the brackish medaka

Primer name	Primer sequence (5' to 3')
Odcftr-F	CTCGCTCTCGATGACAATGA
Odcftr-R	TCCTCTGGCAGTGAGGAGAT
Odcftr-RT-F	TGGATCACTCCACTGCTGAG
Odcftr-RT-R	GGCGGAGAAGAAGTAGGAGG
Odcftr-RACE-F	CCATAACTCTGGTGGTCCTGAGGAAG
Odcftr-RACE-R	CTCTTGGATGACACCACCTCTCTGTC
Odcftr-QPCR-F	TGGATCACTCCACTGCTGAG
Odcftr-QPCR-R	GGCGGAGAAGAAGTATGAGG
β-actin-F	CTGGACTTCGAGCAGGAGAT
β-actin-R	AGGAAGGAAGGCTGGAAGAG

subcloned into the pOSI-T vector (Genemark, Taipei, Taiwan), and amplicons were sequenced for confirmation. β-actin was used as an internal control for all tissues (Kang et al. 2008).

cDNA cloning of a full-length *Odcftr* gene from gills and sequence analysis

cDNA for cloning and rapid amplification of cDNA ends (RACE) was made from total RNA of gills of 4 individuals using a SuperScript III reverse-transcriptase kit (Invitrogen, Carlsbad, CA, USA) and SMART RACE cDNA amplification kit (Clontech) following the manufacturer's protocols. For PCR amplification, 2 μl of cDNA was used as a template in a 50-μl reaction containing 0.25 μM dNTPs, 2.5 units Hot start EX-*Taq* polymerase (Takara), and 0.2 μM of each primer. Primer sets (shown in Table 1) were designed using the Primer3 software (Rozen and Skaletsky 2000). PCR products were subcloned into the pOSI-T vector (Genemark), and amplicons were sequenced to confirm the PCR products. Specific primers for the 5' and 3' RACE were designed according to CFTR partial sequences obtained from the PCR using primer sets listed in table 1. RACE PCR products were also subcloned into the pOSI-T vector (Genemark) and sequenced. Sequence alignments and identities were performed with CLUSTALW. Transmembrane segments were predicted on the TMHMM server vers. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and a phylogenetic tree was constructed using MEGA 4.1 (Tamura et al. 2007). This tree was built using the minimum-evolution method with the pairwise deletion gaps calculating option.

Quantitative real-time (q)PCR

Messenger (m)RNA abundance was quantified with an Applied Biosystems 7000 real-time PCR system (Foster City, CA, USA). PCRs contained 8 μl of cDNA (1000×), 2 μl of either 5 μM of the *Odcftr*-QPCR primer mixture or 5 μM of the β-actin primer mixture (Kang et al. 2008), and 10 μl of 2× SYBR Green PCR MasterMix (Applied Biosystems). The real-time PCRs were performed as follows: 1 cycle at 50°C for 2 min and 95°C for 15 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. Primer sets for the real-time PCR are shown in table 1. Primers for β-actin were used as an internal control in all samples. The occurrence of secondary products and primer-dimers was determined using a melting curve analysis and electrophoresis to confirm that the amplification was specific. One identical control sample was used as an internal control among different groups. For each unknown sample, the comparative Ct method with the formula $2^{-[(Ct_{target\ gene,\ n} - Ct_{\beta\text{-actin},\ n}) - (Ct_{target\ gene,\ c} - Ct_{\beta\text{-actin},\ c})]}$ was used to obtain the corresponding *Odcftr* and β-actin values, where Ct corresponded to the threshold cycle number.

Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) followed by Tukey's pair-wise method, and $p < 0.05$ was set as the significance level. Values were expressed as the mean ± standard error of the mean (SEM) unless stated otherwise.

RESULTS

Immunolocalization of the CFTR in gills

The afferent epithelium of gill filaments from SW- and FW-acclimated medaka was immunostained with an antibody against the CFTR or the NKA α-subunit. Merged images of SW-acclimated medaka revealed localization of the CFTR and NKA α-subunit in the same cells (Fig. 1C). Signals of the CFTR, however, were present in the apical region of NKA-immunoreactive (IR) cells of SW-acclimated medaka (Fig. 1G). On the other hand, there was no CFTR distribution in NKA-IR cells of FW fish (Fig. 1F).

