

Molecular cloning, expression pattern and phylogenetic analysis of Guanine nucleotide exchange factor Vav2 in lamprey, *Lampetra japonica*

HAN Yinglun^{1, 2†}, LI Jun^{1, 3†}, XU Lei^{1, 2†}, MA Qinghua^{1, 2†}, GOU Meng^{1, 2}, PANG Yue^{1, 2}, LIU Xin^{1, 2}, LI Qingwei^{1, 2*}

¹ College of Life Science, Liaoning Normal University, Dalian 116029, China

² Lamprey Research Center, Liaoning Normal University, Dalian 116029, China

³ Freshwater Fisheries Sciences Institute of Liaoning Province, Liaoyang 111000, China

Received 13 March 2016; accepted 28 April 2017

©The Chinese Society of Oceanography and Springer-Verlag Berlin Heidelberg 2017

Abstract

The Guanine nucleotide exchange factor Vav2 (Vav2) is a member of the Vav family that serves as an important regulators for the Rho family of Ras-related GTPases. In the current study, an ortholog (Lj-Vav2) of Vav2 was identified in the lamprey (*Lampetra japonica*). To elucidate the phylogenetic relationship of Vav2, the metazoan genome databases were analyzed to mine the ortholog of Vav. It was found that *Vav2* genes were only existed in vertebrates and Lj-Vav2 was the original one found in agnathans. The evolutionary dynamics of conserved motifs of Vav2 were explored using combined amino acid sequence as markers, and it is revealed that the Calponin homology (CH) domain, Dbl-homologous (DH) domain, Pleckstrin homology (PH) domain, Cysteine-rich (C1) domains, Src homology 3 (SH3) domains and Src homology 2 (SH2) domain were conserved throughout the *Vav2* gene family in vertebrates during gene evolution. Relative quantitative real-time PCR analysis showed that the Lj-Vav2 was distributed in the heart, kidney, supraneural myeloid body, liver, gill and lymphocyte-like cells. The Lj-Vav2 was found to be expressed in these tissues, and the level of which was upregulated in lymphocyte-like cells after the animal was stimulated with LPS. These results indicated that the Lj-Vav2 might be involved in the immune response of lymphocyte-like cells in lamprey. Meanwhile, our findings provided a foundation for further investigation of the function of Lj-Vav2 in the primary vertebrate.

Key words: guanine nucleotide exchange factor Vav2, *Lampetra japonica*, phylogenetic relationship, molecular cloning

Citation: Han Yinglun, Li Jun, Xu Lei, Ma Qinghua, Gou Meng, Pang Yue, Liu Xin, Li Qingwei. 2017. Molecular cloning, expression pattern and phylogenetic analysis of Guanine nucleotide exchange factor Vav2 in lamprey, *Lampetra japonica*. Acta Oceanologica Sinica, 36(12): 24–30, doi: 10.1007/s13131-017-1095-y

1 Introduction

Guanine nucleotide exchange factor Vav2 (Vav2) is a member of the Vav family that serves as a guanine nucleotide exchange factor for the Rho family of Ras-related GTPases (López-Lago et al., 2000). Vav2 participates in the regulation of calcium signals in BCR signaling (Foucault et al., 2005). The Vav2 contains Calponin homology (CH) domain, DBL-homologous (DH) domain, Pleckstrin homology (PH) domain, Cysteine-rich domain (C1), Src homology 3 (SH3) domain, Src homology 2 (SH2) domain and a Src homology 3 (SH3) domain (Romero and Fischer, 1996). The human Vav2 contains three important phosphorylation sites (Tyr142, 159 and 172), which involved in activation of signaling molecules during signal transduction processes (López-Lago et al., 2000). Agnathans, represented by lamprey and hagfish, are agreed to be the oldest vertebrates currently possessing the adaptive immune defenses (Cooper and Alder, 2006). Though T cell receptor (TCR) and B cell receptor (BCR) system

do not exist in jawless vertebrates, it is revealed that lamprey possesses an alternative immune system that could specifically recognize and respond to external pathogens (Cooper and Alder, 2006). This system undergoes germline genomic rearrangements of insertion of diverse leucine-rich-repeat (LRR) modules to generate a large number of different variable lymphatic receptors (VLRs) for resisting the invasion of pathogens. Three types of receptors, VLRA, VLRA and VLRC have been identified in lampreys (Kasamatsu et al., 2010). Recent evidences indicate that VLRA and VLRA are expressed in different cell types that resemble T and B cells of jawed vertebrates, respectively (Guo et al., 2008). At the same time, VLRC is expressed in T-lymphocyte like cells resembles gamma/delta T-cells (Kasamatsu et al., 2010).

