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Evolution of *hes* gene family in vertebrates: *hes5* cluster genes were specifically increased in *Xenopus*

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Abstract

Background

hes genes are chordate homologs of *Drosophila* genes, *hairy* and *enhancer of split*, which encode a basic helix-loop-helix (bHLH) transcriptional repressor with a WRPW motif. Various developmental functions of *hes* genes, including early embryogenesis and neurogenesis, have been elucidated in vertebrates. However, their orthologous relationships remain unclear partly because of less conservation of relatively short amino acid sequences, less conserved synteny, and species-specific gene duplication. This results in complicated gene names in vertebrates, which are not consistent in orthologs. In a previous study, we revealed that *Xenopus* frogs have two clusters of *hes5*, named “the *hes5.1* cluster” and “the *hes5.3* cluster.” The origin has not yet been revealed.

Results

Here, we elucidated the orthologous and paralogous relationships of all *hes* genes of human, mouse, chicken, gecko, zebrafish, medaka, coelacanth, spotted gar, elephant shark, and *Xenopus* frogs (*X. tropicalis* and *X. laevis*) by phylogenic and synteny analysis. Any clusters of *hes5* were not found in amniotes, whereas duplicated *hes5* clusters in teleost were found although not as many genes as *Xenopus*. In addition, *hes5* cluster-like structure was found in the elephant shark genome, but not found in cyclostomata.

Conclusion

These data suggest that the *hes5* cluster existed in the gnathostome ancestor, but was lost in amniotes.

Background

hes genes are chordate homologs of *Drosophila hairy* and *enhancer of split* genes and encode a basic helix-loop-helix (bHLH) transcriptional repressor with a WRPW motif at the C terminus [1]. These genes are known to have various developmental functions, including Notch signaling target and neurogenesis [2], somitogenesis, and early development of the presumptive midbrain-hindbrain boundary (pre-MHB) [3, 4] .

Mammals including human and mouse have seven *hes* genes which form subfamilies [5, 6]. Most of the *hes* homologs in zebrafish are called *her* [7]. As in zebrafish, their orthologs in vertebrates remain unclear partly because the two domains, bHLH and Orange domains, and the WRPW motif at the C terminus are not well conserved, and the sequences are relatively small to compare (their total sizes are about 200 aa). Another possible cause was that the genomes of the model organisms including *Xenopus laevis* (*X. laevis*) and *X. tropicalis* had yet to be sequenced.

Recently, many animal genome analyses, including frogs, *X. laevis* and *X. tropicalis*, have been reported. *Xenopus* includes diploid to dodecaploid species, although polyploidy is considered to be rare in amniotes. *X. tropicalis* has a diploid genome, and *X. laevis* has an allotetraploid genome [8]. The genomic analysis showed that the allotetraploidization was caused by interspecific crosses between two species that have a diploid genome. Thus, *X. laevis* has two subgenomes, called L and S [9,10].

In a previous study, we annotated all *hes* genes of *X. tropicalis* and *X. laevis* by phylogenetic analysis and synteny analysis [11]. In brief, for *X. tropicalis*, we revealed the phylogenetic and synteny relationships of the 18 *hes* genes and renamed them properly. *X. laevis* has 37 *hes* genes including 18 homeologs, one *laevis*-specific gene, *hes5.7*, and a pseudogene, *hes7.4*. Although the number of genes doubled after allotetraploidization, *hes* genes, except for *hes2*, have been conserved in *X. laevis*. In addition, *Xenopus* has more than two paralogs of *hes5*, *hes6*, and *hes7* subfamily genes,

in contrast to human *hes* genes. In particular, the number of *hes5* genes in *Xenopus* is quite high. Interestingly, they form two clusters, which we call “the *hes5.1* cluster” and “the *hes5.3* cluster”. The *hes5.3* cluster is formed with eight genes (*hes5.3* to *hes5.10*).

Clustered genes, such as the Hox gene cluster, the human β -globin gene cluster, and four clustered human growth hormone (hGH)/chorionic somatomammotropin genes, have various functions with unique regulatory mechanisms. The cluster is considered to be formed as a result of gene duplication and divergence [12, 13].