Amino acid alignment of the CFTR

The partial sequence of *Odcftr* (**JQ728537**) was cloned from brackish medaka using original primers designed according to the Japanese medaka databases. A 5046-bp full-length CFTR cDNA encoding an 1503-amino acid protein was isolated from gills of the brackish medaka by RACE-PCR. The cDNA contained 203 bp of the 5' untranslated region (UTR) and 334 bp of the 3' UTR. Examination of the deduced amino acid sequence of the CFTR aligned with those from killifish and humans revealed 12 predicted transmembrane domains (Fig. 2A). The R, NBD1, NBD2, and PDZ domains were identified in the sequence. Eight potential phosphorylation sites were predicted. In addition, conserved regions

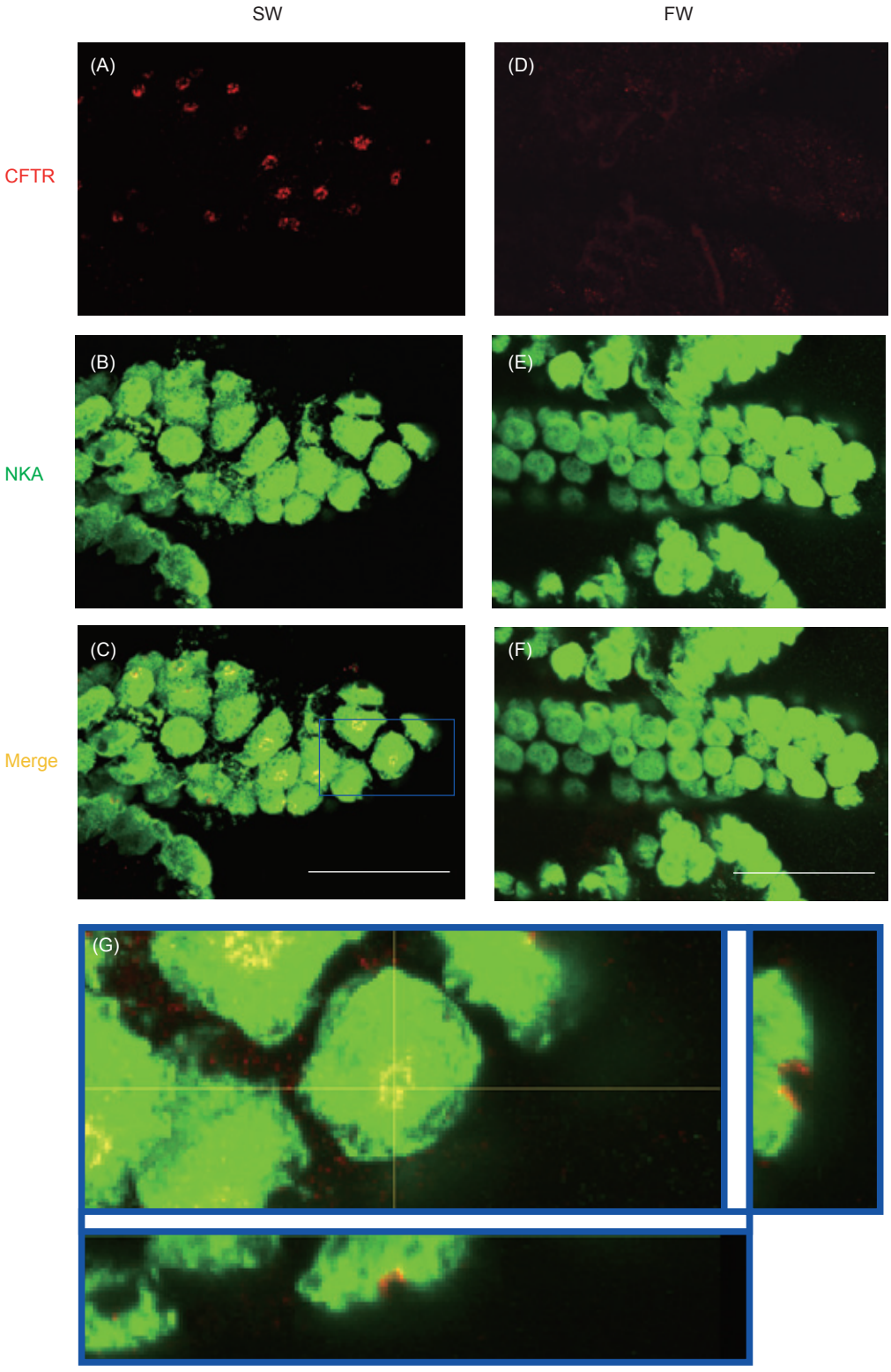

Fig. 1. Confocal micrographs of whole-mount double immunofluorescence staining with anti-cystic fibrosis transmembrane conductance regulator (CFTR) (red; A, D) and anti-Na^+, K^+-ATPase α-subunit (NKA; green; B, E) antibodies on the afferent sides of gill filaments in brackish medaka acclimated to seawater (SW) and fresh water (FW). Merged images (C, F) revealed that the CFTR protein was localized in NKA-immunoreactive (IR) cells in SW medaka but not in FW fish. The magnified merged 3D image (G) of SW fish revealed that CFTR signals were in the apical region of NKA-IR cells. Scale bar = 50 μm.