In contrast to the extensive studies of Vav2 in jawed vertebrates, little is known about the existence and phylogenetic relationship of Vav2 in jawless vertebrates. In the current study, the molecular cloning and characterization of a Vav2 ortholog mo-

Foundation item: The National Basic Research Program of China (973 Program) under contract No. 2013CB835304; the National Marine Public Projects under contract No. 201305016; the National Natural Science Foundation of China (General Program) under contract No. 31601865; the Dalian Science and Technology Program under contract No. 2013E11SF056; the Education Department of the General Scientific Research Project under contract No. L201683651.

*Corresponding author, E-mail: liqw@263.net

†These authors contributed equally to this work.

lecule (*Lj-Vav2*) from the *Lampetra japonica* were reported for the first time. The *Lj-Vav2* could well be the counterpart of jawed vertebrates, which is known to play important roles in lymphocytes signal transduction.

2 Materials and methods

2.1 Animals and stimulation by LPS

The handling of lamprey (*Lampetra japonica*) and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit Number: SYXK2004—0029). Adult lampreys were purchased from Tongjiang section of the Heilongjiang River (Tongjiang City, Heilongjiang Province, China) in December. Adult lampreys (200–220 g in weight) were divided into two groups (20 lampreys per group); Each lamprey of one group was respectively immunized with 0.1 mg of lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) in 0.1 mL PBS and the control lamprey was injected with 0.1 mL PBS only. The animals were immunized at 8-day intervals by four intraperitoneal injections.

2.2 Amplification of the full length cDNA of *Lj-Vav2* by RACE

Based on the analysis expressed sequence tags (EST) of the cDNA library constructed with lamprey lymphocyte-like cells by our laboratory previously, a *Vav2* homolog was found using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). Total RNA was isolated from lamprey lymphocyte-like cells (Wu et al., 2012) using RNAiso (TaKaRa Biotechnology, Dalian, China) reagent following the manufacturer instructions, and dissolved in DEPC-treated water and stored at -80°C . The cDNA was synthesized from 5 μg of total RNA by Reverse Transcriptase M-MLV at 30°C for 10 min, 42°C for 30 min, 70°C for 15 min, 95°C for 5 min, 4°C for 60 min with 3'-coding sequence primer and 5'-coding sequence primer and Random 9 mers primer following the manufacturer instructions (TaKaRa Biotechnology, Dalian, China). The 3' and 5'-end sequences of *Lj-Vav2* were obtained by PCR with outer primers, inner primers (TaKaRa Biotechnology, Dalian, China) and specific primers (Table A1). LA Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China) was used for amplification with the following cycling conditions: 94°C for 3 min, followed by 40 amplification cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 3 min and a final extension step at 72°C for 7 min. Products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. The target band of polymerase chain reaction (PCR) product was isolated and purified, subcloned into pMD19-T vector using DNA Ligation kit (TaKaRa Biotechnology, Dalian, China) and then subjected to DNA sequencing (TaKaRa Biotechnology, Dalian, China).

2.3 Relative quantitative real-time PCR

Total RNAs were extracted from lamprey tissues including lymphocyte-like cells, supraneural myeloid body, gill, heart, liver and kidney using RNAiso reagent (TaKaRa Biotechnology). The total RNAs were treated with DNase I (TaKaRa Biotechnology, Dalian, China), and then subjected to reverse transcription using PrimeScriptTM RT reagent Kit (Perfect Real Time) (TaKaRa Biotechnology, Dalian, China). Relative quantitative real-time PCR experiments were performed with TaKaRa TP800 Real Time PCR System (TaKaRa Biotechnology) using 2 μL cDNA with 16.8 μL SYBR green PCR mastermix (TaKaRa Biotechnology, Dalian, China) and 0.6 μL of each specific primer (Table A1). The efficiency of the primers was analyzed in serial 50-fold dilutions of cDNA by calculating the slope of the regression line of the cycle

thresholds (Cts) versus the relative concentration of cDNA. The *GAPDH* of lamprey was used as the internal control to normalize the starting quantity of RNA. The cycling was performed as follows: 95°C for 30 s, followed by 40 amplification cycles at 95°C for 5 s, 58°C for 30 s, 72°C for 30 s, and a final extension step at 65°C for 15 min. Results were expressed as the mean $\pm$ standard deviation of three independent experiments for each specimen. The differences of gene expression between two groups were analyzed using Student's *t*-test by SPSS statistical software package. The differences were considered statistically significant at $P < 0.05$.

2.4 Identification of Vav

A total of 44 *Vav1/2/3* sequences from vertebrates were obtained from the NCBI (<http://www.ncbi.nlm.nih.gov/>) and the Ensemble genome browser (<http://www.ensembl.org/>). First, a search for Guanine nucleotide exchange factor Vav was performed using Vav and Guanine nucleotide exchange factor Vav as keywords to maximize the number of hit sequences. Second, a number of Vav genomic sequence segments were obtained from the genomic databases by online programs of BLAST and the BLAST-like Alignment Tool (BLAT) in the NCBI and Ensemble genome browser databases. Partial sequences and potential pseudogenes were excluded and some predicted sequences were used to search coding exons at the Ensemble genome browser according to previously verified sequences.