Some of *hes* genes are already known to be indispensable in neurogenesis [4] and the genes are well conserved despite having many genes, forming two clusters at least in *Xenopus*. This implies that the *hes5* cluster may also play an important role during embryogenesis.

To understand the evolution and role of *hes* genes in vertebrates, it is important to reveal the orthologous relationship. In this study, we first elucidated orthologous and paralogous relationships of the *hes* gene family using phylogenic and synteny analyses of human, mouse, chicken, zebrafish, medaka, frogs (*X. tropicalis* and *X. laevis*), *Gekko japonicus*, coelacanth, spotted gar, elephant shark, lamprey, and amphioxus. With this analysis, we revealed that *hes* genes are specifically increased in *Xenopus* and also discussed the evolution of the two *hes5* clusters.

Results

Classification of *hes* genes in sarcopterygian

Previous studies on the annotation of *hes* genes have shown that there are ten *hes5* paralogs in *X. laevis*, which we refer to as “the *hes5.1* cluster” or “the *hes5.3* cluster” [11]. To determine when *hes5* clusters emerged, we first performed a phylogenetic analysis of sarcopterygian *hes5* genes (Fig. 1, Fig. S2; complete tree is shown in Fig. S4). In this

analysis, other *hes* paralogues were included to clarify the outgroup. Maximum likelihood (ML) phylogenetic tree construction revealed that all of the *hes5* genes we observed were assigned in a single clade with high bootstrap percentage (93%; Fig. 1A). In the *hes5* clade, the genes, *Hosa**hes5*, *Mumu**hes5*, *Gaga**hes5chr21-3*, *Geja**hes5sc135-1*, which include human (*Homo sapiens*), mouse (*Mus musculus*), chick (*Gallus gallus*), and gecko (*Gekko japonicus*) genes, formed a single clade. *Gaga**hes5chr21-1* and *Gaja**hes5Sc135-2* also formed a single clade. These two clades were located close to each other in the tree (Fig. 1B, blue letters). Interestingly, *Xenopus hes5.3-5.9* showed a monophyletic group, suggesting that gene duplication of *hes5.3-5.9* occurred independently. In Coelacanth (*Latimeria chalumnae* (Lach)), four putative *hes5* genes were found (red letters). Although one of them formed a clade (*Lachhes5sc00059*), three of these genes (*Lachhes5sc00001* and *Lachhes5sc00319* / *Lachhes5sc00199*) formed a clade with *Xenopus hes5.3-5.9*. This result indicates that three coelacanth genes may be related to the *hes5.3* cluster.

Next, to examine the presence of *hes5* clusters, we analyzed synteny of *hes5* locus in chicken, geckos, and coelacanth genomes (synteny of other *hes* genes are shown in Fig. S1A, C). In the chicken genome, *hes5* genes were located on a single chromosome, chromosome 21 (Fig. 2B). In gecko, synteny around *hes5* was observed in scaffolds 135 and 31595 (Fig. 2C). In coelacanth, we found four *hes5* genes in scaffold00199, 00001, 00319, and 00059 (Fig. 2D). *Hes5chr21-1-3* genes in chick, *hes5sc135-1~2*, and *hes5-like* genes in gecko, and *hes5sc00319* gene in coelacanth were all located next to *pank4*, suggesting that these genes correspond to the *hes5.1* cluster (orange background). In chicken and gecko, however, there were no *hes5* genes between *nol9* and *zbtb48*, defined as *hes5.3* cluster genes in *Xenopus* (blue background). In contrast, the coelacanth *hes5sc00199* gene was located near *nol9*. This result suggested that coelacanth *hes5sc00199* may be homologous to the *hes5.3* cluster gene. In coelacanth Sc00001, a

hes5 gene was found near *chd5* (Fig. 2D). In *Xenopus*, the *chd5* gene (*chd5-like*) was located next to *rnf207* near the *hes5* clusters, suggesting the relevance of the coelacanth gene to the *hes5* clusters. *Lachhes5c00059* was found near *ppil2*, which is located on the 1st chromosome in *Xenopus*, indicating that the syntenic property was different from other *hes5* genes. Phylogenetic analysis also indicated that *Lachhes5sc00059* was first divided in the *hes5* gene family, suggesting a distinct evolution of this gene. Together with these results, it is suggested that the *hes5* genes of both chicken and gecko correlate more with the *hes5.1* cluster, whereas coelacanth *hes5* genes are located in both *hes5.1* and *hes5.3* clusters.