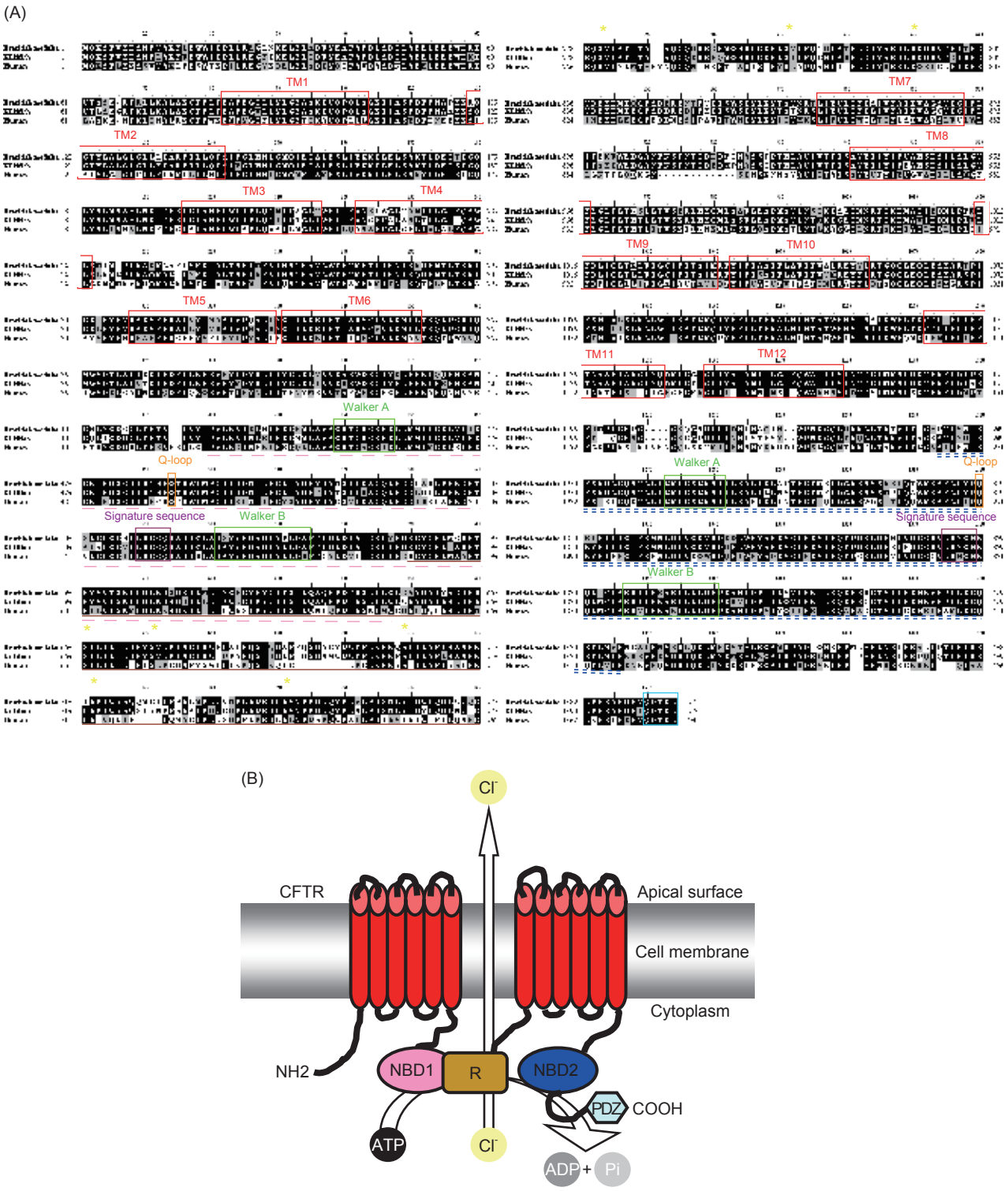

Fig. 2. (A) Amino acid sequence alignment of the brackish medaka cystic fibrosis transmembrane conductance regulator (CFTR) (**JQ728537**) compared to the CFTR of killifish (**AAC41271**) and humans (**AAC13657**). Gaps (dashed) were introduced to optimize the alignment. All amino acids identical or similar to those of brackish medaka CFTR are shaded. Twelve putative transmembrane segments (TM1-TM12) are indicated (red box). The continuous line (brown) indicates the R domain. Two nucleotide-binding domains of NBD1 and NBD2 are respectively represented by single (pink) and double blue dotted lines. Eight potential phosphorylation sites are labeled with asterisks (yellow). Q-loop (orange), signature sequence (purple), Walker A, and Walker B (green) regions are identified in the medaka CFTR sequence. The PDZ domain is marked by a blue box. (B) Putative CFTR topology of the brackish medaka.

of the CFTR, including the Q-loop, signature sequence, Walker A, and Walker B, were also found in this fish sequence. The putative CFTR topology of brackish medaka is shown in figure 2B.

Phylogenetic analysis of full-length amino acid sequences of the CFTR

CFTRs from teleosts can be classified according to the phylogenetic tree (Fig. 3). Numerous isoforms of the CFTR were found in different species. The full-length amino acid sequence of the CFTR of brackish medaka showed 97% identity to that of the Japanese medaka, 85%-80% identities to those of the killifish, seabass, pufferfish, fugu, and stickleback, 78% identity to that of salmon, and 56%-54% identities to those of other vertebrates. Only 26% identity was found between sequences of medaka CFTR and the outgroup, ABCC4, from humans.

Tissue distribution of *Odcftr* genes detected by the RT-PCR in brackish medaka

The RT-PCR analysis of different organs revealed that *Odcftr* (888 bp) was mainly localized in the gills, kidneys, intestines, ovaries, and testes of brackish medaka (Fig. 4). Low levels of the *Odcftr* gene were found in the brain, eyes, and liver.

Odcftr mRNA abundance quantified by a real-time PCR

Branchial *Odcftr* mRNA levels of the brackish medaka acclimated to SW, 50% SW, and FW

