Extensive Expansion of the Claudin Gene Family in the Teleost Fish, *Fugu rubripes*

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In humans, the claudin superfamily consists of 19 homologous proteins that commonly localize to tight junctions of epithelial and endothelial cells. Besides being structural tight-junction components, claudins participate in cell–cell adhesion and the paracellular transport of solutes. Here, we identify and annotate the claudin genes in the whole-genome of the teleost fish, *Fugu rubripes* (*Fugu*), and determine their phylogenetic relationships to those in mammals. Our analysis reveals extensive gene duplications in the teleost lineage, leading to 56 claudin genes in *Fugu*. A total of 35 *Fugu* claudin genes can be assigned orthology to 17 mammalian claudin genes, with the remaining 21 genes being specific to the fish lineage. Thus, a significant number of the additional *Fugu* genes are not the result of the proposed whole-genome duplication in the fish lineage. Expression profiling shows that most of the 56 *Fugu* claudin genes are expressed in a more-or-less tissue-specific fashion, or at particular developmental stages. We postulate that the expansion of the claudin gene family in teleosts allowed the acquisition of novel functions during evolution, and that fish-specific novel members of gene families such as claudins contribute to a large extent to the distinct physiology of fishes and mammals.

[Supplemental material is available online at www.genome.org. The sequence data from this study have been submitted to GenBank under accession nos. AY554341–AY554396. The following individuals kindly provided reagents, samples, or unpublished information as indicated in the paper: T. Suzuki.]

The function of epithelial and endothelial cells as barriers between different compartments of tissues and organs is dependent on their ability to establish and maintain cell–cell adhesion through tight junctions (TJ; Tsukita et al. 2001). The TJ also prevents the diffusion of proteins and lipids between the apical and basolateral plasma membrane domains of epithelial and endothelial cells and is thus important for the generation and maintenance of membrane polarity in these cell types (van Meer et al. 1986). Furthermore, TJ form a continuous intercellular seal that restricts and regulates the paracellular transport of water, small solutes, and immune cells (Tsukita and Furuse 2000, 2002; Heiskala et al. 2001).

Claudins, a family of ~20 homologous proteins that span the plasma membrane four times (see Fig. 1A; Furuse et al. 1998a; Morita et al. 1999a; Heiskala et al. 2001; Gonzalez-Mariscal et al. 2003), are structural components of TJ and major determinants of the fibrils observed by cryo-electron microscopy of freeze-fractured TJ (Furuse et al. 1998b). Homo- and heterotypic interactions between claudins on paired strands of adjacent cells provide the intercellular seal responsible for the function of TJ as paracellular diffusion barriers (Furuse et al. 1999; Inai et al. 1999). On the cytosolic face of the membrane, claudins are tethered to the actomyosin cytoskeleton via interaction with scaffolding proteins such as the zonula occludens (ZO) proteins (Itoh et al. 1999; Gonzalez-Mariscal et al. 2003). Besides epithelia and endothelia, claudins are also found at sites of intimate cell–cell contact in other cell types such as Schwann cells (Morita et al. 1999b) and keratinocytes (Brandner et al. 2002; Tsukita and Furuse 2002).

Tissue- and cell-type-specific expression as well as the variability in homo- and hetero-oligomerization of individual claudins on neighboring cells are thought to determine the composition of the resulting intercellular complex and, in turn, the characteristic permeability properties observed for different epithelia and endothelia (Heiskala et al. 2001; Tsukita et al. 2001). While the intercellular seal provided by TJ acts as a barrier for the diffusion of large molecules, it allows the selective paracellular transport of water and, depending on the particular claudins, small solutes. Claudins 2 and 4, for example, have been linked to intercellular Na^+ and K^+ permeability (Van Itallie et al. 2001; Amasheh et al. 2002), whereas claudin 16 is critical for renal resorption of Ca^{2+} and Mg^{2+} (Simon et al. 1999; Muller et al. 2003), indicating that claudins can function as highly selective paracellular ion channels. Consistent with specific functions for individual claudins, mutations in an increasing number of claudins are associated with distinct diseases in humans or animal models (Gow et al. 1999; Simon et al. 1999; Weber et al. 2000; Wilcox et al. 2001; Furuse et al. 2002), supporting the idea that individual claudins mediate specific functions.

In contrast to the ~20 claudin genes present in mammals (Heiskala et al. 2001; Tsukita and Furuse 2002), invertebrates such as *Caenorhabditis elegans* or *Drosophila melanogaster* only possess four to five claudin-related genes (Asano et al. 2003; Behr et al. 2003). This, together with the frequently observed tissue- and cell-type-restricted expression of individual claudins in mammals (Heiskala et al. 2001), indicates that the evolution of increasingly complex tissues and organs was paralleled with the expansion of the claudin gene family, possibly to accommodate new or overlapping functions. The recent completion of the sequence of the human (Lander et al. 2001; Venter et al. 2001) and the Japanese pufferfish, *Fugu rubripes* (*Fugu*; Aparicio et al. 2002) genomes allows a detailed comparison of the complexity and organization of claudin genes between mammals and teleosts, the two most distant bony vertebrate lineages. *Fugu* has a compact genome of only 365 Mb (the smallest known vertebrate genome), yet it possesses a repertoire of genes similar to that of

