# Molecular cloning, characterization, and expression of pannexin genes in chicken

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**ABSTRACT** Pannexins (Panx) are a family of proteins that share sequences with the invertebrate gap junction proteins, innexins, and have a similar structure to that of the vertebrate gap junction proteins, connexins. To date, the Panx family consists of 3 members, but their genetic sequences have only been completely determined in a few vertebrate species. Moreover, expression of the Panx family has been reported in several rodent tissues: Panx1 is ubiquitously expressed in mammals, whereas Panx2 and Panx3 expressions are more restricted. Although members of the Panx family have been detected in mammals, their genetic sequences in avian species have not yet been fully elucidated. Here, we obtained the full-length mRNA sequences of chicken *PANX* genes and evaluated the homology of the amino acids from these sequences with those of other species. Furthermore, *PANX* gene expression in several chicken tissues was investigated based on mRNA levels. *PANX1* was detected in the brain, cochlea, chondrocytes, eye, lung, skin, and intestine, and *PANX2* was expressed in the brain, eye, and intestine. *PANX3* was observed in the cochlea, chondrocytes, and bone. In addition, expression of *PANX3* was higher than *PANX1* in the cochlea. Immunofluorescent staining revealed PANX1 in hair cells, as well as the supporting cells, ganglion neurons, and the tegmentum vasculosum in chickens, whereas PANX3 was only detected in the bone surrounding the cochlea. Overall, the results of this study provide the first identification and characterization of the sequence and expression of the PANX family in an avian species, and fundamental data for confirmation of Panx function.

**Key words:** pannexin, gap junction, chicken, inner ear, cloning

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## INTRODUCTION

The pannexin (**Panx**) family consists of integral membrane glycoproteins that share sequences with the invertebrate gap junction proteins, innexins, and have similar structures to those of the vertebrate gap junction proteins, connexins (Bhalla-Gehi et al., 2010). The Panx family consists of 3 members, Panx1, Panx2, and Panx3, which have been shown to exist in both human and mouse genomes (Panchin et al., 2000; Yen and Saier, 2007; D'hondt et al., 2009). Although the homology between Panx and connexins is not high, the 2 vertebrate gap junction proteins share structural and functional similarities (Panchin et al., 2000; Yen and Saier, 2007). Specifically, both proteins have 4 transmembrane domains, 2 extracellular loops, 1 intracellular loop, and cytoplasmic N- and C-termini (Baranova et al., 2004), which enable formation of a hexameric pore complex known as a hemichannel. Panx possess extracellular loops of 50 to 60 amino acids, whereas connexins possess relatively small extracellular loops (~30 amino acids). The size of the loops may be influenced by the strength of the interaction between opposing hemichannels (Shestopalov and Panchin, 2008).

The function and expression of the Panx family have been reported in several tissues of mammals (Baranova et al., 2004; Shestopalov and Panchin, 2008; Penuela et al., 2013). Panx1 was detected in the brain, spinal cord, heart, skeletal muscle, eye, ovary, liver, lung, blood vessel endothelium, and other tissues in rats and mice (Panchin et al., 2000; Bruzzone et al., 2003; Baranova et al., 2004; Ray et al., 2005, 2006; Vogt et al., 2005; Weickert et al., 2005). Panx1 is essential to the mediation of intracellular signaling events, which


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occur through $Ca^{2+}$-mediated adenosine triphosphate (**ATP**) release. This release of ATP was recently shown to occur between supporting cells and hair cells in the cochlea of the inner ear (Zhao et al., 2005). Panx2 and Panx3 are not as widely expressed as Panx1 (Penuela et al., 2013). Specifically, Panx2 is predominantly detected in the brain, although it has also been found to be coexpressed with Panx1 in portions of the central nervous system, including the retina, cerebral cortex, hippocampus, and cerebellum in rodents (Bruzzone et al., 2003; Baranova et al., 2004; Sohl et al., 2005). Panx3 has been found to be associated with the regulation of chondrocyte proliferation and differentiation. Thus, it is restrictively expressed in mouse cartilage, where it plays a prominent role in mechanical load resistance and skeletal support of structures and skin (Iwamoto et al., 2010). The expression of all 3 Panx was recently reported in rodent cochleae (Tang et al., 2008; Wang et al., 2009).

However, members of the PANX family have not yet been cloned in avian species, and its expression has not been reported. Here, we identified *PANX* gene family sequences and characterized their expression in several tissues, including the brain, cochlea, liver, chondrocytes, kidney, heart, eye, lung, skin, intestine, bone, and spleen in chickens. In addition, their expression in chicken cochleae was analyzed by real-time PCR and immunohistochemistry analysis.

## MATERIALS AND METHODS

### Ethics Statement

The animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kyungpook National University, and approved by the Committee on the Ethics of Animal Experiments of the Kyungpook National University.

### Birds

Fertilized White Leghorn (*Gallus gallus domesticus*) eggs were purchased from a poultry farm (Sibligol Egg Farm, Daegu, South Korea) and incubated at 37°C with constant humidity. Embryos were investigated at embryonic (**E**) d 13 (E13), E15, and E18. Chicken embryos were euthanized by hypothermia followed by decapitation.