2.5 Sequence alignments, phylogenetic and conserved motif analyses

All Vav amino acid sequences were aligned with ClustalX (<http://www.ebi.ac.uk/Tools/clustalw/>) using default settings except for identity matrix set for protein weight matrix. Result of multiple sequence alignments was converted into mega format and directly imported to MEGA 4.0 software for constructing phylogenetic tree (Kumar et al., 2008). A Neighbor-Joining (NJ) tree was constructed based on pair-wise deletion of gaps/missing data and the distance matrix of amino acids model with 1 000 bootstrap replicates. Conserved motif analyses of *Vav2* were performed online using the MEME system version 4.9.0 (<http://meme.nbcr.net/meme/>) (Bailey and Elkan, 1994). The default settings of minimal and maximal motif widths and the number of different motifs were defined as 6, 30, and 30, respectively.

3 Results and discussion

3.1 Identification of *Lj-Vav2* in lamprey

A sequence fragment was found in a lymphocyte-like cDNA library which has been constructed and performed EST sequencing previously in our laboratory. Through PCR followed by 5'-RACE and 3'-RACE, the full-length cDNA of *Lj-Vav2* with 6 568 bp nucleotides was obtained (NCBI accession number: KM052165), which contained a 2 511-bp open reading frame (ORF), a 746-bp 5'-untranslated region (UTR) and a 3 311-bp 3'-UTR, encoding a polypeptide of 837 amino acids with an estimated molecular mass of 97.14 kDa. The signal sequence and the transmembrane domain do not exist in *Lj-Vav2*, which indicate it may be an intracellular protein. Multiple sequence alignments of *Lj-Vav2* with other Vav2 revealed that it had 75%–81% sequence ortholog with Vav2 of other vertebrates. As a result of this high conservation in amino acid sequence, the *Lj-Vav2* also had six domains that are characteristic of Vav2, namely the CH, DH, PH, C1, SH3 and SH2 domain. In addition, three important phosphorylation Sites Y141 (corresponding to Y142 of human Vav2), Y160 (corresponding to Y159 of human Vav2) and Y173 (corresponding to Y172 of human Vav2) also exist (Fig. 1). Based on