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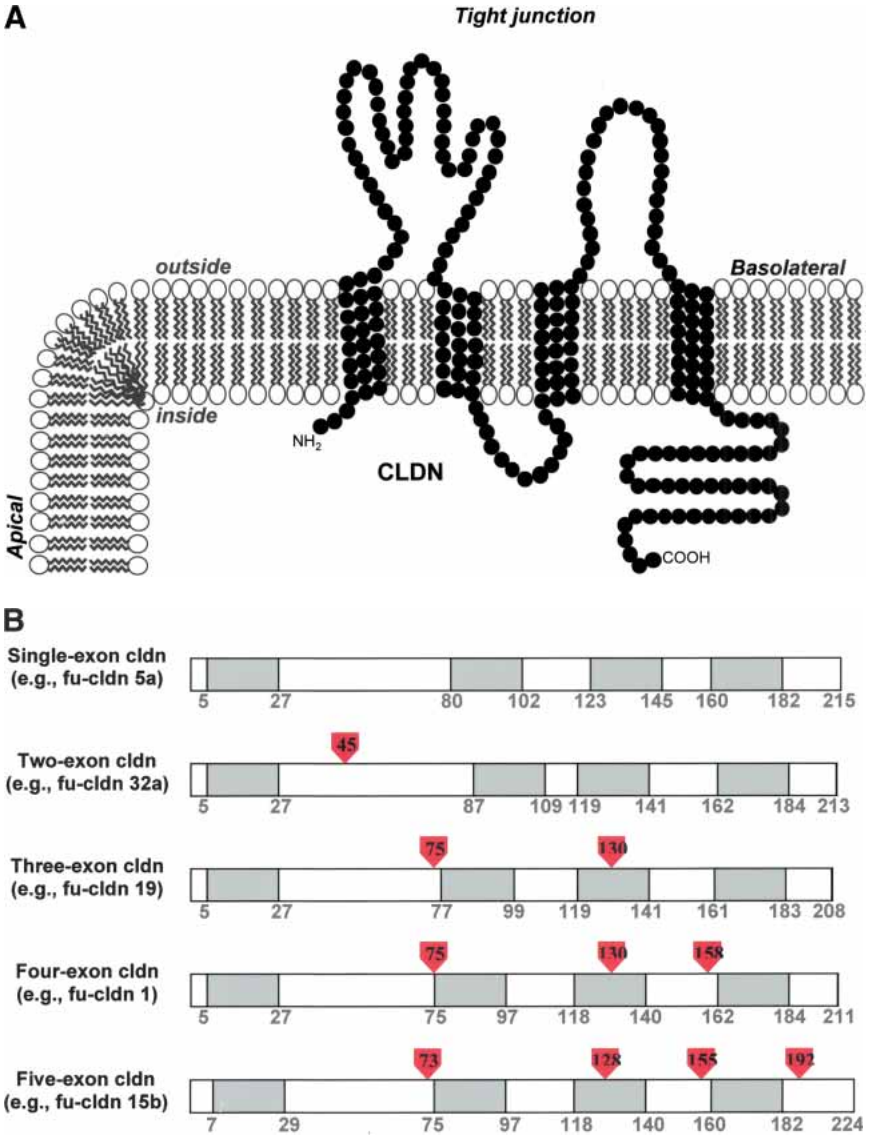


Figure 1 Topology and exon–intron organization of *Fugu* claudins. (*A*) Membrane topology of claudins. A diagram of the typical topology of claudins in the lipid bilayer and their location at the tight junction between the apical and lateral plasma membrane of epithelial cells is shown. (*B*) Exon–intron structure of typical *Fugu* claudin genes. The approximate location of introns in claudin genes encoded by one, two, three, or four exons is shown based on a typical example. Amino acids are numbered and show the boundaries of the four transmembrane domains (shaded in gray), which are typical for claudins. Intron position is shown by the red arrowheads (the numbers within correspond to the location in relation to the amino acid sequence).

human (Brenner et al. 1993). The compactness of the *Fugu* genome is attributed to its short introns and compressed intergenic regions because of a paucity of repetitive sequences (Venkatesh et al. 2000), making it an ideal vertebrate genome for characterizing genes and their regulatory regions.

Here, we report on the annotation of the claudin genes in *Fugu* and their expression profile, and the evolution of the vertebrate claudin gene family.

RESULTS AND DISCUSSION

Claudin Gene Prediction and Annotation

A search of the complete genome sequence of *Fugu* (Aparicio et al. 2002), and annotation and analyses of the claudin genes identified a total of 56 claudin gene sequences, distributed on 31 scaffolds (Table 1). The annotated sequences of all the *Fugu* claudin genes have been submitted to GenBank (accession numbers AY554341–AY554396). The 56 claudin genes found in *Fugu* represents a substantial increase when compared with the total number of claudin genes in humans or mouse, consistent with the previously observed duplication of many genes in *Fugu* (Taylor et al. 2001, 2003; Aparicio et al. 2002; Williams et al. 2002a,b; Yu et al. 2003). The presence of additional genes in *Fugu* and other teleosts such as zebrafish has led to the hypothesis that a whole-genome duplication occurred in the teleost lineage (Amores et al. 1998; Taylor et al. 2003; Christoffels et al. 2004). However, in contrast to the duplication observed for most other genes in *Fugu* and zebrafish, the 56 claudin genes represent an almost threefold increase as compared with the 20 claudin genes in mammals, and cannot be accounted for by a single genome duplication event.

Of the 56 *Fugu* claudin genes, 24 are located independently on different scaffolds, and the remaining 32 are found in seven clusters containing two or more genes each (Table 1). Of particular interest are gene duplications on scaffolds 102 and 302, each containing 10 claudin genes spread over a distance of ~23 kb. Of these 20 genes, 17 are closely related to human claudin 3 and 4 (see below), which are located 60 kb apart on human Chromosome 7q11.23.

Of the 56 claudin genes, 36 are encoded in a single exon, three by two exons, and the remaining 17 by three or more exons (Table 1). The location of introns in relation to the four transmembrane domains that are typical for claudins is shown for representative genes encoded by one or more exons (Fig. 1B). Of the 18 orthologs characterized so far in zebrafish (Kollmar et al. 2001), 10 claudin genes are encoded by a single exon and six by multiple exons, like their orthologs in *Fugu* (Table 1). Thus, it is likely that most of the duplications have occurred early in the teleost linage and are shared by related subgroups of this lineage. In contrast, only five of the 19 human genes are encoded by one exon, with one ortholog being encoded by two exons and the rest by three exons or more. Of the 32 orthologous genes shared between *Fugu* and humans, 20 *Fugu* genes have the same number of exons as their human counterparts. Thus, the claudin loci in *Fugu* and humans appear to be very dynamic in terms of "gain" and "loss" of spliceosomal introns.

Phylogenetic Analysis of Claudin Genes

A total of 109 claudin genes identified from *Fugu*, human, mouse, and zebrafish were used to generate a phylogenetic tree, using a claudin-related gene from *Ciona* as an outgroup. Because a large number of diverse genes were involved, several preliminary rounds of alignment and tree construction with the complete set

Table 1. Nomenclature, Scaffold Location, and Exon/Intron Organization of *Fugu* Claudin Genes