Comparison of *hes* genes between teleosts and *Xenopus*

It is known that whole genome duplication (WGD) occurred 500 million year ago in the common ancestor of vertebrates. In addition to this, in teleost, another WGD occurred 3.7 million years ago after divergence from the common ancestor of gnathostomata [14, 15]. Thus, in teleost genome, the two loci having similar gene order to each other, which are called doubly conserved synteny, are often found. [16]. In zebrafish (*Danio rerio*) and medaka/Japanese ricefish (*Oryzias latipes*), *hes* genes have not been well characterized as mammalian orthologue. Indeed, many genes that seem to be *hes* were described as "*her*" genes. Therefore, we attempted to identify the orthologous relationship of teleost *hes* genes based on their amino acid sequences. By our phylogenetic analysis, we found that many zebrafish and medaka "*her*" genes formed a clade with the *Xenopus hes* subfamily genes (Fig. 3A; complete tree is shown in Fig. S5; detailed gene annotations are shown in Table S1). Zebrafish *her6* and medaka *her6/her6.2* formed a single clade with *Xenopus hes1*. Medaka *her4* and zebrafish *her9* were located near *Xenopus hes4* clade. *Orlaher8.2* and *Dareher8.2/8a* showed high similarity with *Xenopus hes6.2*. *Dareher13*, *Darehes6*, *Orlahes62of2*, and *orlahes6* belonged to a clade with

Xenopus hes6.1/human *hes6* genes. The genes annotated as *hes3* and *hes2* in zebrafish, medaka, and *Xenopus* belonged to each homologous clade. *her5/7* of both medaka and zebrafish belonged to *Xenopus hes7.1* clade, whereas zebrafish *her1*, *her11*, and medaka *her7* belonged to the *Xenopus hes7.3* clade.

In *hes5*, many homologous genes, *Dareher4.1-her4.4*, two *Dareher4.2*, *Dareher2/12/15.1-15.2*, and *Orlaher4.2/12* were found in both zebrafish and medaka (Fig. 3B). We examined if the *hes5* cluster first appeared in the common ancestor of Teleostei and Sarcopterygii. However, *Dareher4.1-4.4*, *Orlaher4.2*, and *Dareher15/12/2* and *Orlaher12* formed teleost-specific monophyly among the large *hes5* clade. These results indicated that it is not clear which *Xenopus* subclade zebrafish/medaka *hes5* belongs to. Thus, we next performed synteny analysis around *her4.1-4.4/12* and *her2/15.1-15.2* to clarify whether these genes formed clusters similar to *Xenopus hes5.1* and *hes5.3* clusters (the synteny of other teleost *hes* gene were shown in Fig. S1B, D).

In the zebrafish genome, the *her4.1-4.4/12* cluster and *her2/15.1-15.2* cluster were present on chromosomes 23 and 11, respectively. *dnajc11* and *rnf207* genes were found in the genomic region around the clusters. In addition, *icmt*, *kcnab2*, *nol9*, and *chd5* genes, which are located in *Xenopus hes5* locus, were also found on either chromosome 23 (DRE23) or chromosome 11 (DRE11). These results suggested that DCSs were found in the *hes5* region of the zebrafish genome. Near the *her2/15* cluster on DRE11, *dnajc11*, which is located near *Xenopus hes5.3* cluster, was found (Fig. 4A). However, other typical features of the *hes5.3* cluster were not observed in the locus. For instance, *nol9* or *zbtb48* was not located near the *hes2/15* locus.