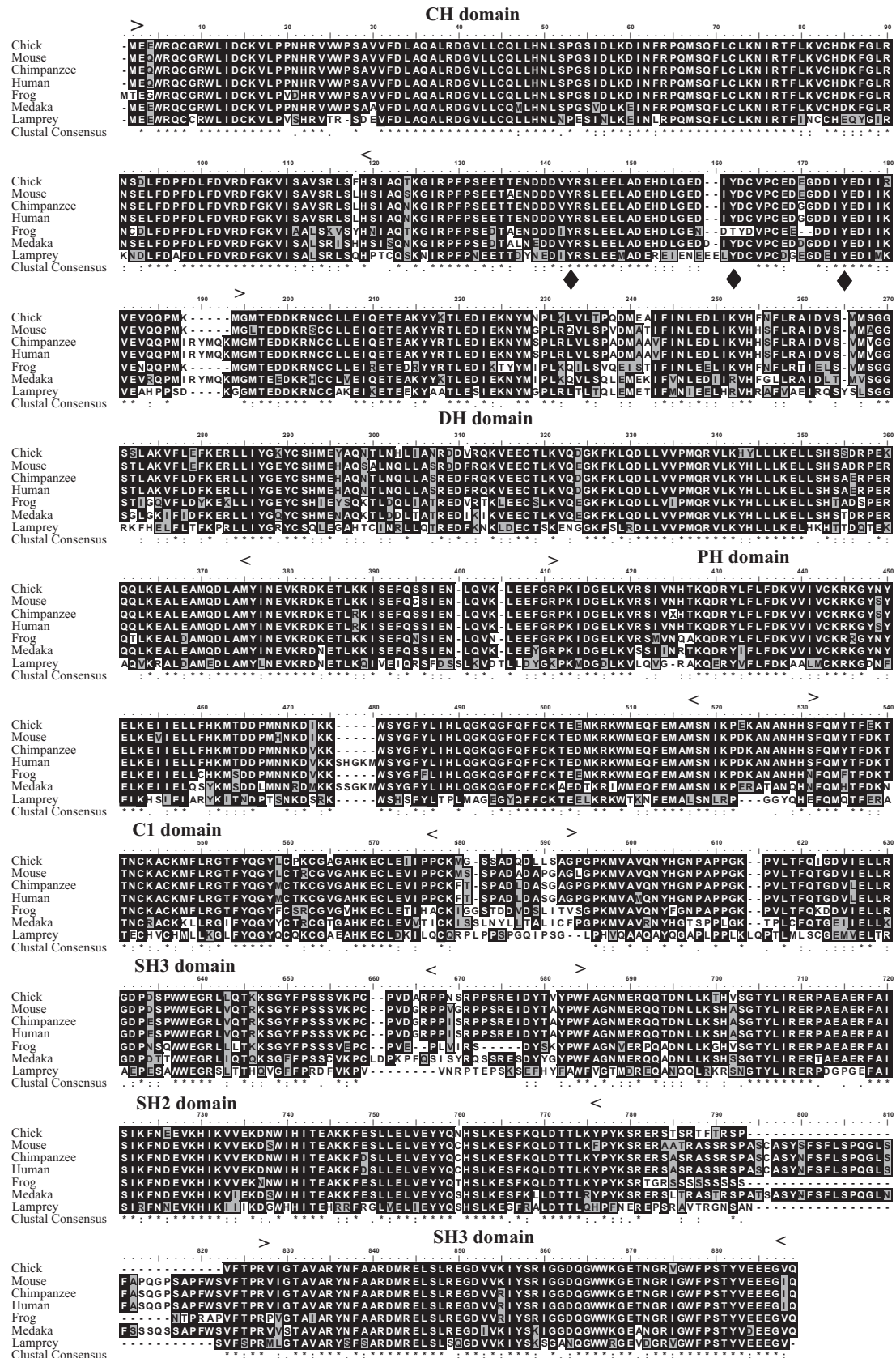


Fig. 1. Sequence alignment of Lj-Vav2 with other typical Vav2 using Clustal X. The GenBank accession numbers are as follows: *Homo sapiens*: NP_001127870; *Pan troglodytes*: XP_003312439; *Rattus norvegicus*: NP_001100033; *Gallus gallus*: NP_989473; *Xenopus laevis*: NP_001079955; *Danio rerio*: XP_002666022; and *Lampetra japonica*: KM052165. The rhombus marks the three conserved tyrosine residues. Identical (asterisk) and similar (colon) residues are indicated.