### RNA Isolation and Sequence Analysis

Total RNA was extracted to analyze the regional expression and full mRNA sequence of *PANX* gene family in the chicken brain, cochlea, liver, chondrocytes, kidney, heart, eye, lung, skin, intestine, bone, and spleen of E13, E15, and E18 using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracted total RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), after which PCR was carried out to analyze *PANX* expression in different tissues of the E13, E15, and E18 samples. The following primers were used for this analysis: *PANX1* (forward: 5′-CTC ACC TCT CTT CGG ACC TG-3′; reverse: 5′-TGA TGG TGC AGA GAA ACT CG-3′), *PANX2* (forward: 5′-CTG CAA AAA CCT CAC ACA GCA A-3′; reverse: 5′-TAC TTG TCG TAG CGG TCG TG-3′), and *PANX3* (forward: 5′-CCT CCT GCT CTT GTT CTT GG-3′; reverse: 5′-CTG CAG GGC ACT GTA GTA GAT GA-3′). In addition, glyceraldehyde-3-phosphate dehydrogenase (forward: 5′-GAC AAC TTT GGC ATT GTG GA-3′; reverse: 5′-CCA CAA CAC GGT TGC TGT AT-3′) was used as a positive control. The PCR was conducted by subjecting the samples to the following conditions: initial denaturation at 95°C for 2 min, followed by 28 cycles of amplification by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 50 s, and final extension at 72°C for 5 min. The amplified cDNA fragments were then separated on 2% agarose gels and visualized by ethidium bromide staining.

We conducted reverse transcription (**RT**) PCR and rapid amplification of cDNA ends (**RACE**) to determine the full-length mRNA sequences, including the 5′ and 3′ untranslated regions (**UTR**) of the chicken *PANX* gene family. To accomplish this, 5′ RACE was performed using a GeneRacer Kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 4 μg of chicken cochlea RNA with *PANX1* and *PANX3*, and brain RNA with *PANX2* was treated with calf intestinal phosphatase and tobacco acid pyrophosphatase to remove the 5′ phosphates and the 5′ cap structure, respectively. The RNA oligonucleotide bound to the RNA was then removed from the 5′ cap structure using T4 DNA ligase. Next, the first-strand cDNA was synthesized using SuperScript III reverse transcriptase, after which the original RNA template was removed using ribonuclease A. The GeneRacer 5′ primer (5′-CGA CTG GAG CAC GAG GAC ACT GA-3′), which is homologous to the RNA oligonucleotide, was ligated to the 5′ end and amplified using a reverse gene specific primer: *PANX1* (5′-AGC GCC AGA ACA GAC ATG GT-3′), *PANX2* (5′-TCC TGG CAA GGC ATC TTT TA-3′), and *PANX3* (5′-GAG AGC CCA AAG GGA CTT GG-3′). To obtain the 3′ ends, an oligo dT bound adaptor primer was added to the total RNA of the chicken cochlea and brain. The first-strand cDNA was then synthesized using a high-capacity cDNA reverse transcription kit (Applied Biosystems), after which PCR was accomplished using a 5′ gene specific forward primer [*PANX1* (5′-GGA ATT CGG GCA TTC TCA AAG-3′), *PANX2* (5′-CAC CCC TCA TCC TCA AAA G-3′), *PANX3* (5′-ATG CCG TCA GTG CCT TGC-3′)] and a homologous adaptor primer. Following PCR, the cDNA was extracted from

the agarose gel using a gel extraction kit (Elpis Biotech, Daejeon, South Korea). Next, the extracted cDNA samples were cloned into the pGEM-T easy T/A cloning vector (Promega, Madison, WI) at 4°C overnight, after which they were transformed into competent *Escherichia coli* cells. Positive clones were subsequently selected and plasmid DNA was extracted using a miniprep kit (Elpis Biotech). Sequences of cloned cDNA for UTR and direct RT-PCR products were analyzed using a sequencer (3130xl, Applied Biosystems). Nucleotide sequence alignment was performed using the CLC sequence viewer program, version 6 (CLC Bio, Aarhus, Denmark).

### Phylogenetic Analysis of Amino Acid Sequence

The ORF Finder (Open Reading Frame Finder, http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to predict the start and stop codons of the 6 isoform of *Panx* gene family. The amino acid sequences of human (PANX1: NP_056183.2, PANX2: NP_443071.2, PANX3: NP_443191.1), mouse (Panx1: NP_062355.2, Panx2: NP_001002005.2, Panx3: NP_766042.2), rat (Panx1: NP_955429.1, Panx2: NP_955441.2, Panx3: NP_955430.1), and cow (Panx1: NP_001232854.1, Panx2: NP_001095695.1, Panx3: NP_001137556.1) Panx were retrieved from the National Center for Biotechnology Information (**NCBI**) database (http://www.ncbi.nlm.nih.gov/), and the maximum likelihood phylogeny of Panx homologous with those of different species was determined using the CLC sequence viewer program, version 6 (CLC Bio, Aarhus, Denmark). Transmembrane domains were predicted using the TMHMM server, v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Finally, a phylogenetic tree was constructed based on each member of the *Panx* gene family using the MEGA software, v. 5.05 (Tamura et al., 2011).