S/No.	*Fugu* scaffold number	*Fugu* claudin number	No. of exons in *Fugu*	No. of exons in human ortholog[a]	Zebrafish ortholog[a] exon structure
1	2	fu-cldn 5a	1	1	—
2		fu-cldn 33c	1	—	—
3	8	fu-cldn 7a	4	4	zf-cldn 7/M
4		fu-cldn 3c	1	1	—
5		fu-cldn 3d	1	1	zf-cldn c/S
6		fu-cldn 27c	1	—	—
7		fu-cldn 27d	3	—	—
8	102	fu-cldn 28b	1	—	zf-cldn e/S
9		fu-cldn 28c	1	—	—
10		fu-cldn 29a	1	—	zf-cldn d/S
11		fu-cldn 29b	1	—	—
12		fu-cldn 30c	1	—	—
13		fu-cldn 30d	1	—	zf-cldn a/b/S
14	107	fu-cldn 18	4	5	—
15	137	fu-cldn 23b	1	—	—
16	161	fu-cldn 12	2	3	zf-cldn 12/S
17	196	fu-cldn 7b	4	4	zf-cldn 7/M
18	281	fu-cldn 23a	1	—	—
19	300	fu-cldn 14a	2	3	—
20		fu-cldn 3a	1	1	zf-cldn h/S
21		fu-cldn 3b	1	1	—
22		fu-cldn 5c	1	1	—
23		fu-cldn 6	1	1	zf-cldn j/S
24	302	fu-cldn 13	1	—	—
25		fu-cldn 27a	1	—	—
26		fu-cldn 27b	1	—	zf-cldn f/S
27		fu-cldn 28a	1	—	zf-cldn e/S
28		fu-cldn 30a	1	—	—
29		fu-cldn 30b	1	—	zf-cldn a/b/S
30	415	fu-cldn 20a	1	1	—
31	457	fu-cldn 31	1	—	zf-cldn g/S
32	552	fu-cldn 1	4	4	zf-cldn 19/M
33	628	fu-cldn 2	1	2	—
34	641	fu-cldn 20b	1	1	—
35	654	fu-cldn 11b	3	3	zf-cldn 11/M
36	696	fu-cldn 32b	1	—	—
37	812	fu-cldn 15b	5	5	—
38		fu-cldn 8a	1	1	—
39	863	fu-cldn 8b	1	1	—
40		fu-cldn 8c	1	1	—
41		fu-cldn 8d	1	1	—
42	1289	fu-cldn 11a	3	3	zf-cldn 11/M
43	1321	fu-cldn 10d	4	5	—
44		fu-cldn 10e	3	5	—
45	1339	fu-cldn 10a	4	5	—
46	2207	fu-cldn 32a	2	—	zf-cldn l/S
47	2235	fu-cldn 5b	1	1	—
48	2355	fu-cldn 19	3	4	—
49	2558	fu-cldn 26	4	—	—
50	2732	fu-cldn 25	4	—	zf-cldn 10/M
51	2743	fu-cldn 14b	1	3	—
52		fu-cldn 33b	1	—	—
53	2835	fu-cldn 15a	4	5	—
54	2840	fu-cldn 10b	4	5	—
55		fu-cldn 10c	4	5	—
56	3054	fu-cldn 33a	1	—	—

Each of the predicted 56 *Fugu* claudin genes was named according to its human ortholog (see Fig. 1), with alphabetical suffixes if there was more than one *Fugu* ortholog. If a human ortholog could not be identified, the identity of such genes was confirmed after phylogenetic analysis, and new numbers were assigned from 25 onward. The scaffolds on which the different *Fugu* claudin genes are located are listed. The exon–intron structures of *Fugu* genes were predicted based on their homology to known claudin genes from other organisms, and, where the boundaries were not clear, the intron acceptor and donor sites and the corresponding open reading frames were determined by visual inspection. The number of exons encoding the human orthologs is provided for reference. Where data were available, zebrafish orthologs were classified into single (S) or multiple (M) exon genes (Kollmar et al. 2001).
[a]Where applicable/data available.

and subsets of genes were computed, allowing us to divide the genes into two major subgroups and subsequently generate their trees separately (Fig. 2A,B). For convenience, the claudin gene family was grouped into nine classes (Class I to Class IX) of related genes. The orthology of *Fugu* genes was assigned based on their phylogenetic relationships to the known claudin genes

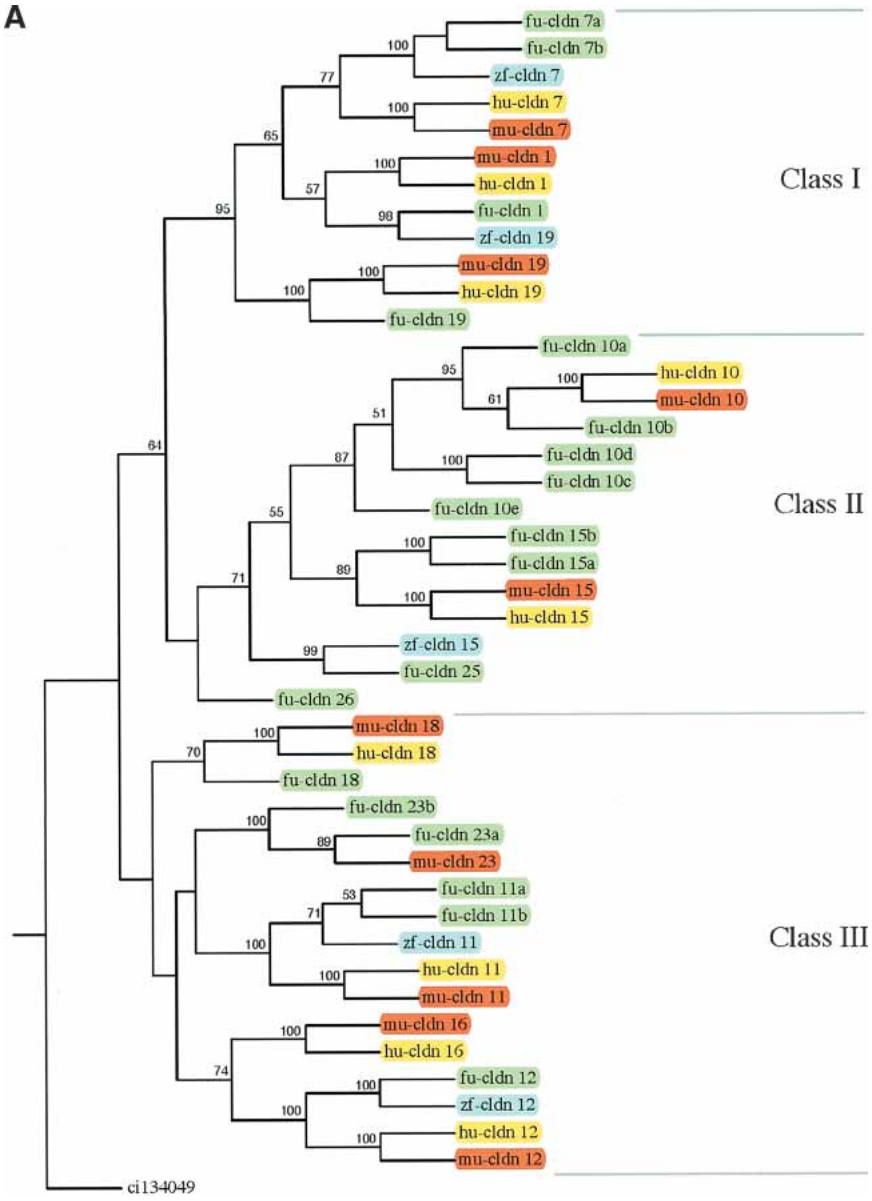


Figure 2 (Continued on next page)

from humans and mouse. Duplicate copies of genes are denoted by an alphabetic suffix (a, b, etc.). Novel genes found only in *Fugu* were given numbers starting from 25. Overall, 37 *Fugu* claudin genes were found to be orthologous to 17 mammalian claudin genes, with the rest being unique to the fish lineage. *Fugu* does not contain the ortholog for mammalian claudin 16. With few exceptions, *Fugu* claudin genes encoded by a single exon clustered together (Classes I–III), whereas those with multiple exons grouped to Classes IV–IX. Single-exon claudin genes also have more paralogs, suggesting retrotransposition of a first copy followed by tandem duplications as a possible mechanism for this expansion.