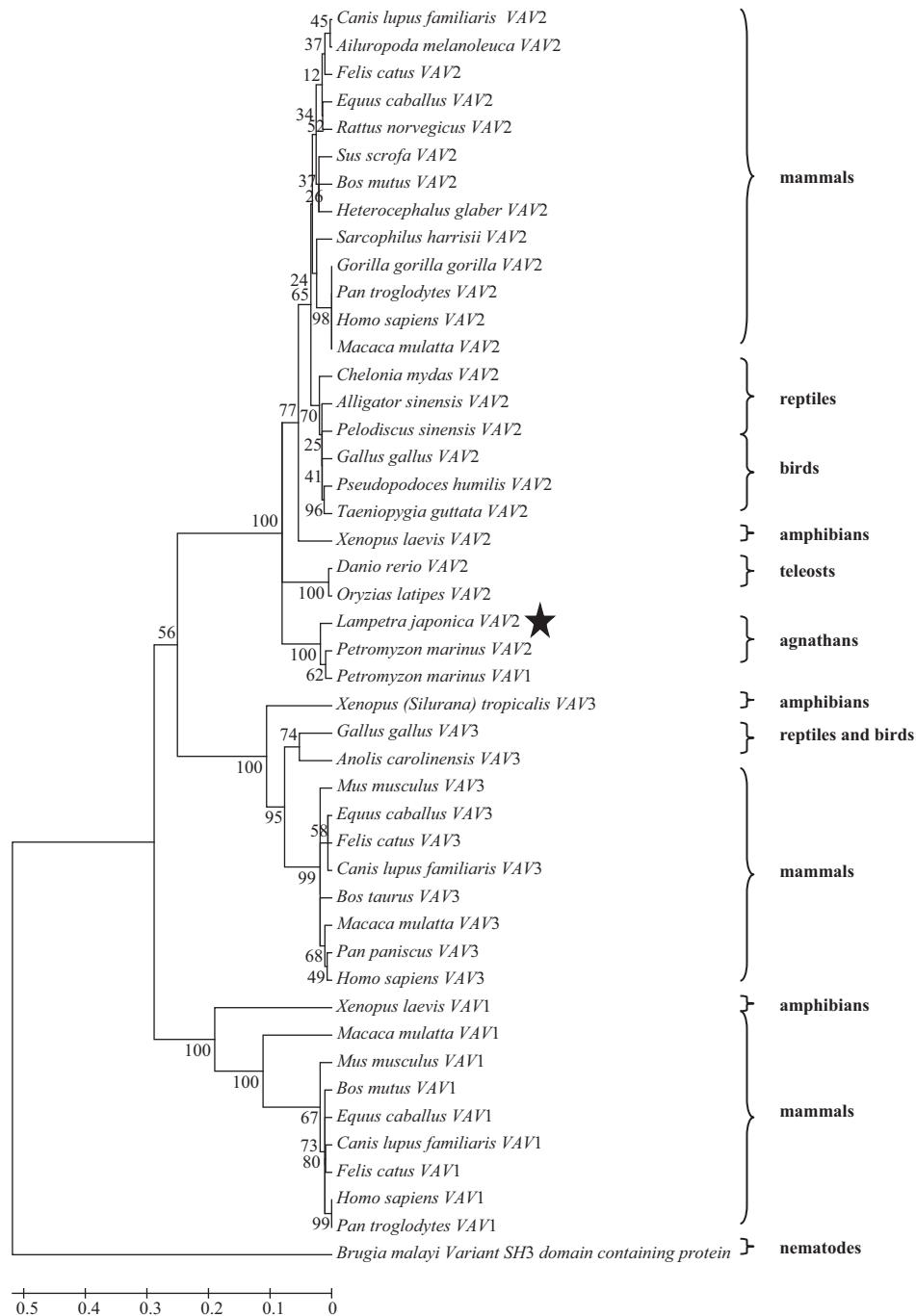


Fig. 2. Phylogenetic tree reconstructed with 27 Vav2 ortholog based on the NJ method. Bootstrap values are indicated as percentages at the nodes. Mammals, reptiles, birds, amphibians, teleosts, agnathans and nematodes clades are delineated by vertical brackets at the right. The bar indicates genetic distance. The GenBank accession numbers are as follows: *Pseudopodoces humilis*: XP_005529272; *Taeniopygia guttata*: XP_002199325; *Gallus gallus*: NP_989473; *Pelodiscus sinensis*: XP_006123810; *Rattus norvegicus*: NP_001100033; *Heterocephalus glaber*: XP_004849050; *Maylandia zebra*: XP_004538472; *Chelonia mydas*: XP_007061074; *Pan troglodytes*: XP_003312439; *Gorilla gorilla gorilla*: XP_004048874; *Sus scrofa*: XP_001927176; *Homo sapiens*: NP_001127870; *Macaca mulatta*: NP_001252562; *Oryzias latipes*: XP_004075103; *Equus caballus*: XP_001917927. *Xenopus (Silurana) tropicalis*: NP_001039156; *Sarcophilus harrisii*: XP_003757521; *Xenopus laevis*: NP_001079955; *Ailuropoda melanoleuca*: XP_002920752; *Canis lupus familiaris*: XP_005625211; *Danio rerio*: XP_002666022; *Felis catus*: XP_006939691; *Bos mutus*: XP_005897504; *Alligator sinensis*: XP_006038960; *Ornithorhynchus anatinus*: XP_001505586; *Lampetra japonica*: KM052165; *Brugia malayi*: XP_001894233; *Homo sapiens*: NP_001073343; *Pan paniscus*: XP_003822283; *Macaca mulatta*: XP_001083337; *Bos Taurus*: XP_002686208; *Canis lupus familiaris*: XP_537047; *Felis catus*: XP_006935050; *Equus caballus*: XP_005610610; *Mus musculus*: NP_065251; *Anolis carolinensis*: XP_003220125; *Gallus gallus*: NP_996745; *Xenopus (Silurana) tropicalis*: XP_002932225; *Pan troglodytes*: XP_512321; *Homo sapiens*: NP_001245135; *Felis catus*: XP_003981777; *Canis lupus familiaris*: XP_005633021; *Equus caballus*: XP_005611552; *Bos mutus*: XP_005890772; *Mus musculus*: NP_001157287; *Macaca mulatta*: XP_002801087; and *Xenopus laevis*: NP_001086991.

these protein sequence characteristics, we concluded that Vav2 existed in Agnathans which occupied the most ancient taxonomic position in vertebrates.