On DRE23, the *her4.1-4.4/12* cluster was located between *emc1* and *icmt* (Fig. 4A). The *icmt* gene was located near the *hes3* gene in *Xenopus* (Fig. 4B). No *her/hes* gene was detected between *zbtb48* and *nol9*, as in the chicken genomes (Fig. 2B). From these results, it was difficult to determine whether the *her4.1-4.4/12* cluster corresponds to

Xenopus hes5.1 cluster or *hes5.3* cluster. It should be pointed out that the sequence homology of the zebrafish genes with *Xenopus hes5* genes appeared to be higher for the *hes5.1* cluster genes than for the *hes5.3* cluster genes (Table 1), suggesting that *her2/15* and *her 4.1-4.4/12* genes of zebrafish might correspond to the *hes5.1* cluster genes in *Xenopus*.

In medaka, the *her7* gene was found to be located near *grik5*, which was located near the *hes5.1* cluster in *Xenopus* (Fig. 4C). However, phylogenetic analysis showed that *OLA her7* was in the *Xenopus hes7.1* subclade (Fig. 3A). Conversely, *OLA her4.4* and *her12* were located around these genes, *espn*, *acot7*, and *hes2.2*, which are near the *hes5.3* cluster locus in *Xenopus*, even though no genes were located between *nol9* and *zbtb48* (Fig. 4D). A gene order similar to the *Xenopus hes5* region was also observed in chromosome 1 in medaka, but no *hes*-related genes were found in the locus (Fig. 4D).

Classification of *hes* genes in gnathostomata

To determine the origin of the *hes5* cluster, we carried out phylogenetic analysis with spotted gar (*Lepisosteus oculatus*), elephant shark (*Callorhynchus milii*), lamprey (*Petromyzon marinus*), and amphioxus (*Branchiostoma floridae*) (Fig. 5A, B, S3; the complete tree is shown in Fig. S6). As a result, genes of *hes7* and *hes5* were clearly separated from the other genes with high bootstrap values. First, we counted the number of *hes* genes in these species with the exception of *hes7*- and *hes5*-classified genes, although the bootstrap values were low. Spotted gar was considered to have two *hes3*, two *hes7*, and three *hes6* (Fig. 5A, shown in red letter). Elephant shark had one *hes1*, *hes2*, *hes4*, and *hes6* (Fig. 5A, shown in blue letter). In lamprey, there were one *hes4*, three *hes2*, and one *hes3* (Fig. 5A, shown in purple letter). In amphioxus, the *hes A-G* gene was found, but not in any of the *hes* subfamily clades (Fig. 5A, shown in green letter).

In the *hes5* clade, both spotted gar and elephant shark possessed four genes (Fig. 5B), but all the genes were separately classified from the *Xenopus* genes (Fig. 5B, red and blue letters). This feature was different from other *hes*-related genes (Fig. 5A). In lamprey and amphioxus, any *hes5* gene was not found (Fig. 5B). These results indicated that the phylogenetic analysis could not identify the homologous relationship of *hes5* genes between *Xenopus* and gar/elephant shark.

Next, we compared the gene order around *Xenopus hes5* cluster region in spotted gar and elephant shark. In the spotted gar linkage group (LG) 25, four clustered *hes5-like* genes were located next to *pank4*, but no *hes5* genes were found near *nol9* (Fig. 6B). This suggests that gar had a *hes5* cluster, and the cluster was closer to the *hes5.1* cluster than to the *hes5.3* cluster in *Xenopus*. In contrast, three of the four genes in elephant shark were clustered near *nol9* on KI635912.1 (Fig. 6C). This suggested that the clustered genes might be related to the *hes5.3* cluster in *Xenopus*. In addition, the gene named *her3* was located near *pank4*, which is located near the *hes5.1* cluster in *Xenopus*, on HMISc93. Although the gene may have been given a wrong name because the sequence lacking WPRW domain, the synteny analysis suggested that the gene might be the homolog of *hes5*, and thus, the *hes5.1* cluster might be conserved in elephant shark. Another *hes5* gene in elephant shark was located next to *ppil2*. The order was conserved in coelacanth (Fig. 2D), but not in the *Xenopus hes5* cluster. This suggests the possibility that the common ancestor of teleost and cartilaginous fishes had another *hes5* next to *ppil2*, but later lost the gene.