### Real-Time PCR

The cDNA synthesized from E13 and E18 of chicken cochlea samples was amplified by real-time PCR using the following primers: *PANX1* (forward: 5′-CTC ACC TCT CTT CGG ACC TG-3′, reverse: 5′-TGA TGG TGC AGA GAA ACT CG-3′), *PANX2* (forward: 5′-GCT GCA CGG TGC TTA GCA ATT A-3′, reverse: 5′-ACT TCG CCG AGG AGC CAA TA-3′), and *PANX3* (forward: 5′-CAC ACG GCT CTA GTA CAT-3′, reverse: 5′-GAG AGC CCA AAG GGA CTT GG-3′). Each PCR mix contained synthesized cDNA, 5 μ*M* primers, and 6.25 μL of 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with a total volume of 12.5 μL. The PCR was conducted using a StepOne Plus real-time PCR machine (Applied Biosystems) by subjecting samples to the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Normalization and relative quantification was conducted using the $2^{-\Delta\Delta Ct}$ method, and the results were analyzed using the StepOne software, v.2.1. All analyses were performed in triplicate.

### Immunohistochemical Staining

The cochlea duct of the E18 chicken was used for immunohistochemical staining. Following dissection, the tissues were immediately fixed in 4% paraformaldehyde solution for 12 h at 4°C, after which they were decalcified in 10% EDTA solution for 24 h at 4°C, cryoprotected in 30% sucrose solution for 24 h, and then embedded in optimal cutting temperature compound. Next, sections with thicknesses of 10 μm were cut using a cryostat (Microm HM505N, Carl Zeiss, Walldorf, Germany), permeabilized in PBS, and treated with 0.1% triton X-100 containing 5% normal goat serum for PANX1 (chicken) and 5% normal donkey serum for PANX3 for 1 h at room temperature. The tissues were subsequently incubated for 3 h at room temperature in the presence of rabbit anti-Panx1 (1:1,000, YoungIn Frontier, Seoul, South Korea) and goat anti-Panx3 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. After a 15 min wash with 3 changes of PBS, the tissues were incubated in the dark for 1 h in Alexa Fluor 488 conjugated donkey anti-goat IgG (1:200, Invitrogen, Life Technologies) and goat anti-rabbit (1:1,000, Invitrogen, Life Technologies). Following the second antibody incubation, the tissues were stained with 4′,6-diamidine-2-phenylindole dihydrochloride (Roche Diagnostics, Indianapolis, IN) for 5 min to visualize the cell nuclei. After completely washing out the second antibodies with PBS, the tissues were mounted with a fluoromount (Sigma, Saint Louis, MO) and observed using a Zeiss fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

## RESULTS

### Expression Analysis of the PANX Gene Family in Chicken

The RT-PCR analysis was conducted to determine the tissue expression profile of the *PANX* gene family using equivalent amounts of total chicken mRNA from 12 chicken tissues (brain, cochlea, liver, chondrocytes, kidney, heart, eye, lung, skin, intestine, bone, and spleen). *PANX1* was strongly expressed in the brain, eye, and skin, and weakly in the cochlea, chondrocytes, lung, and intestine (Figure 1). *PANX2* was expressed in the brain, eye, and intestine, whereas *PANX3* mRNA was strongly expressed in the cochlea and bone, and present at a lower level in the chondrocytes and eye (Figure 1). We conducted RT-PCR from 12 tissues at E13, E15, and E18, and the results showed a similar pattern in the 3 stages.

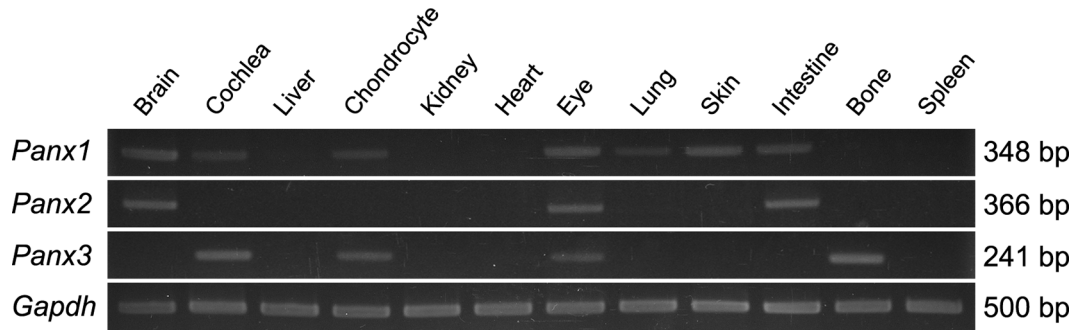


**Figure 1.** The mRNA expression based on reverse transcription PCR of pannexins (*PANX*) *1*, *2*, and *3* in 12 tissues of an embryonic d 13 chicken. *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