3.2 Phylogenetic analyses of Vav2 proteins in vertebrates

In order to explore the evolutionary history of vertebrates Vav2, the metazoan genome databases were analyzed to mine the ortholog of Vav with online programs of BLAST and BLAT. The phylogenetic tree was constructed by NJ method with 44 ortholog identified from agnathans to mammalian, and the Variant SH3 domain containing protein of nematodes (*Brugia malayi*) which contains the CH, DH, PH, C1, SH2 and SH3 domains (34% identity with human Vav2) was used as the out group. At the same time, two Vav EST sequences were also found in the genome of sea lamprey (*Petromyzon marinus*). The topology of the resulted NJ tree (Fig. 2) showed that, Vav of jawed vertebrates were unequivocally grouped into several clusters relatively in accordance with their evolutionary position. The first one (named Cluster I) included Vav2 from mammals to agnathans, the second one (named Cluster II) included Vav3, and the third one (named Cluster III) included Vav1 and the fourth (named Cluster IV) includes the Variant SH3 domain containing protein only. Cluster I could be further classified into three subgroups, the first one included Vav2 from mammals, reptiles, birds and amphibians. The second one included teleosts. The third one included Lj-Vav2 and two Vav EST sequences were also found in the genome of sea lamprey. Phylogenetic analysis indicated that the Lj-Vav2 was clustered as the out group of Vav2 from jawed vertebrates and its origin was far earlier than the one from the common ancestor of jawed vertebrates. Considering that Agnathans, represented by lamprey and hagfish, are agreed to be the oldest vertebrates possessing the primary lymphocyte-like cells which take the function of adaptive immune defenses (Guo et al., 2008), the origination of Vav2 seems highly related with the emergence of lymphocytes. Though the evolution pattern of the Vav2 family is in accordance with the classical interpretations of the origin of species, there is a short genetic distance (above 0.2) between

Vav2 from jawless and jawed vertebrates. From Fig. 2, the common ancestor gene of vertebrate Vav2 possessed far genetic distance with the Variant SH3 domain containing protein from nematodes, which illustrated that the origin of the Vav2 gene can be traced back to invertebrates.

3.3 Conservation of Vav2

In order to explore the evolutionary dynamics of conserved motifs of the Vav2 family, amino acid sequences of Vav2 from jawless and jawed vertebrates were analyzed by the MEME Suite. There are total 35 conserved motifs identified from vertebrate Vav2s (Table A2). According to the arrangement of the motifs along the protein sequences of the Vav2s, Motifs 13, 16, 22, 23 and 32 represent CH domain, Motifs 6, 8, 14, 20, 27, 30 and 35 indicate the DH domain, Motifs 1, 9, 18, 19 and 24 comprise the PH domain, cysteine-rich domain is made up of Motifs 2 and 17, Motifs 3, 5, 12 and 15 represent SH3 domain and the SH2 domain is made up of Motifs 4, 7, 10 and 34 (Table 1). Above 80% (22 of the 27 motifs) of the motifs that represent six domains of Vav2 exist ubiquitously in all vertebrate sequences, especially those motifs that represent PH, C1, SH2 and SH3 domains are nearly 100% identical in all vertebrate sequences. The other four motifs (Motif 32 in CH domain, Motifs 27 and 35 in DH domain and Motif 34 in SH2 domain) exist only in Vav2s of jawed vertebrates. These results revealed that domains PH, C1, SH2, SH3, CH and DH of Vav2 are conserved throughout the Vav2 gene family in jawless and jawed vertebrates during gene evolution and the short insertion seems to be the mechanism of gene molecular evolution. The same mechanism was also found among those motifs that belong to none of the five domains. For example, Motifs 25 and 28 appear in Vav2s from reptiles and mammals, Motif 31 exist in Vav2s from reptiles, birds and mammals, and Motif 29 exist in Vav2s from fishes, reptiles, birds and mammals.

3.4 Expression pattern of Lj-Vav2 in adult tissues

LPS is a major component of the outer membrane of Gram-negative bacteria and has been used in mammals, amphioxus,

Table 1. Type and distribution of conserved motifs discovered among typical Vav2 homologues from mammals, reptiles, bird, amphibians, teleosts and agnathans using the MEME system

Species	Motifs																																				
<i>H. sapiens</i>	16*	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>P. troglodytes</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>F. catus</i>		33	12	13	22	26	21		6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33		5	3		
<i>S. harrisii</i>	16	32	23	13	22	26			6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>E. caballus</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>S. scrofa</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>R. norvegicus</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>O. anatinus</i>	16	32	23	13	22	2	30	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17		12	15		7	4	34	10	33	25	5	3	
<i>G. gallus</i>	16	32	23	13	22	26	21		6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33		5	3		
<i>T. guttata</i>	16	32	23	13	22	26	21		6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33		5	3		
<i>C. mydas</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33	25	5	3		
<i>P. sinensis</i>	16	32	23	13	22	26	21	28	6	30	20	27	14	35	8	11	24	9	18	1	19	2	17	31	12	15	29	7	4	34	10	33		5	3		
<i>X. laevis</i>	16	32	23	13	22	26	21		6	30	20	27	14		8	11	24	9	18	1	19	2	17		12	15		7	4	34	10	33		5	3		
<i>X. tropicalis</i>	16	32	23	13	22	26	21		6	30	20	27	14		8	11	24	9	18	1	19	2	17		12	15		7	4	34	10	33		5	3		
<i>M. zebra</i>	16	32	23	13	22	26	21		6	30	20	27	14	35	8	11	24	9	18	1	19	2	17		12	15	29	7	4	34	10	33		5	3		
<i>O. latipes</i>	16	32	23	13	22	26	21		6	30	20	27	14	35	8	11	24	9	18	1	19	2	17		12	15	29	7	4	34	10	33		5	3		
<i>L. japonica</i>	16		23	13	22	26	21		6	30	20		14		8	11	24	9	18	1	19	2	17		12	15		7	4		10	33		5	3		
Protein domains ¹⁾	—CH— - - - —DH— —PH— —C1— - —SH3— - —SH2— - - —SH3—																																				