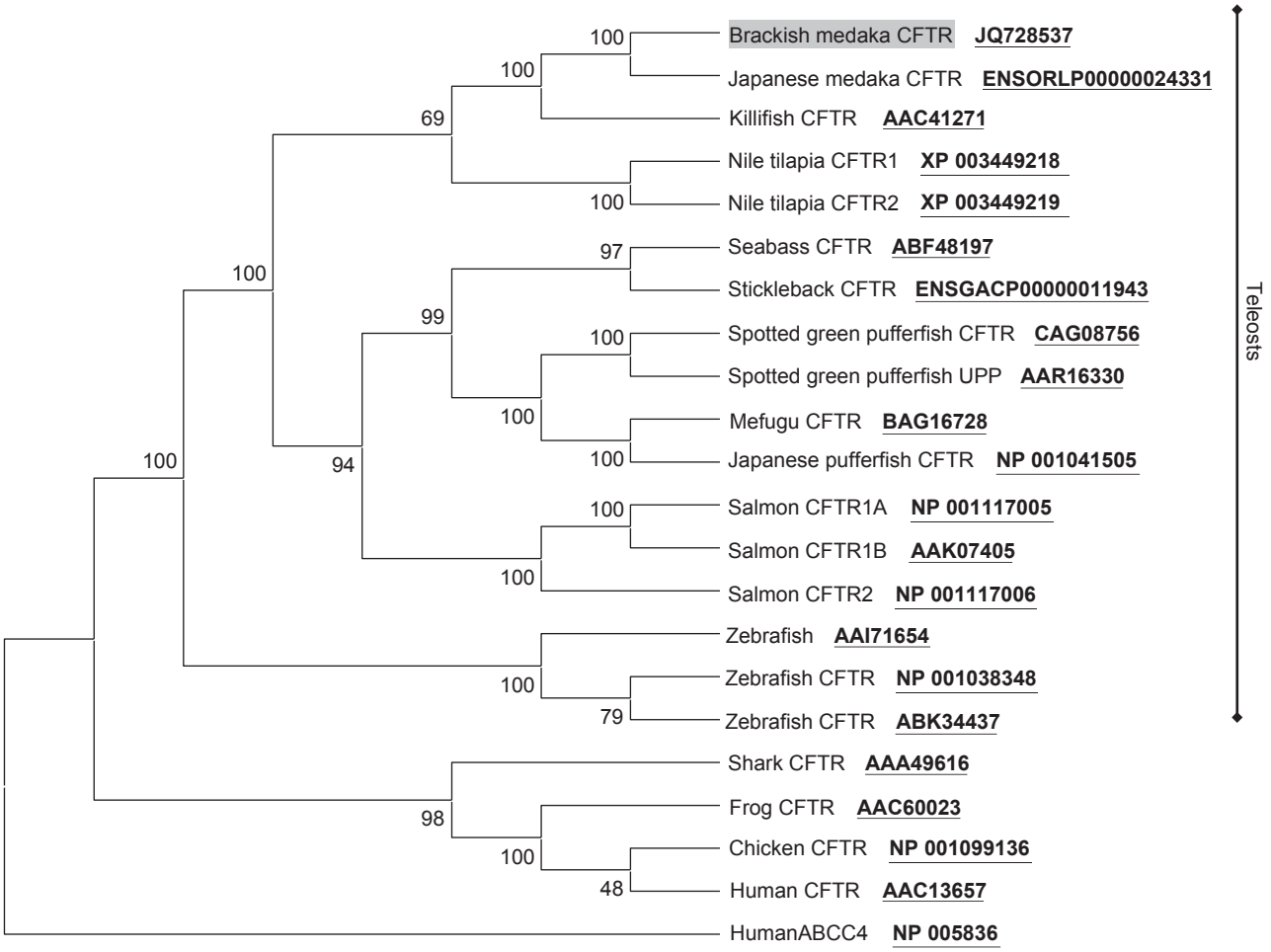

Fig. 3. Phylogenetic analysis of full-length amino acid sequences of the cystic fibrosis transmembrane conductance regulator (CFTR) from different species, shown as a phylogenetic tree according to the minimum-evolution method with 1000 bootstrap replicates. The brackish medaka CFTR is shaded. The ABCC4 protein of humans was used as an outgroup to root the tree. Branches were found to be different sub-tree regions. Numbers at the nodes are bootstrap values for 1000 replications, showed as percentages.

significantly differed among the groups (Fig. 5). The highest *Odcftr* mRNA level was found in SW-acclimated individuals, and the lowest *Odcftr* mRNA level was in the FW group. Those of the SW and 50% SW groups were about 7- and 5-fold higher, respectively, than the FW group. The level in SW fish was thus significantly higher (approximately 1.2-fold) than that in the 50% SW group. Therefore, branchial *Odcftr* mRNA abundances in brackish medaka significantly dropped with decreasing environmental salinities.