Multiple Duplications of the Ancestral Claudin 3 and Claudin 4 Gene Cluster in Teleosts

The phylogenetic tree for the Class V group of claudin genes (Fig. 1B) reveals that mammalian claudin 3 and 4 are duplicate genes that arose in the mammalian lineage. The close linkage of claudin 3 and claudin 4 genes on human Chromosome 17 indicates that they arose as the result of tandem duplication (Fig. 3). In *Fugu*, 17 claudin genes found in clusters on scaffolds 102 and 302 (Class V and VI genes) are closely related to mammalian claudin 3 and 4 (Fig. 2B). The genomic organization of the *Fugu* genes suggests that the *Fugu* ortholog of the human claudin 3 and 4 genes has undergone several successive rounds of *cis* and *trans* duplications to give rise to two clusters of 17 genes. Because orthologs for some of these *Fugu* genes are also present in zebrafish (Fig. 2B), a distantly related teleost to *Fugu*, the duplications appear to have occurred very early during the evolution of teleosts, implying that the majority of teleosts possess this complement of claudin genes.

The results of the gene prediction and phylogenetic analysis also offer insight into the possible order of duplications that occurred in scaffolds 102 and 302 (Fig. 4). With the exception of *cldn5c*, *cldn6*, and *cldn13*, the remaining 17 genes are all closely related to human *CLDN3* and *CLDN4*, and the pair of claudin genes that are direct paralogs in these scaffolds can be identified. Chromosomal fragments represented by scaffold 102 or 302 could have been more ancient, and intrachromosomal duplications (dotted arrows) on the ancestral chromosomal fragment had already resulted in the presence of at least seven genes. A single duplication event, presumably the postulated whole-genome duplication, then caused a duplication of this fragment (dotted lines). This event is supported by the conservation of the order and orientation of paralogous genes on the two scaffolds. The paralogs of claudin 29a and 28c (scaffold 302) were subsequently lost on scaffold 302, whereas claudin 29a duplicated once more within scaffold 102.

Besides the extensive duplication of genes on scaffolds 102 and 302, there are several other *Fugu* duplications that presumably arose as part of the proposed whole-genome duplication in the teleost lineage. These include fu-claudin 7a and 7b (Class I); claudin 15a and 15b (Class II); claudin 11a and 11b (Class III); claudin 20a and 20b (Class IV); claudin 5a and 5b (Class VII); claudin 8c and 8d (Class VIII); and claudin 33a and b (Class IX).

Independent Claudin Gene Duplication in Mammals

Independent gene duplications have occurred in the mammalian lineage as well. For example, human claudin 17 and 8 (Class VIII) are the result of gene duplication in the mammalian lineage. Similarly, mammalian claudin 3 and 4 (Class V) and claudin 9 and 6 (Class VII) are the results of duplications in the mammalian lineage.

Independent Claudin Gene Loss in Mammals and Teleosts

In addition to gene duplications, claudin genes have been lost in both mammalian and fish lineages. From the phylogenetic tree