Note: Each number represents a specific motif. ¹⁾ The protein domains are indicated with segments marked with their initial abbreviations: CH represents Calponin homology domain, DH DBL-homologous domain, PH Pleckstrin homology domain, C1 Cysteine-rich domain, SH3 Src homology 3 domain, and SH2 Src homology 2 domain. The dash means that the corresponding motif belongs to none of the five domains.

fish and lamprey for immunological studies (Tsutsui et al., 2007; Huang et al., 2011). The expression pattern of *Lj-Vav2* was examined using relative quantitative real-time PCR with total RNA extracted from different tissues of LPS treated lampreys (Fig. 3). The expression level of *Lj-Vav2* was higher in liver than in other tissues in the control (treated with PBS). For the LPS-stimulated group, the expression level of *Lj-Vav2* transcript reached the highest in the lymphocyte-like cells, which was about seven folds increase compared with the control ($P < 0.05$). The significant up-regulation in lymphocyte-like cells when treated with LPS indic-

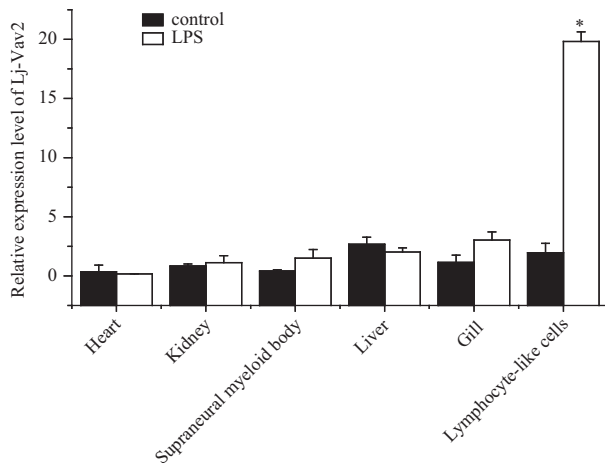


Fig. 3. *Lj-Vav2* mRNA expression was significantly upregulated in the lymphocyte-like cells after treatment with LPS. The *Lj-Vav2* mRNA levels were determined using relative quantitative real-time PCR in various tissues. Total RNA was extracted from the lymphocyte-like cells, supraneural myeloid body, gill, heart, liver and kidney of lampreys after stimulation with LPS. Lamprey GAPDH served as an internal control to calibrate the cDNA template for all of the samples, and PBS served as a negative treatment control. The significant differences ($p < 0.05$) in *Lj-Vav2* mRNA expression between the challenged group and the control group are indicated with asterisks.

ated that *Lj-Vav2* is involved in the lamprey immune response after LPS stimulation.

In conclusion, a novel member of Vav2 was identified in lamprey. Phylogenetic analysis of the Vav2 indicated that the *Lj-Vav2* could be regarded as a primary type of Vav2 in vertebrates and an ortholog of Vav2 in jawed vertebrate. The *Vav2* gene came into existence after multiple gene duplications that took place in the emergence of jawless vertebrates. The *Lj-Vav2* is involved in the immune response of lymphocyte-like cells in lamprey. Since the accurate function of *Lj-Vav2* remains unknown till now, more thorough studies are needed to elucidate this speculation.

References

- Bailey T L, Elkan C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol*, 2: 28–36
- Cooper M D, Alder M N. 2006. The evolution of adaptive immune systems. *Cell*, 124(4): 815–822
- Foucault I, Le Bras S, Charvet C, et al. 2005. The adaptor protein 3BP2 associates with VAV guanine nucleotide exchange factors to regulate NFAT activation by the B-cell antigen receptor. *Blood*, 105(3): 1106–1113
- Guo Peng, Hirano M, Herrin B R, et al. 2008. Dual nature of the adaptive immune system in lampreys. *Nature*, 459(7248): 796–801
- Huang Huiqing, Huang Shengfeng, Yu Yingcai, et al. 2011. Functional characterization of a ficolin-mediated complement pathway in amphioxus. *J Biol Chem*, 286(42): 36739–36748
- Kasamatsu J, Sutoh Y, Fugo K, et al. 2010. Identification of a third variable lymphocyte receptor in the lamprey. *Proc Natl Acad Sci USA*, 107(32): 14304–14308
- Kumar S, Nei M, Dudley J, et al. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform*, 9(4): 299–306
- López-Lago M, Lee H, Cruz C, et al. 2000. Tyrosine phosphorylation mediates both activation and downmodulation of the biological activity of Vav. *Mol Cell Biol*, 20(5): 1678–1691
- Romero F, Fischer S. 1996. Structure and function of Vav. *Cell Signal*, 8(8): 545–553
- Tsutsui S, Nakamura O, Watanabe T. 2007. Lamprey (*Lethenteron japonicum*) IL-17 upregulated by LPS-stimulation in the skin cells. *Immunogenetics*, 59(11): 873–882
- Wu Fenfang, Zhao Jing, Chen Liyong, et al. 2012. A novel BTK-like protein involved in immune response in *Lethenteron japonicum*. *Immunol Lett*, 146(1–2): 57–63