DISCUSSION

Ion transporters exhibit specific distributions and expressions of molecular functions in various epithelial cells. Two marker proteins, V-ATPase and aquaporin 2 (AQP2), were respectively used to classify kidney epithelial cells into intercalated and principle cells in the collecting ducts of mammals (Brown et al. 2009). According to review articles, the well-known marker protein, NKA, was applied to observe cell bodies of ionocytes in gills of various teleosts (Hwang and Lee 2007, Kaneko et al. 2008, Hwang et al. 2011). On the other hand, apical membranes of gill ionocytes in some teleostean species were labeled with concanavalin A or peanut lectin agglutinin (Goss et al. 2001, Lee et al. 2003, Lin et al. 2006). However, the lectines could not detect gill ionocytes of brackish medaka acclimated to SW and FW according to our preliminary data (data not shown). Shen et al. (2011) determined Cl^- effluxes in ionocytes of the Japanese medaka (*O. latipes*) yolk membrane by the scanning ion-selective electrode technique. The current model of gill ionocytes in SW teleosts proposes that the CFTR is expressed in apical membranes of epithelial ionocytes and transports Cl^- from the cytoplasm of cells into external media (Marshall 2002, Hirose et al. 2003, Hwang and Lee 2007, Evans 2008, Hwang et al. 2011).

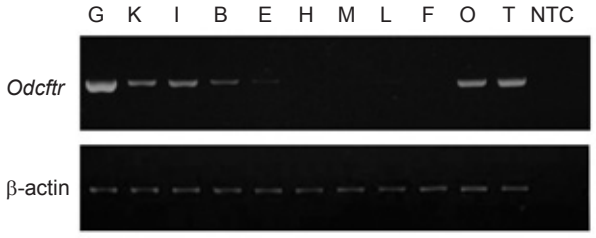

Fig. 4. Tissue distribution of *Odcftr* genes detected by an RT-PCR in brackish medaka. β-actin was used as an internal control. B, brain; E, eye; F, fin; G, gill; H, heart; I, intestine; K, kidney; L, liver; M, muscle; O, ovary; T, testis; NTC, no template control.

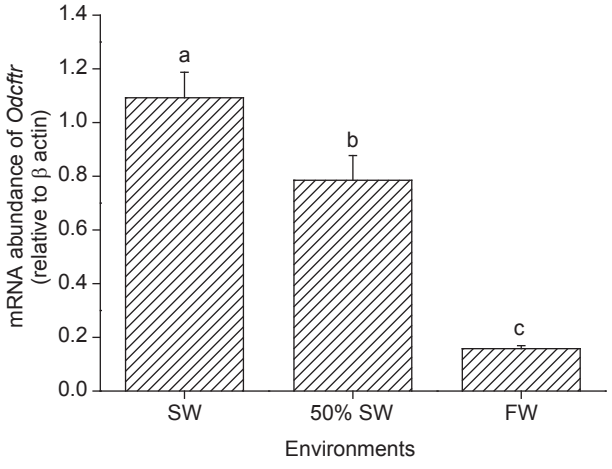

Fig. 5. mRNA levels of *Odcftr* in gills of brackish medaka acclimated to sea water (SW), 50% SW, or fresh water (FW) (n = 5 for all groups). Branchial mRNA abundances of brackish medaka significantly decreased in low environmental salinities. Different letters indicate a significant difference ($p < 0.05$) using Tukey's multiple-comparison test following a one-way ANOVA. Values are the mean ± S.E.M.

The CFTR, one of the ATP-binding cassette proteins, forms an anion channel with a conductance of 7-10 pS (Nagel 1999). Riordan et al. (1989) first identified the gene sequence of the CFTR of human epithelial cells. In mammalian studies, numerous symptoms of cystic fibrosis were caused by mutations of the CFTR protein, including abnormal expression, channel operation, and protein trafficking (Frelet and Klein 2006). Denning et al. (1992) first used an anti-human CFTR antibody (24-1, R&D Systems) to detect expression of the mammalian CFTR protein with Western blotting. The epitope of the CFTR antibody was reported and identified to be a DTRL sequence according to SPOTs technology (Marshall et al. 1994). The CFTR was electrophysiologically found in apical membranes of ionocytes in the killifish (*Fundulus heteroclitus*, Marshall et al. 1995). Singer et al. (1998) subsequently cloned the gene sequence of the CFTR homologue of killifish. Marshall et al. (2002) first detected the CFTR protein in gills of the killifish with the CFTR antibody. Previous studies demonstrated that the CFTR was observed in apical membranes of SW-exposed ionocytes rather than FW-exposed cells in gills of the mudskipper (*Periophthalmodon schlosseri*, Wilson et al. 2000), killifish (*F. heteroclitus*, Marshall et al. 2002, Katoh and Kaneko 2003), Hawaiian