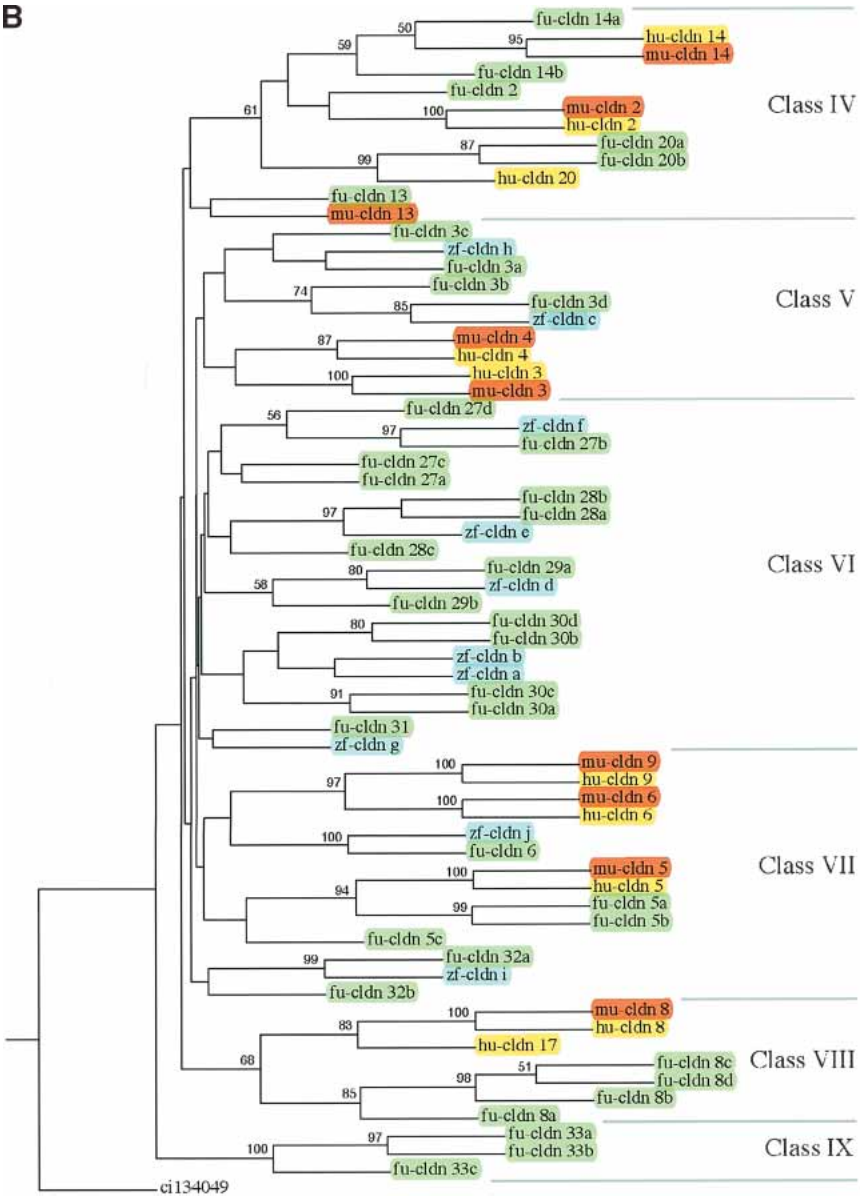


Figure 2 Consensus phylogenetic tree of claudin proteins. The trees were generated using 109 claudin protein sequences from human (hu, yellow), mouse (mu, red), *Fugu* (fu, green), and zebrafish (zf, blue) and using a related gene from *Ciona* (ci) as an outgroup gene. To reduce the complexity of phylogenetic trees, claudin genes were first divided into two groups of related proteins based on the results of preliminary phylogenetic analysis, and phylogenetic trees were generated separately for the two groups. Based on the origin and phylogenetic relationships, vertebrate claudin genes were classified into nine classes (*A*, Class I–III; *B*, Class IV–IX). Bootstrap values >50% are given.

(Fig. 1A,B), it can be inferred that orthologs of *Fugu* claudin 32 (Class VII) and claudin 25 (Class II) have been lost in the mammalian lineage. Interestingly, whereas the ortholog of *Fugu* claudin 13 (Class IV) is present in mouse, there is no ortholog in humans. On the other hand, the fish ortholog of mammalian claudin 16 (Class III) has been lost in the *Fugu* lineage.

Tissue- and Developmental-Stage-Specific Expression of *Fugu* Claudin Genes

Given the unexpected expansion of the claudin gene family in *Fugu*, it was of interest to determine how many of these genes are actually expressed, and how many of the duplicate copies have an identical expression pattern, which could suggest redundant function. For this purpose, we did RT-PCR using primers specific to each gene (see Supplemental material) to check the expression in various adult *Fugu* tissues (Fig. 5A). Surprisingly, most of the *Fugu* claudin genes were found expressed in a more or less tissue-specific manner (Table 2), indicating that they are functional and have distinct expression patterns. *cldn12* and *cldn26* were expressed in all *Fugu* tissues analyzed. Expression of human *CLDN12* has been reported for brain, prostate, colon, and uterus (Heiskala et al. 2001).

Transcripts for only 10 *Fugu* claudin genes (*cldn5c*, *cldn10a*, *cldn10e*, *cldn14a*, *cldn15b*, *cldn18*, *cldn20b*, *cldn27c*, *cldn33a*, and *cldn33c*) could not be detected in the tissues analyzed. With the exception of *cldn27c*, *cldn33a*, and *cldn33c*, all these genes have human orthologs that are expressed in brain, lung, and various other tissues (*CLDN5*); brain (*CLDN10*); heart (*CLDN14*); intestine (*CLDN15* and *CLDN20*); or lung and stomach (*CLDN18*; Table 2; Heiskala et al. 2001). Because, except for *cldn18*, these claudin genes have undergone duplication in teleost and duplicated genes were expressed in the corresponding tissues (i.e., *cldn5b* and *cldn10b* in brain, *cldn14b* in heart, *cldn15a* in intestine), it is conceivable that *cldn5c*, *cldn10a*, *cldn10e*, *cldn14a*, and *cldn15b* are pseudogenes. Alternatively, however, the claudins we failed to detect may be expressed in tissues other than those analyzed, or during earlier stages of *Fugu* development. Indeed, three out of the 10 claudins (i.e., *cldn5c*, *cldn27c*, and *cldn33c*) not detected in the adult tissues analyzed were expressed in an early somite stage of the *Fugu* embryo (Fig. 5B; Table 2), indicating that at least some of the claudin genes are subject to developmental regulation.

All the remaining *Fugu* claudin genes showed a more or less restricted tissue-specific expression pattern. Interestingly, many *Fugu* claudins were only detected in organs that are in direct contact with the aquatic environment of fish. Expression of *cldn1* and *cldn10c* was restricted to eyes, gills, and skin; *cldn8d* and *cldn28b* to gills and skin; and *cldn13* was only found in gills. Several other claudins with a restricted expression pattern were detected in gills (*cldn19*, *cldn33b*, and *cldn27d*). Given the importance of claudins for the permeability characteristics of epithelia, it is likely that these claudins are involved in regulating the exchange of specific solutes with the aqueous environment. *cldn1*, expressed in the skin of *Fugu*, is found in the epidermis of mammals, where it is crucial for preventing water loss across the skin and dehydration (Furuse et al. 2002). Other claudin genes showing a restricted expression profile are *cldn10d* (intestine), *cldn11a* (brain, heart, kidney, testis), *cldn11b* (liver), *cldn23b* (intestine, muscle), *cldn15a* (intestine, kidney), and *cldn29a* (ovary and testis). In addition to gills and skin, heart, kidney, and intestine express the largest number of different claudin genes. Similar to gills and skin, kidney and

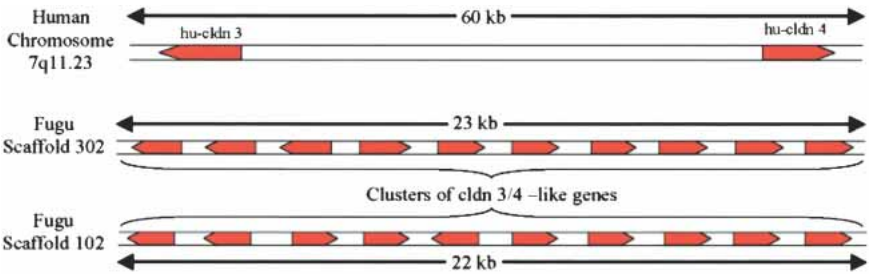


Figure 3 Extensive duplication of an ancestral claudin 3 and 4 gene in *Fugu*. The human *CLDN3* and *CLDN4* locus on Chromosome 7 is compared with the *cldn3*/*cldn4*-like gene loci on scaffolds 102 and 302 of *Fugu*. The location and orientation of the genes are indicated.

intestine are also involved in maintaining osmotic homeostasis in fishes.

Because *Fugu* claudin genes with a similar tissue distribution may encode conserved regulatory elements in their 5′ noncoding region, we selected, as an example, two claudin genes with a similar expression profile, *cldn1* and *cldn10c* (Table 2), for promoter analysis (Loots et al. 2002). The two claudin genes shared three highly conserved regions that may encode potential regulatory elements for tissue- and/or stage-specific expression in their 5′ part (Fig. 6A). In contrast, two closely related claudin genes with a different tissue distribution, *cldn11a* and *cldn11b* (Table 2), did not share conserved promoter regions (Fig. 6B), suggesting that their promoters have diverged considerably since the duplication such that they either share the expression domains of the parent gene ("subfunctionalization") or have acquired novel expression domains ("neofunctionalization").