Appendix:

Table A1. Primers used to amplify the full cDNA of *Lj-Vav2*

Name	Sequences
Cloning of the full-length cDNA	
5'-RACE of <i>Lj-Vav2</i>	5'-GAACGAGGGAGGCTGGCAGGGGGGG-3'
3'-RACE of <i>Lj-Vav2</i>	5'-GGATTGTTGCTGCGCTGGTGAAATCT-3'
Analysis of real-time PCR	
<i>Lj-Vav2</i> (Forward)	5'-TTGAAGGTGGATACTCTGTTGGAT-3'
<i>Lj-Vav2</i> (Reverse)	5'-TTTGTGCTGGTCGGGTCGTTGGT-3'
<i>GAPDH</i> (Forward)	5'-ACCAACTGCCTGGCTCCT-3'
<i>GAPDH</i> (Reverse)	5'-TCTTCTGCGTTGCCGTGT-3'

Table A2. Conserved motifs discovered among Vav2 amino acid sequences from agnathans, fishes, amphibians, birds and mammals using the MEME system

Motif ¹⁾	Width	Best possible match
1	33	WSYGFYLIHLQKGQGFQFFCKTE[DE]MKRKWM
2	42	HH[NS]FQM[YH]TF[DE]KTTNCKAC[KR]MFLRGTFYQGY
3	39	V[KR]IYSRIGGDQGWKGE[TA]NGR[IV]GWFPSTYV
4	36	RFAISIKFN[DE]EVKHIKVVEKD[NS]WIHTEAK
5	30	VFTPRVIGTAVARYNFAARDMRELSLREGD
6	33	DDKRNCCLLEIQETEAKYY[RK]TLEDIEKNYM
7	33	YPWFAGNMERQQ[TA]DNLLKSHASGTYLIRER
8	33	ECTLKVQ[DE]GKFKLQDLLVPMQRLKYHLL
9	36	VNHTKQDRYLFLFDKVVIVCKR[KR]GY[NS]YELK
10	33	ELVEYYQ[CS]HSLKESFKQLDTTLKYPYKSRE
11	37	HS[TAS]DRPE[RK]QQLKEALEAMQDLAMYINEVKR
12	30	GPKMVAVQNYHGNPAPPGKPVLTFTQTDVI
13	33	CLKNIRTFCLKVCHDKFGLRNS[ED]LFDPFDLF
14	42	KERLLIYGEYCSHME[HY]AQNTLN[QH]L[LI]A[ST]RED
15	43	RGDP[ED]S[QP]WWEGR[L]VIL[QT][RK]KSGYFPSSSVKPC
16	33	ME[EQ]WRQCGRWLIDCKVLPPNHRVWPSAVV
17	30	CTKCG[VA]GAHKECLE[VI][IT]PPCKI
18	27	[IV]IELLFHKMTDDPMNNKD[VI]KK
19	18	EQFEMAMSNKP[DE]KA
20	21	M[AE]A[IV]FINLEDLIKVH
21	15	IYDCVPCEDGDDIY
22	39	DVRDFGKVISA[VL]SRLS[LH]HSIAQ[NT]KGIRPFP
23	30	GVLCCQLLHNLSPGSIDLKDINFRPQMSQF
24	33	ETLKKISEFQSSIENLQVKLEE[FY]GRPKIDG
25	42	PASCASYNFSFLSPQGL[SN]F[AS]SQG[SP]S[AT]PFWS
26	24	ET[TA]ENDDDVYRSLEELADEHD
27	30	RAIDVSM[AS]GGS[TS]LAKVFL[DE]F
28	21	DIKVEVQQPMIRYMQKMGMT
29	18	RPP[IN]SRPPSREIDYT
30	23	NPL[RK]LVL[ST]P[AQ][DE]
31	23	S[PS]AD[LQ]DA[SL][GN]AG
32	8	FDLAQALR
33	20	[AT]SR[AT][SF][ST]RS
34	6	KFESLL
35	9	[FV]RQKVE

Note: ¹⁾ Numbers correspond to the motifs described in Table 1.