## Sequence Analysis and Characterization of Chicken PANX Gene Family

To investigate the full-length cDNA of the chicken *PANX* family, the brain was used for *PANX1* and *PANX2*, and the cochlea was used for *PANX3*. The full-length cDNA of the chicken *PANX* gene family was obtained from RACE and RT-PCR. Two mRNA isoforms of the chicken *PANX1* gene, *PANX1–1* and *PANX1–2,* were detected. The 5′ UTR of *PANX1–1* and *PANX1–2* were 14 and 249 bp, respectively, whereas the 3′ UTR of *PANX1–1* and *PANX1–2* were both 559 bp; therefore, the nucleotide sequences representing the complete cDNA of the chicken *PANX1–1* and *PANX1–2* were 1,875 and 1,735 bp, respectively (Figure 2A). The *PANX1–2* isoform of *PANX1* was found to have an alternative start codon region because of a 140-bp sequence deletion between c.195 and c.335 of *PANX1–1* (Figure 2A). The chicken *PANX2* gene was found to have 3 mRNA isoforms, *PANX2–1*, *PANX2–2*, and *PANX2–3*, with cDNA sequences of 2,404, 2,330, and 2,079 bp, respectively. The *PANX2–2* isoform had the longest ORF length, including the 148-bp 5′ UTR and the 241-bp 3′ UTR (Figure 2B). The *PANX2–1* isoform contained a 74-bp insert at c.1826 of the *PANX2–2* isoform, changing the stop codon position, whereas the *PANX2–3* isoform had a 251-bp deletion between c.375 and c.625 of the *PANX2–2* isoform, transferring the start codon position (Figure 2B). Only one form was identified in *PANX*3, for which the full length of cDNA was 1,452 bp, including a 77 bp 3′ UTR and 145 bp 5′ UTR (Figure 2C).

## Multiple Amino Acid Sequence Alignment and Phylogenetic Tree

The ORF of the chicken PANX family were obtained using the ORF finder program of the NCBI website. The putative proteins had the following length: 433 amino acids for PANX1, 645 amino acids for PANX2, and 409 amino acids for PANX3. The deduced amino

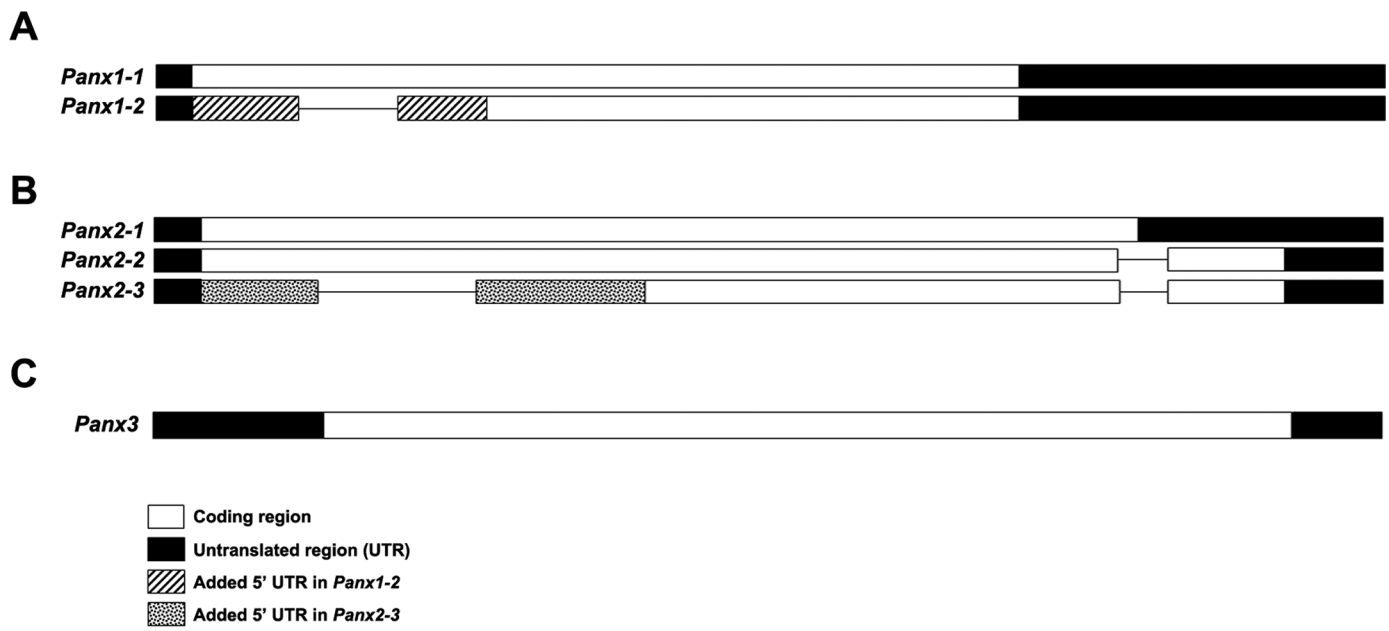


**Figure 2.** Schematic representation of the reverse-transcription (RT) PCR product and type of mRNA of pannexins (*PANX*) *1*, *2*, and *3* in chickens. (A, B, C) Two, three, and one type of *PANX1*, *PANX2*, and *PANX3* of mRNA exist in chickens, respectively.

acid sequences of the PANX family were aligned with previously reported human, mouse, rat, and cow sequences available in the NCBI database. Multiple sequence alignments indicated that chicken PANX1, 2, and 3 had an average of 70% homology at the amino acid level with Panx1, 2, and 3 in human (70, 80, and 70%), mouse (68, 81, and 68%), rat (67, 81, and 69%), and cow (67, 70, and 70%). Among the 3 Panx, Panx2 showed the greatest similarity between mammals and chickens.