The variability of the expression profiles observed even for closely related *Fugu* genes suggests that the duplicated claudin genes may have acquired new and unique functions. Comparison of the tissue expression of *Fugu* and mammalian claudin orthologs did not reveal an extensive correlation, possibly reflecting the acquisition of similar functions by some of the different teleost claudins. Thus, although the specificity and functionality of *Fugu* claudins diverged from their human orthologs, it is possible that the overall functionalities were both compensated and/or expanded by the duplicated claudins. Duplication of claudin genes with the retention of their function may also have enabled teleosts, by introducing evolutionary modifications in the promoter regions, to evolve new temporal and/or spatial expression patterns of a particular claudin gene.

In conclusion, a total of 56 unique claudin genes were identified and analyzed in *Fugu*. The phylogenetic analysis revealed that the claudin genes have undergone unprecedented expansion in the teleost lineage, leading to an almost threefold higher number of claudins in *Fugu* as compared with mammals. Although some of the additional genes arose as a result of the proposed whole-genome duplication in the fish lineage, a substantial number of new genes are the result of tandem duplications in the fish lineage. In addition to extensive tandem duplications, some of the claudin genes that were present in the ancestral vertebrate have been lost in the fish lineage. Independent duplications and deletions have also occurred in the mammalian lineage, albeit on a smaller scale as compared with the fish lineage. The bulk of the 56 *Fugu* claudin genes are expressed in a more or less tissue-specific fashion, suggesting that individual members of the expanded families may have acquired new or overlapping functions in teleost fish. These results show that claudins are a dynamic family of genes in vertebrates and have continued to evolve after the divergence of the common ancestors of the mammalian and fish lineages. The new genes in each lineage may have acquired novel functions, reflecting the unique tissues and cell types in the two distant vertebrates. It is likely that the large number of novel claudin genes found in fishes are involved in the exchange of specific ions with the aquatic environment and maintenance of osmotic balance. The expression of a large number of claudin genes in the gills and kidney, which play a major role in these functions in fishes, supports this view.

METHODS

Gene Prediction

The human claudin protein sequences, 19 in all, were retrieved from the National Center for Biotechnology Information (NCBI). They were searched against the "draft" *Fugu* genome database (3rd assembly) at http://www.fugu-sg.org using the TBLASTN program to identify homologous claudin loci in the *Fugu* genome. The "draft" *Fugu* genome sequence comprises 8597 scaffolds each >2 kb long, and represents 95% of the nonrepetitive portion of the 365-Mb *Fugu* genome (Aparicio et al. 2002). The identified *Fugu* gene loci on the scaffolds were then searched against the NCBI human genome protein database using BLASTX to confirm that they, in fact, code for claudins, and to identify their putative human orthologs and obtain an initial closest human claudin match. The exon–intron structure of *Fugu* genes was predicted by their homology to known claudin genes from other organisms, and where the boundaries were not clear, the intron

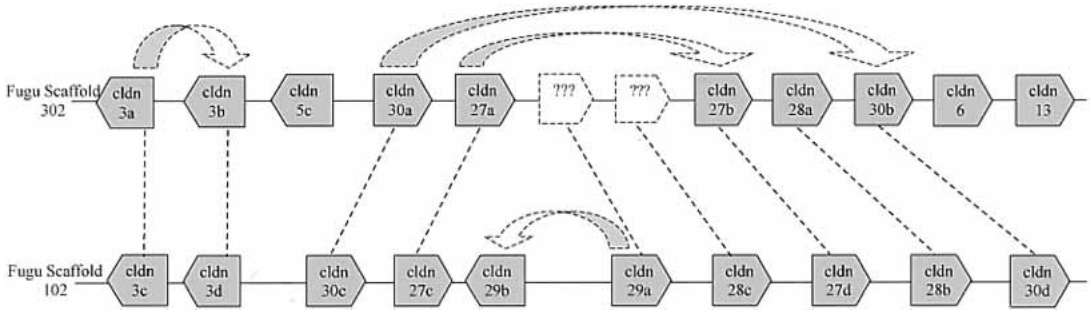


Figure 4 Proposed order of duplication of the claudin 3- and 4-like genes in *Fugu*. The location and orientation of the different genes on the two scaffolds are indicated. The dotted arrows denote intrachromosomal gene duplications; the dotted lines indicate duplication of the chromosomal segment, presumably as a result of whole-genome duplication.