Evolutionary phylogenetic relationships of *hes* gene in gnathostomes

To further confirm the classification, we performed a phylogenetic analysis with human *hey* genes, which are reported to be close to *hes* genes, as an outgroup (Fig. 7A: complete tree is shown in Fig. S7) [17]. As it can be seen from the low bootstrap values

in the *hes* clades, the phylogenetic tree was not solved well. However, as we already discussed above, eight zebrafish genes (*her4.1-4.4*, *her12*, *her15.1-15.2*), two medaka genes (*Orlaher4.2/12*), and three gar genes formed a single clade with *Xenopus hes5.1/5.2* (Fig. 7B, B'). However, monophyletic groups including *Xenopus hes5.3-5.10*, three coelacanth *hes5* (LachSc00001/00119/00319), Zebrafish *her2*, and one gar *hes5* (LeocLG25-1) were formed (Fig. 7B, B'), even though LachSc00319 and LeocLG25-1 showed syntenic similarity with the *hes5.1* cluster (Fig. 2D, Fig. 6B).

Discussion

Phylogenetic analysis showed that the *hes5* genes were absent in lamprey and amphioxus (Fig. 5). However, eight hairy genes have been reported in amphioxus, four of which have conserved gene expression patterns in vertebrates (in the central nervous system, presomitic mesoderm, somites, notochord, and gut) [18]. Some instances have been reported that the gene names of *hes* and the function were mismatched [19]. It remains unclear why *hes5* was specifically absent in lamprey and amphioxus, but other *hes* genes might substitute for *hes5* function in these species.

We found that elephant shark possessed *hes5* (Figs. 5 and 6). Interestingly, synteny analysis indicated that three *hes5* genes might be the orthologue of the *hes5.3* cluster in *Xenopus*. Together with the result that the putative *hes5* gene (*her3*) existed near *pank4* in the shark, it is thought that a common ancestor of gnathostomata acquired both *hes5.1* and *hes5.3* genes. In spotted gar, the *hes5.3* cluster was not found (Fig. 6B). One of the possibilities is that, after divergence into cartilaginous fishes and neopterygii, *hes5* near *nol9* was translocated to the locus next to *pank4*. Another possibility is that *her3* (= *hes5.1* cluster gene) was duplicated, and three *hes5-like* genes (= *hes5.3* cluster gene) in elephant shark were lost in the spotted gar. Unfortunately, we have not yet obtained

direct evidence for these possibilities from phylogenetic analysis (Fig. 5).

The synteny of both *hes5.1* and *hes5.3* cluster seemed to be maintained in both teleost and neopterygian, even though the gene orders of these species in these loci were highly divergent (Figs. 4 and 6). In addition, although no cluster was formed probably due to the insufficient scaffold connection, many *hes5* genes were found in coelacanth (Fig. 2), suggesting that the prototype of the *hes5.1/hes5.3* clusters would be retained in the common ancestor of amphibians and sarcopterygians. However, all amniote *hes5* genes seemed to be the *hes5.1* cluster genes, and not *hes5.3* cluster genes (Figs. 1 and 2), suggesting that the *hes5.3* clusters was lost after branching into amniotes. We further examined the number of exons in the coding regions of each *hes5* gene. In both *X. tropicalis* and *X. laevis*, almost all *hes5* consisted of three exons, except for *hes5.8*. On the other hand, *hes5* genes of many actinopterygian including zebrafish, medaka, and spotted gar genes possessed two exons in coding region (Table 2). This might reflect that *hes5* genes in actinopteryzoa and osteichthyes were increased in an independent manner.