Phylogenetic analyses of the amino acid sequences of the full coding region of the Panx family revealed close genetic relationships among all mammals, whereas chicken sequences showed significant genetic differences from mammal sequences in each of the Panx. The closest relationship between Panx among species was between human and rat sequences, followed by mouse, cow, and chicken sequences (Figure 3). Panx1 and Panx3 were most closely related to the common ancestor of the 3 Panx (Figure 3).

## Expression of PANX Family in Chicken Cochlea

Real-time PCR was performed to compare the relative expression of *PANX* family members in the cochlea at E13 and E18. As shown in Figure 4, expression of *PANX1* and *PANX3* differed significantly. Specifically, *PANX2* was barely expressed compared with *PANX1* and *PANX3* (data not shown). The expression of *PANX3* was highest among the 3 *PANX* gene family during the embryonic development and much greater than that of *PANX1* at E13 and E18. These findings were consistent with those of RT-PCR analysis. Comparison of tissues from E13 and E18 using real-time PCR revealed no significant difference in expression (Figure 4). We also conducted real-time PCR from the cochlea at E15, and the results showed a similar pattern in the E13 and E18.

*PANX* family expression in the cochlea at E18 was investigated based on evaluation of protein levels by immunofluorescent staining. The results showed a markedly different expression pattern between *PANX1* and *PANX3*, whereas no labeling was observed in *PANX2*, which was similar to the results obtained by real-time PCR. PANX1 was detected in the tegmentum vasculosum (Figure 5A, 5B), hair cells, supporting cells (Figure 5A, 5C), and ganglion neurons (Figure 5A, 5D), whereas PANX3 was observed in the bone surrounding the cochlea (Figure 6). In addition, PANX1 and PANX3 were both weakly detected in the tectorial membrane. We also performed immunohistochemistry in the cochlea for PANX1 and PANX3 at earlier stages, E11 and E13, with the results showing a similar pattern (data not shown).

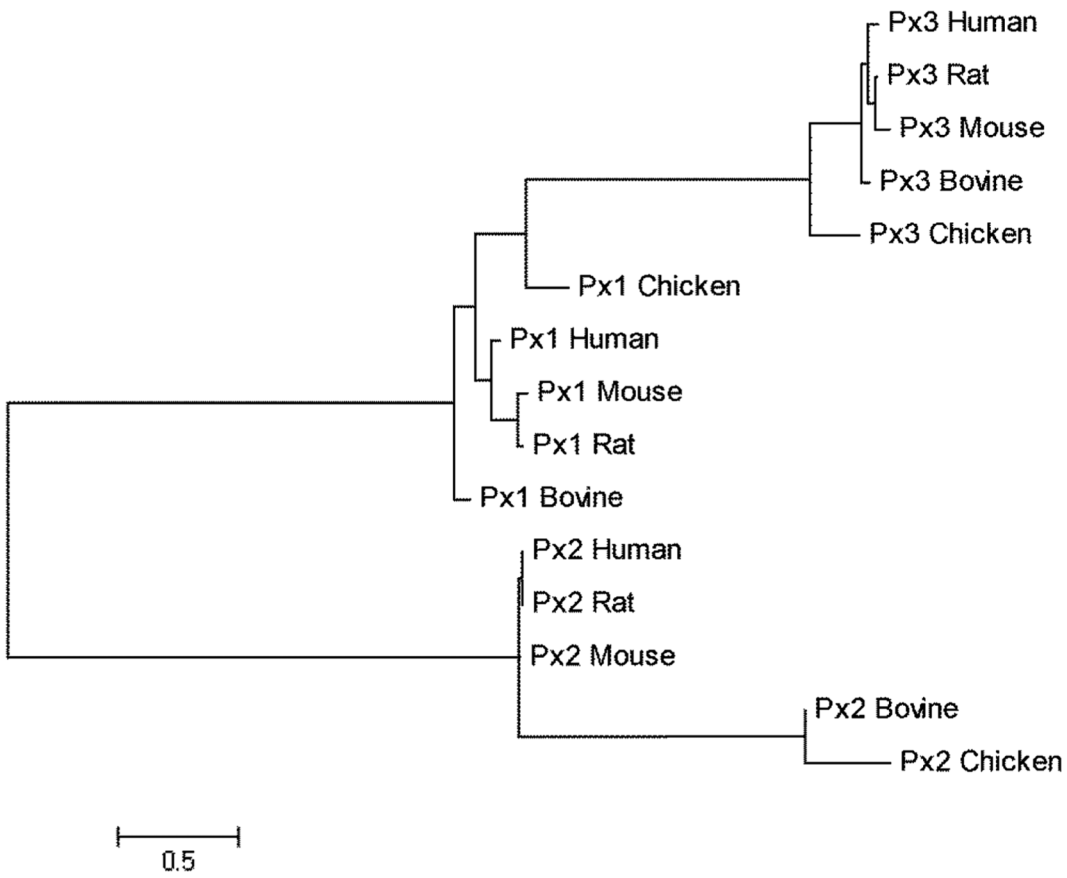


**Figure 3.** Phylogenetic tree analysis showing evolutionary distance from several species in the pannexin family, and distance of 3 pannexin genes from several species.