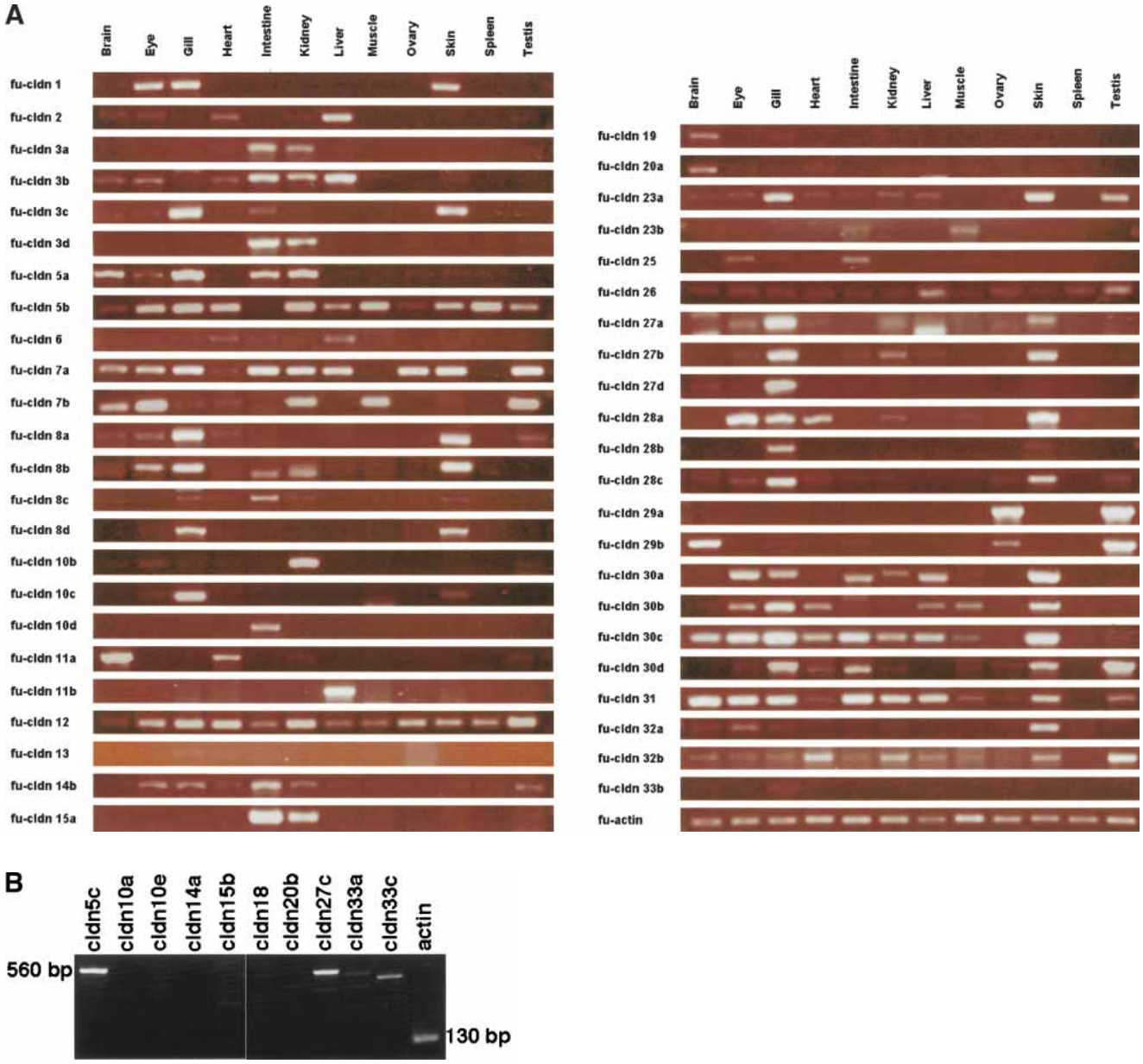


Figure 5 Expression profiling of the *Fugu* claudin genes. Expression of the different claudin genes in (*A*) adult tissues and (*B*) an early somite embryonic stage of *Fugu* was analyzed by semiquantitative RT-PCR. The amplification of fu-actin was monitored as a positive control.

acceptor and donor sites and the corresponding open reading frames were identified by manual inspection of the sequence.

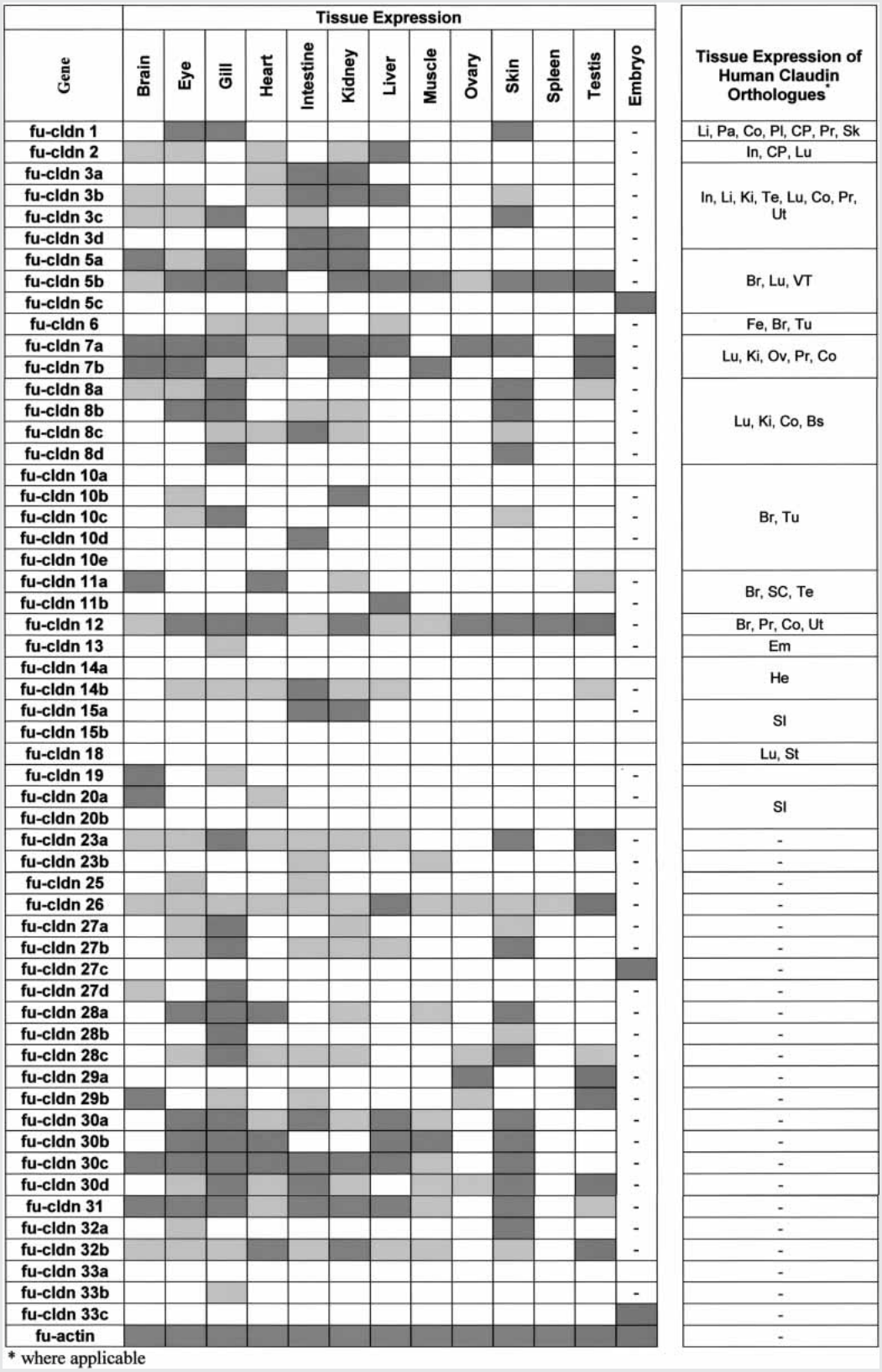
Phylogenetic Analysis and Promoter Region Characterization

The predicted *Fugu* claudin protein sequences, together with those of human, and those available for mouse and zebrafish, were aligned using CLUSTALW (version 1.8.2) with default parameters (Thompson et al. 1994). The columns of alignment that contained gaps were removed and the tree topologies were generated using the PHYLIP (version 3.6) programs PROTDIST, NEIGHBOR, and CONSENSE (Felsenstein 1989). In all, 1000 bootstrap samples were used to test the reliability of the clustering within phylogenetic trees by repeated pseudo sampling with replacements of sites from the aligned data sets. Paralogs obtained aligned well in CLUSTALW. Promoter region analysis was carried out using the rVISTA software (Loots et al. 2002), with a threshold of ≥50% identity across 75-bp windows.