In this study, we showed that the number of *hes5* genes is specifically high in *Xenopus*, especially the number of *hes5.3* cluster genes. To estimate the duplication process, comparison of the transcriptional direction among *hes5* genes may be considered important [11]. As we previously reported, the directions of *hes5.5*, *5.6*, *5.7*, and *5.9* are the same. Phylogenetic analysis also indicated that these genes were closely mapped in the tree (Fig. 1A), suggesting that these genes may share a common origin and may be tandemly duplicated in *Xenopus*. Phylogenetic analysis also indicated that *hes5.1*, *hes5.2*, and *hes5.10* showed high similarity (Fig. 1B, 3B, 5B, and 7B). This result suggests another possibility that *hes5.10* duplicated from *hes5.1/5.2* (or vice versa).

It is known that *hes5* functions downstream of Notch signaling and inhibits neuronal differentiation [20, 21]. RNA-seq analysis revealed that the expression of

almost all *hes5* genes is high during the gastrula and neurula stages in *Xenopus* [11]. These results suggests that the function of *hes5* is conserved between mouse and *Xenopus*. How *hes5* works in neurogenesis should be investigated, and this may elucidate the significance of the high number of *hes5* genes in *Xenopus*.

Conclusion

In this study, to understand the evolutionary process of *hes* genes, we estimated the evolutionary origins of two *hes5* clusters. Although the *hes5* gene was found in other jawed vertebrates, the number of *hes5* genes was highest in *Xenopus* (Fig. 8). The rudiment of the two clusters was found in elephant shark, suggesting that ancestral species of chondrichthyans might have these clusters. In addition, we reorganized the orthologous relationship of *hes* genes in vertebrates using phylogenic and synteny analyses. These findings go a step further in the research on the function of all *hes* genes in vertebrates as well as the understanding of the evolutionary process of large gene clusters.

Methods

Protein sequencing comparison

A multiple alignment of protein sequence of *hes* genes were visualized with MUSCLE [22].

Phylogenic analysis

Phylogenetic analysis was performed using RAxML (v8.2.0) [23]. Multiple alignments of protein sequence were carried out using MAFFT (v7.221) [24] with the `-auto` strategy.

Unaligned regions were trimmed with TrimAl (v1.2rev59) [25] using the `–gappyout` option and phylogeny trees were constructed by the maximum likelihood method with PROTGAMMAAUTO.

Annotation of Genes: GenBank accessions of *hes* genes

Genomic synteny of *hes* genes was analyzed using genome assemblies of *X. laevis*(v9.1), *X. tropicalis* (v9) from *Xenopus* genome project (<http://viewer.shigen.info/Xenopus/>).

Other species Gene ID and accession number for these analysis are from NCBI (<https://www.ncbi.nlm.nih.gov/>) and Ensembl (<https://asia.ensembl.org/index.html>).

hes5 genes are as follows: *hes5chr21-1* ID: 419390. *hes5chr21-2* ID: 107057363. *hes5chr21-3* ID: 419392. *hes5sc135-1* ID: 107122264. *hes5Sc135-2* ID: 107122267. *Lach hes5Sc00059* ID: 102346872. *hes5Sc00199* ID: 102346203. *Lepisosteus oculatus hes5 LG25-1* ID: 102684766. *hes5LG25-2* ID: 102684967. *hes5 LG25-3* ID: 102683774. *hes5LG25-4* ID: 102685165. *Callorhinchus milii hes5Sc221* ID: 103188596. *hes5Sc58-2* ID: 103181452. *hes5Sc58-3* ID: 103181453. *hes5Sc58-1* ID: 103181414.

Author's contribution

Experiments were planned by A.K., T.Y., M.T. and T.M., and conducted by A.K., T.M. The manuscript was prepared by A.K., T.Y., M.T. and T.M.

Ethics approval

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Fig. 1. Phylogenetic analysis of *hes* genes in sarcopterygian

A. The outline of phylogenetic tree in *hes* gene family. Complete tree was shown in Fig. S1. **B.** The phylogenetic tree was constructed based on the amino acid sequences using the ML method. Bootstrap support values for nodes are indicated (n=100). Evolutionary analysis was conducted in RAxML. Blue and red letters indicate amniote and coelacanth genes, respectively. The abbreviation of animals are as follows: Hosa; human, Mumu; Mouse, Gaga; Chicken, Geja; Japanese gecko, Xetr; *X.tropicalis*, Xela; *X.laevis*, Lach; Coelacanth.