## DISCUSSION

Gap junctions are intercellular communication channels that facilitate ion exchange between cells, small molecules, and signaling molecules (Tang et al., 2008). The *Panx* genes have received attention for their potential to create gap junctions. The full sequences, molecular characteristics, and expression of the *Panx* gene family have been described for mammalian species, including humans and mice; however, little is known about the full sequence and expression of the *PANX* gene family in avian species, which are evolutionarily close. Thus, we conducted this study to identify the full length of the *PANX* gene family, analyzed the expression patterns in chicken, and carried out evolutionary analysis.

Mammalian *Panx* gene families have been reported to have one mRNA isoform (Bruzzone et al., 2003); however, human *PANX2* was reported to have 2 mRNA isoforms because of the insertion of a sequence that rearranged the ORF of *PANX2* and changed the start codon, reducing the ORF length (Baranova et al., 2004). In the present study, the chicken *PANX* gene family was found to have one or more mRNA isoforms, with the chicken *PANX2* gene having 3 mRNA isoforms (*PANX2–1*, *2–2*, and *2–3*). Among these isoforms, *PANX2–3* had a 251-bp deletion in the predicted exon 3 relative to the *PANX2–2* isoform, which changed the start codon position, producing a small protein that lacks the 3 transmembrane domains. Nevertheless, the *PANX2–3* isoform can be used to predict the creation of normal PANX2 protein, because it is similar to cow Panx2 mRNA and protein.

We performed amino acid alignment of human, mouse, rat, cow, and chicken sequences for comparison with Panx of other species. Panx2 showed the greatest homology and exhibited 200 to 300 more amino acids in the C-terminal region because of hydrophilic extension. The additional amino acids likely convey unique functions to Panx2 regulation (Yen and Saier, 2007). The Panx family has 2 conserved cysteine residues that form intramolecular disulfide bonds and are essential for intercellular docking in extracellular domains (Abascal and Zardoya, 2013; Penuela et al., 2013). Additionally, several conserved cysteines essential for intercellular docking of the gap junction to opposing cell membranes exist in the extracellular loops (Unger et al., 1999; Falk, 2000). In the present study, almost every cysteine of the chicken PANX family was found to be conserved, indicating that the *PANX* family mRNA sequences identified by this study were accurate.

As shown in the phylogenetic tree of Panx families of vertebrates based on human, mouse, rat, cow, and chicken sequences, these families evolved from a common ancestor. In each Panx clade, avian species diverged first and mammals separated later. Moreover, *Panx1* and *Panx3* were closest to the common ancestor among the 3 *Panx* genes. While the evolution of vertebrate Panx occurred, 2 rounds of duplication were observed, leading first to Panx2 and then to Panx1 and Panx3, indicating that the homology of Panx1 and Panx3 was higher to each other than to Panx2 (Baranova et al., 2004; Penuela et al., 2007; Fushiki et al., 2010). Moreover, vertebrate Panx families did not divide into 3 clades according to species classification, but rather according to paralogs of protein families. In other words, the divergence of Panx families may have occurred earlier than species differentiation of vertebrates (Fushiki et al., 2010; Abascal and Zardoya, 2013).

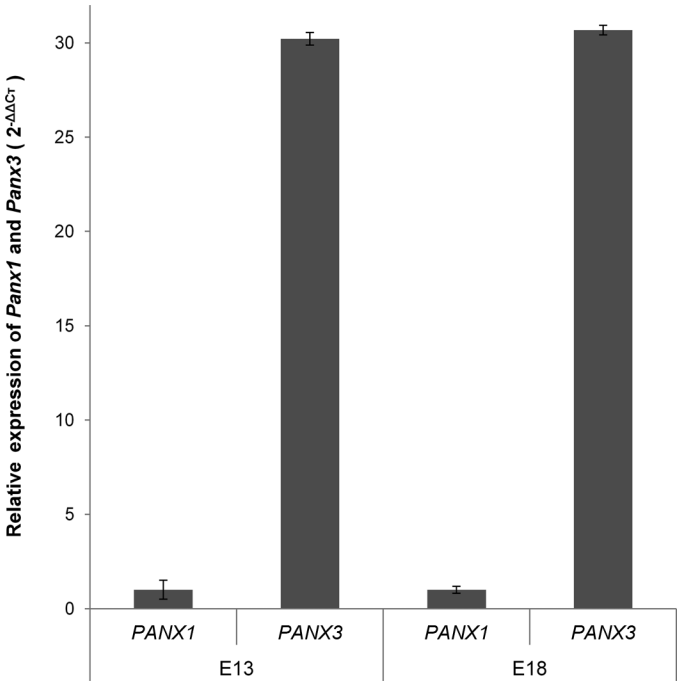


**Figure 4.** The expression of pannexin (*PANX*) *1* and *3* mRNA levels in the chicken embryonic cochleae as determined by real-time PCR. Each bar indicates the mean values ± SEM of at least 3 experiments. In addition, statistical analysis using SPSS (SPSS Inc., Chicago, IL) showed significant differences between *PANX1* and *PANX3* of both embryonic d (E) 13 and 18 ($P < 0.05$).