goby (*Stenogobius hawaiiensis*, McComick et al. 2003), tilapia (*O. mossambicus*, Hiroi et al. 2005; *Sarotherodon melanotheron*, Ouattara et al. 2009), Japanese eel (*Anguilla japonica*, Tse et al. 2006), seabass (*Dicentrarchus labrax*, Bodinier et al. 2009), spotted green pufferfish (*Tetraodon nigroviridis*, Tang et al. 2011), and milkfish (*Chanos chanos*, Tang et al. 2011). Apical signals of the gill CFTR were also found to be salinity-dependent in apical membranes of ionocytes of the brackish medaka stained with the CFTR antibody. Based on the Z-axial micrograph, the CFTR-IR signals invaginated to form an orifice like the hole-type morphology observed by scanning electron microscopy in apical openings of NKA-IR cells/ ionocytes of the brackish medaka (Kang et al. 2013). IR signals of the Na^+, K^+, $2Cl^-$ cotransporter/ NKCC immunostained with an mAb (T4, DSHB) were respectively found in apical and basolateral regions of gill ionocytes in FW and SW fish (Wu et al. 2003, Katoh et al. 2008, Kang et al. 2010). Therefore, the results of this study confirmed the Cl^- secretory model and revealed that the CFTR antibody with unique apical signals can be used as a marker of SW ionocytes in the brackish medaka.

To our knowledge, the full-length amino sequences and protein distributions of the CFTR stained with the CFTR antibody in gills of only 3 teleostean species, killifish (*F. heteroclitus*), seabass (*D. labrax*), and pufferfish (*T. nigroviridis*), were reported (Marshall et al. 2002, Bodinier et al. 2009, Tang et al. 2011). The deduced amino acid sequence of the CFTR from the brackish medaka showed high similarities to CFTR sequences of other teleosts (Fig. 3). Based on the deduced protein sequence of the CFTR from the brackish medaka (Fig. 2A), its molecular weight was predicted to be 169 kDa. A similar immunoreactive band of the CFTR detected in killifish gills was about 160-170 kDa (Shaw et al. 2008 2010). The deduced amino acid sequence of the medaka CFTR was recognized to exhibit numerous homologous domains or motifs compared to CFTR sequences of humans and killifish (Fig. 2B). The conserved regions indicated that the medaka CFTR might have similar molecular functions as the human CFTR. In the 12 transmembrane domains (TMDs), the predicted segments of TM6 and TM12, which were shown to line the channel pore (McDonough et al. 1994, Cheung et al. 1996), showed the highest identity with those of the human CFTR. The conservation of 4 cytoplasmic loops (CLs) which link to the TMDs on the cytoplasmic side of the membrane also showed high levels of similarity compared to other species. In the human CFTR, CL1 and CL2 are involved in determining the open probability and conductive states of the channel (Xie et al. 1995 1996), while CL3 and CL4 regulate the channel's opening and closing (Seibert et al. 1996a b). Meanwhile, the CFTR sequence alignment also revealed 2 nucleotide-binding domains (NBDs), NBD1 and NBD2. In each NBD of the medaka CFTR, the Walker A and B motifs and hallmarks of ATP-binding proteins were identical to the same motifs in the human CFTR. Atwell et al. (2010) defined the feature of the ABC family to which the CFTR belongs as a conserved LSGGQ signature motif with NBDs. The LSGGQ motif was also found in the medaka CFTR. Based on this structural and functional evidence, the medaka CFTR could require association of NBDs in a head-to-tail dimer, sandwiching ATP molecules between the Walker A and signature LSGGQ motifs in ATP sites, as in the human CFTR (Gadsby et al. 2006, Oswald et al. 2006). In addition, the R domain of the medaka CFTR retained 8 consensus protein kinase A (PKA) phosphorylation sites, similar to 9 sites for the R domain of the mammalian CFTR (Riordan et al. 1989, Marshall et al. 1991). Hence the R domain of the medaka CFTR might play similar roles in regulating channel activation through phosphorylation by cAMP-dependent protein kinases (Gadsby and Nairn 1999). Meanwhile, the site of the most frequent mutation in the human CFTR, F508 (Denning et al. 1992), was retained in the medaka CFTR. The C-terminal QDTRL sequence was the PDZ (PSD-95, Dlg, and ZO-1) domain, which is required for CFTR polarization to the apical plasma membrane and interaction with the PDZ domain-containing protein, EBP50 (NHERF) (Moyer et al. 2000). The PDZ domain was also highly conserved in CFTR sequences of brackish medaka. Analyses of the medaka CFTR sequence (Fig. 2) indicated that the CFTR antibody that recognizes the DTRL sequence of the human CFTR could also bind to the medaka CFTR protein localized in gill ionocytes.