Fig. 2. Syntenic analysis of *hes* gene locus in sarcopterygian

The syntenies in frog (A), chicken (B), gecko (C) and coelacanth (D) are shown. Chromosome number is described as “XLA7L” in each panel. Pentagon arrows show genes with the 5’ to 3’ direction. A magenta one shows *hes* gene and a broken lined one indicates a pseudogene. Orange and blue square shows *hes5-1* and *hes5-3* cluster region, respectively. The broken lined circle shows “partial” *hes* gene.

Fig. 3. Phylogenetic analysis of teleost *hes* genes

The phylogenetic tree was constructed by ML method. *Hes* genes except *hes5* (**A**) and only *hes5* genes (**B**) were presented. Complete tree was shown in Fig. S2. Red and blue letters indicate zebrafish/medaka and human *hes* genes, respectively. Hosa, human; Xela, *Xenopus laevis*; Xetr, *Xenopus tropicalis*; Dare, zebrafish; Orla, medaka.

Fig.4. Comparison with *hes* gene locus among *Xenopus*, zebrafish and medaka

Chromosome number is described as “DRE23”. Pentagon arrows show genes a gene with

5' to 3' direction. The magenta one shows *hes* gene. Broken arrow means same region on DRE23. DRE, zebrafish; OLA, medaka; XLA, *Xenopus laevis*.

Fig. 5. Phylogenetic analysis of *hes* genes of several jawed vertebrates

The phylogenetic tree was constructed by ML method. *Hes* genes except *hes5* (A) and only *hes5* genes (B) of spotted gar, elephant shark, lamprey and amphioxus were presented. Blue, red, purple and green letters indicate spotted gar, elephant shark, lamprey and amphioxus, respectively. Hosa, human; Xetr, *Xenopus tropicalis*; Leoc, spotted gar; Cami, elephant shark; Pema, lamprey; Brfl, amphioxus.

Fig.6. Comparison with *hes* gene locus among *Xenopus*(A), spotted gar(B) and elephant shark (C)

Pentagon arrows show genes with the 5' to 3' direction. The magenta one shows *hes* gene and broken lined one is a pseud gene. Orange square shows *hes5-1* cluster region. Orange and blue square shows *hes5.1* and *hes5-3* cluster region, respectively.

Fig. 7. Comprehensive phylogenetic analysis of *hes* genes except *hes5* (A) and *hes5* genes (B) in jawed vertebrate

Evolutionary analysis was conducted in RAxML. Human HEYL, *X.tropicalis* hey2 and sponge (*Amphimedon queenslandica*) HEY1-like gene sequences were used as an outgroup. For easy comprehension, the outline of B is described in B'. Hosa, human; Mumu, Mouse; Gaga, Chicken; Geja, Japanese gecko; Xetr, *Xenopus tropicalis*; Lach, Coelacanth; Dare, zebrafish; Orla, medaka; Leoc, spotted gar; Cami, elephant shark; Amqu, sponge.

Fig.8. Evolutionary acquisition of *hes5* genes and *hes5* cluster

The tree shows the phylogenetic relationship of jawed vertebrate. The list shows the number of paralogous *hes5* genes which synteny conserved with *hes5.1* cluster and *hes5.3* cluster genes and the number of paralogous *hes5* genes derived from other *hes5* gene.

Table 1. Amino acid sequence identities of *Xenopus hes5* genes with *hes5* genes of zebrafish

Amino acid sequence identities of zebrafish (Dare) *hes5* genes are in the left column and that of *Xenopus tropicalis hes5* genes are in the right column.

Table 2. The list of number of exon, protein size and conserved *Xenopus hes5* synteny of *hes5* genes

Orthologous *hes5* genes are in the left column and the number of exon, protein size and *Xenopus* conserved synteny in the right column. X1, X2 means isoform.