Different expression patterns of the Panx family were observed in mammals in previous studies. Panx1 has been reported to be ubiquitously expressed (Wang et al., 2009), and it has been found in tissues of the brain (Bruzzone et al., 2003; Baranova et al., 2004; Vogt et al., 2005), spinal cord (Bruzzone et al., 2003), testis (Turmel et al., 2011), eye (Dvoriantchikova et al., 2006), and spleen (Abascal and Zardoya, 2013) in rodents. In contrast, Panx2 has only been reported to be expressed in the brain and spinal cord (Bruzzone et al., 2003; Baranova et al., 2004; Vogt et al., 2005; Zappala et al., 2007), whereas Panx3 was detected in the skin, cochlea, chondrocytes, and bone (Penuela et al., 2007, 2008; Wang et al., 2009; Iwamoto et al., 2010). Because the chicken PANX family showed similar expression patterns to that of the mammalian family, it is likely that Panx plays an important role in the tissues that were expressed.

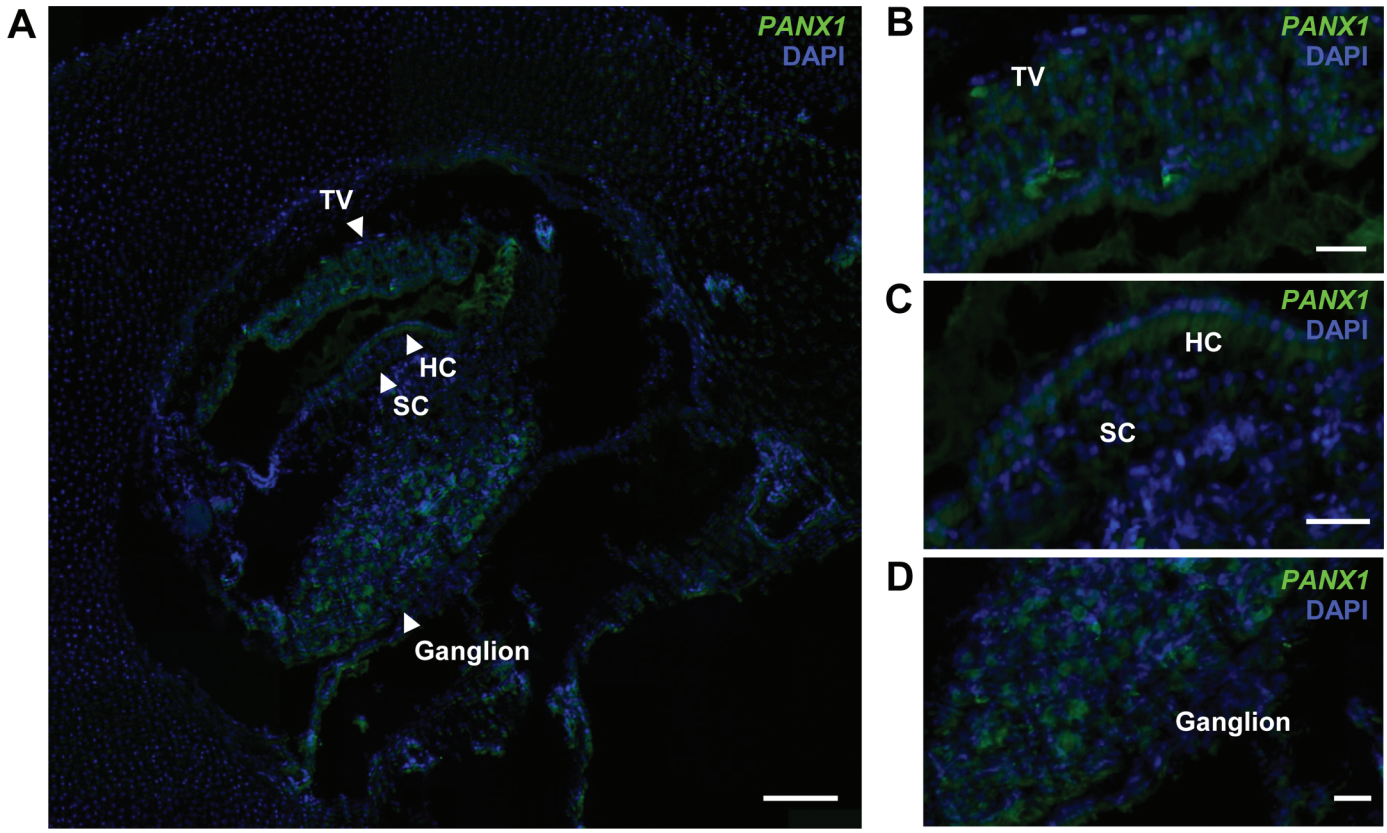


**Figure 5.** Immunofluorescent staining for pannexin (*PANX*) 1 in the chicken cochlea at embryonic d (E) 18. (A) *PANX1* was detected in the chicken cochlea at (B) tegmentum vasculosum (TV), (C) hair cells (HC), supporting cells (SC), and (D) ganglion. Scale bars: 200 μm (A); 50 μm (B–D).