Nomenclature

Each of the predicted *Fugu* claudin genes was numbered (Claudin 1, Claudin 2, etc.) according to its human ortholog, with alphabetical suffixes (Claudin 1a, Claudin 1b, etc.) if there was more than one *Fugu* ortholog. If a human ortholog could not be identified unambiguously, the identity of such genes was confirmed after phylogenetic analysis and a new number commencing from 25 onward was assigned. Zebrafish claudin genes were named in accordance with a previous study (Kollmar et al. 2001).

Table 2. Tissue and Developmental Expression of *Fugu* Claudin Genes



Gene	Tissue Expression													Tissue Expression of Human Claudin Orthologues*
	Brain	Eye	Gill	Heart	Intestine	Kidney	Liver	Muscle	Ovary	Skin	Spleen	Testis	Embryo	
fu-cldn 1													-	Li, Pa, Co, Pl, CP, Pr, Sk
fu-cldn 2													-	In, CP, Lu
fu-cldn 3a													-	In, Li, Ki, Te, Lu, Co, Pr, Ut
fu-cldn 3b													-	
fu-cldn 3c													-	
fu-cldn 3d													-	
fu-cldn 5a													-	Br, Lu, VT
fu-cldn 5b													-	
fu-cldn 5c														
fu-cldn 6													-	Fe, Br, Tu
fu-cldn 7a													-	Lu, Ki, Ov, Pr, Co
fu-cldn 7b													-	
fu-cldn 8a													-	Lu, Ki, Co, Bs
fu-cldn 8b													-	
fu-cldn 8c													-	
fu-cldn 8d													-	
fu-cldn 10a														Br, Tu
fu-cldn 10b													-	
fu-cldn 10c													-	
fu-cldn 10d													-	
fu-cldn 10e														
fu-cldn 11a													-	Br, SC, Te
fu-cldn 11b													-	
fu-cldn 12													-	Br, Pr, Co, Ut
fu-cldn 13													-	Em
fu-cldn 14a														He
fu-cldn 14b													-	
fu-cldn 15a													-	SI
fu-cldn 15b														
fu-cldn 18														Lu, St
fu-cldn 19													-	
fu-cldn 20a													-	SI
fu-cldn 20b														
fu-cldn 23a													-	-
fu-cldn 23b													-	-
fu-cldn 25													-	-
fu-cldn 26													-	-
fu-cldn 27a													-	-
fu-cldn 27b													-	-
fu-cldn 27c														-
fu-cldn 27d													-	-
fu-cldn 28a													-	-
fu-cldn 28b													-	-
fu-cldn 28c													-	-
fu-cldn 29a													-	-
fu-cldn 29b													-	-
fu-cldn 30a													-	-
fu-cldn 30b													-	-
fu-cldn 30c													-	-
fu-cldn 30d													-	-
fu-cldn 31													-	-
fu-cldn 32a													-	-
fu-cldn 32b													-	-
fu-cldn 33a														-
fu-cldn 33b													-	-
fu-cldn 33c														-
fu-actin														-

* where applicable

Overview of the tissue expression data of different claudin genes derived from Figure 5. A gray box indicates that the particular claudin is expressed in the respective tissue; a darker-shaded box indicates a higher relative expression as compared with a lighter-shaded one. Expression of human and/or mouse orthologs is indicated. (Li) liver; (Pa) pancreas; (Co) colon; (Pl) placenta; (CP) choroidal plexus; (Pt) prostate; (Sk) skin; (In) intestine; (Lu) lung; (Ki) kidney; (Te) testis; (Ut) uterus; (Br) brain; (VT) various tissues; (Fe) fetal tissues; (Tu) tumors; (Ov) ovaries; (Bs) breast; (SC) spinal cord; (Em) embryonic tissues; (He) heart; (SI) small intestine; (St) stomach.

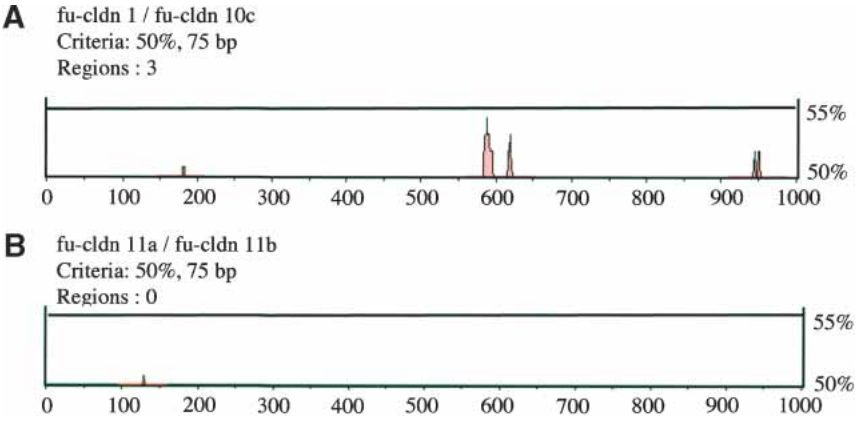


Figure 6 Promoter region analysis for two pairs of *Fugu* claudin genes with either similar (*A*) or distinct (*B*) tissue distributions. The 5′-noncoding regions of two claudin genes with a similar (*cldn1* and *cldn10c*; Table 2) or different (*cldn11a* and *cldn11b*; Table 2) expression profile were analyzed. Red areas show regions along the 5′-noncoding sequence that share a homology of 50% or more.

Gene Expression Profiling

cDNA from early-somite-stage *Fugu* embryos was provided by Tohru Suzuki (National Research Institute of Aquaculture, Fisheries Research Agency, Mei-ken 516-0193, Japan). Total RNA from various adult *Fugu* tissues (brain, eye, gill, heart, intestine, kidney, liver, muscle, ovary, skin, spleen, testis) was extracted using Tizol reagent (GIBCO BRL), and the first-strand cDNA was synthesized using the SMART RACE cDNA amplification kit (Clontech). First-strand cDNA was used as template for RT-PCR together with gene-specific primers (see Supplemental material). A 130-bp fragment of actin cDNA was amplified as an internal positive control using the primers ACTF (5′-AACTGGGAYGA CATGGAGAA-3′) and ACTR (5′-TTGAAGGTCTCAAACATGAT-3′). The PCR comprised an initial denaturation step for 2 min at 95°C followed by 35 cycles of denaturation (30 sec at 95°C), annealing (60 sec at 55°C), and extension (60 sec at 72°C), and a final elongation step of 5 min at 72°C. The PCR products were separated by gel electrophoresis (1.5% agarose gel at 160 V) in the presence of ethidium bromide and visualized under ultraviolet light. The identity of PCR products was confirmed by sequencing representative PCR fragments.

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