Figures

Fig.1

Figure 1

Phylogenetic analysis of hes genes in sarcopterygian A. The outline of phylogenetic tree in hes gene family. Complete tree was shown in Fig. S1. B. The phylogenetic tree was constructed based on the amino acid sequences using the ML method. Bootstrap support values for nodes are indicated (n=100). Evolutionary analysis was conducted in RAXML. Blue and red letters indicate amniote and coelacanth

genes, respectively. The abbreviation of animals are as follows: Hosa; human, Mumu; Mouse, Gaga; Chicken, Geja; Japanese gecko, Xetr; X.tropicalis, Xela; X.laevis, Lach; Coelacanth.

Fig.2

Figure 2

Syntenic analysis of hes gene locus in sarcopterygian The syntenies in frog (A), chicken (B), gecko (C) and coelacanth (D) are shown. Chromosome number is described as “XLA7L” in each panel. Pentagon arrows show genes with the 5’ to 3’ direction. A magenta one shows hes gene and a broken lined one indicates a pseudogene. Orange and blue square shows hes5-1 and hes5-3 cluster region, respectively. The broken lined circle shows “partial” hes gene.

Fig.3

Figure 3

Phylogenetic analysis of teleost hes genes The phylogenetic tree was constructed by ML method. Hes genes except hes5 (A) and only hes5 genes (B) were presented. Complete tree was shown in Fig. S2. Red and blue letters indicate zebrafish/medaka and human hes genes, respectively. Hosa, human; Xela, Xenopus laevis; Xetr, Xenopus tropicalis; Dare, zebrafish; Orla, medaka.

Fig.4

Figure 4

Comparison with hes gene locus among Xenopus, zebrafish and medaka. Chromosome number is described as "DRE23". Pentagon arrows show genes a gene with 5' to 3' direction. The magenta one shows hes gene. Broken arrow means same region on DRE23. DRE, zebrafish; OLA, medaka; XLA, Xenopus laevis.

Fig.5

Figure 5

Phylogenetic analysis of *hes* genes of several jawed vertebrates. The phylogenetic tree was constructed by ML method. *Hes* genes except *hes5* (A) and only *hes5* genes (B) of spotted gar, elephant shark, lamprey and amphioxus were presented. Blue, red, purple and green letters indicate spotted gar, elephant shark, lamprey and amphioxus, respectively. Hosa, human; Xetr, *Xenopus tropicalis*; Leoc, spotted gar; Cami, elephant shark; Pema, lamprey; Brfl, amphioxus.

Fig. 6

Figure 6

Comparison with hes gene locus among *Xenopus*(A), spotted gar(B) and elephant shark (C) Pentagon arrows show genes with the 5' to 3' direction. The magenta one shows hes gene and broken lined one is a pseud gene. Orange square shows hes5-1 cluster region. Orange and blue square shows hes5.1 and hes5-3 cluster region, respectively.

Fig. 7

Figure 7

Comprehensive phylogenetic analysis of hes genes except hes5 (A) and hes5 genes (B) in jawed vertebrate Evolutionary analysis was conducted in RAxML. Human HEYL, X.tropicalis hey2 and sponge (Amphimedon queenslandica) HEY1-like gene sequences were used as an outgroup. For easy comprehension, the outline of B is described in B'. Hosa, human; Mumu, Mouse; Gaga, Chicken; Geja,

Japanese gecko; Xetr, *Xenopus tropicalis*; Lach, *Coelacanth*; Dare, zebrafish; Orla, medaka; Leoc, spotted gar; Cami, elephant shark; Amqu, sponge.

Fig.8

Figure 8

Evolutionary acquisition of hes5 genes and hes5 cluster The tree shows the phylogenetic relationship of jawed vertebrate. The list shows the number of paralogous hes5 genes which synteny conserved with hes5.1cluster and hes5.3 cluster genes and the number of paralogous hes5 genes derived from other hes5 gene.

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