Panx family expression of the inner ear cochlea has been reported in rodents (Tang et al., 2008; Wang et al., 2009); however, the accurate function of the Panx family in the cochlea is unclear. Previous studies of Panx1 expression in rodent cochleae revealed that it was observed in various regions, including inner and outer sulcus cells, interdental cells of the spiral limbus, the spiral prominence of the lateral wall, Claudius cells,

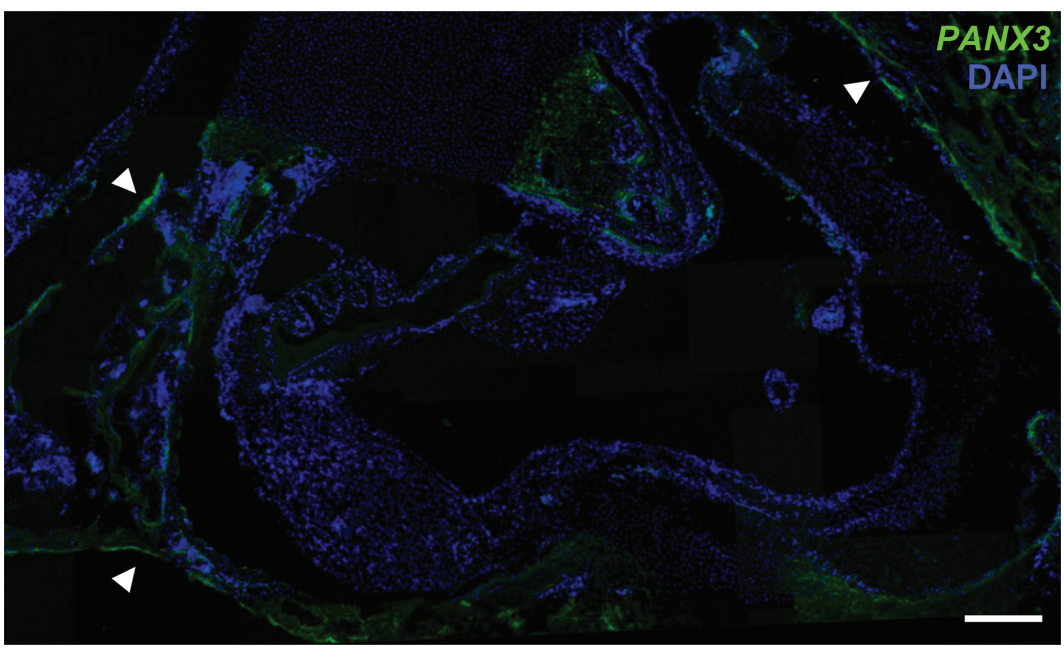


**Figure 6.** Immunofluorescent staining for pannexin (*PANX*) 3 in the chicken cochlea at embryonic d 18. The *PANX3* expression is only present in the bone surrounding the cochlea. The arrows indicate the bone. Scale bar: 200 μm.

pillar cells, Hensen cells, and Boettcher cells of the organ of Corti (Tang et al., 2008; Wang et al., 2009). Outer sulcus cells participate in $K^+$ transport and regulate the endolymphatic $K^+$ concentration (Marcus and Chiba, 1999); thus, Panx1 may play a role in maintenance of inner ear homeostasis (Wang et al., 2009). Panx1 was detected in the hair cells, supporting cells, and ganglion neurons, as well as the tegmentum vasculosum in chickens. Previous studies suggested that supporting cells of the avian cochlea might contribute to the maintenance of high endolymphatic $K^+$ concentration via electrophysiological analysis (Ninoyu et al., 1987; Runhaar et al., 1991; Sauer et al., 1999). Therefore, it is possible that PANX1 also plays a role in maintenance of avian cochlea homeostasis. The tegmentum vasculosum, one of the expression regions of PANX1, is functionally quite similar to the stria vascularis of mammals (Ishiyama et al., 1970; Cotanche and Sulik, 1982; Cotanche et al., 1987; Hossler et al., 2002b). It is well known that the stria vascularis and tegmentum vasculosum show a high level of $Na^+$, $K^+$-ATPase activity (Kuijpers et al., 1967; Kujpers, 1974; Hossler et al., 2002a). Additionally, the Panx family has been shown to act as hemichannels that release ATP into the extracellular space and to function as gap junctions in osteoblasts (Bruzzone et al., 2005; Ishikawa et al., 2011). Therefore, it is likely that $Na^+$, $K^+$-ATPase might be regulated by Panx, which regulate the release of ATP. In this study, PANX3 in chickens was expressed in the surrounding bone of the cochlea and found to be present in levels equal to those seen in mammals (Wang et al., 2009). Panx3 was recently reported to be a novel target protein for runt-related transcription factor 2 (Runx2), which is a key protein for bone formation (Bond et al., 2011). In addition, Panx3 promotes osteoblast differentiation through the hemichannel, which enables the release of ATP and activation of the PI3-Akt pathway in the membrane, as well as the gap junction, thereby enabling the conveyance of $Ca^{2+}$ between adjacent cells (Ishikawa et al., 2011). Overall, our results indicate that Panx3 has equivalent patterns to those in mammalian and avian species, indicating that it plays important roles in bone development.

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