Expression of the *cftr* gene was found in the brain, gills, opercular epithelium, and posterior intestines of SW killifish by Northern blots (Singer et al. 1998). In addition to the tissues studied in the killifish, the eyes, liver, and gonads were also investigated in this study (Fig. 4). The *Odcftr* gene was mostly distributed in gills of the brackish medaka, like in other studied teleosts (Singer et al. 1998, Scott et al. 2004, Tse et al. 2006, Mackie et al. 2007, Bondinier et al. 2009). Our

preliminary data, however, did not reveal a single immunoreactive band at 160-170 kDa of total protein lysates from gills of the brackish medaka by Western blotting with the CFTR antibody. This result implies that the antigen of the medaka CFTR might be altered to a form to which the antibody could not bind in the denatured sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) protocol of Western blotting (data not shown). Since mRNA abundances of the CFTR were correlated with protein levels in gills of killifish (*F. heteroclitus*), seabass (*D. labrax*), and Japanese eel (*A. japonica*) when acclimated to environments with different salinities (Scott et al. 2004, Tse et al. 2006, Bondinier et al. 2009), detection of gene abundances by an RT-PCR or a real-time PCR could feasibly evaluate the functional expression of the CFTR in the brackish medaka. In gills of the brackish medaka, expression of *Odcftr* mRNA was salinity-dependent (Fig. 5). Similar genetic profiles were also found in gills of the Atlantic salmon (*Salmo salar*, Singer et al. 1998 2002, Mackie et al. 2007), killifish (Scott et al. 2004), Japanese eel (*A. japonica*, Tse el at. 2006), and seabass (*Dicentrarchus labrax*, Bondinier et al. 2009). In killifish, numerous glucocorticoid- and osmotic-responsive elements were identified in the promoter sequence of the *cftr* gene (Singer et al. 2008). On the other hand, *cftr* gene expression was reported to mediate the cortisol pathway in gills of the Atlantic salmon (Singer et al. 2003, Kiilerich et al. 2007). Therefore, the *Odcftr* gene could be induced by plasma cortisol and translated to salinity-dependent proteins detected by the CFTR antibody in gill ionocytes of the brackish medaka (Fig. 1) upon exposure to environments with increasing salinity.

In summary, this study elucidated salinity-dependent cellular and genetic expressions of the CFTR in branchial ionocytes of the brackish medaka. Since the CFTR was specifically localized in apical membranes of ionocytes with a Cl^- secretory capacity in gills of the brackish medaka, the present study demonstrated that the CFTR would be a good marker protein for studying the hypo-osmoregulatory mechanism of teleosts exposed to environments with higher salinities. This marker protein could identify SW ionocytes and be applied to evaluate the physiological situation of marine fish in ecophysiological and ecotoxicological studies. In future work, the regulatory pathways of the branchial CFTR in brackish medaka will be examined. The promoter region of the CFTR sequence will be characterized to clarify the transcribed mechanism of the salinity-dependent CFTR in gills of brackish medaka.

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