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Short Communication

Ribosomal Protein Genes S23 and L35 from Amphioxus *Branchiostoma belcheri tsingtauense*: Identification and Copy Number

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Abstract The complete cDNA and deduced amino acid sequences of the ribosomal proteins S23 (AmphiS23) and L35 (AmphiL35) from amphioxus *Branchiostoma belcheri tsingtauense* were identified in this study. AmphiS23 cDNA is 546 bp long and encodes a protein of 143 amino acids. It has a predicted molecular mass of 15,851 Da and a pI of 10.7. AmphiL35 cDNA comprises 473 bp, and codes for a protein of 123 amino acids with a predicted molecular mass of 14,543 Da and a pI of 10.8. AmphiS23 shares more than 83% identity with its homologues in the vertebrates and more than 84% identity with those in the invertebrates. AmphiL35 is more than 63% identical to its counterparts in the vertebrates and more than 52% identical to those in the invertebrates. Southern blot analysis demonstrated the existence of 1–2 copies of the S23 gene and 2–3 copies of the L35 gene in the genome of amphioxus *B. belcheri tsingtauense*. This is in sharp contrast to the presence of 6–13 copies of the S23 gene and 15–17 copies of the L35 gene in the rat genome. It is clear that the housekeeping genes like S23 and L35 underwent a large-scale duplication in the vertebrate lineage, reinforcing the gene/genome duplication hypothesis.

Key words amphioxus; ribosomal protein; S23; L35; copy number

Ribosomes are the RNA-protein organelles that catalyze the sequential addition of amino acids to the carboxyl end of the growing polypeptide chain, according to the blueprints encoded by mRNA [1]. Each ribosome comprises two subunits: a large (L) and a small (S) subunit. In eukaryotes, the large 60S subunit is composed of three ribosomal RNAs (rRNAs) and nearly 50 ribosomal proteins, whereas the small 40S subunit consists of one rRNA and approximately 30 proteins [2]. Ribosomal proteins are highly conserved proteins encoded by the housekeeping genes, as their activity is required for the growth and maintenance of all cell types [3]. Information contained in the sequences of ribosomal proteins can contribute to unraveling their evolution and function.

The eukaryotic ribosomal protein S23, known as S12 in bacteria and as either S12 or S23 in *Archaea* [4], appears to be involved in the translation initiation step of protein synthesis [5]. The ribosomal protein L35 is found to bind to both initiator and elongator tRNAs [6,7]. The gene encoding S23 has been identified in several organisms such as mammals (GenBank accession No. AAS55902 for *Chinchilla lanigera*; AAH70221 for *Homo sapiens*; CAA54584 for *Rattus norvegicus*; AAS59430 for *Sus scrofa*), amphibians [8], teleosts [9], insects (GenBank accession No. AAV34880 for *Bombyx mori*; BAD26702 for *Plutella xylostella*), nematodes [10,11], and annelids (GenBank accession No. CAC14789 for *Lumbricus rubellus*). The gene encoding L35 has been isolated from organisms including mammals [12], birds [13], reptiles (GenBank accession No. AAR10441 for *Ophiophagus hannah*), amphibians [12], teleosts [14,15], insects (GenBank accession No. AAV34846 for *Bombyx mori*), and nematodes (GenBank accession No. AAA28216 for *Caenorhabditis elegans*). Amphioxus or lancelet, a basal chordate, has been widely known as the "living fossil" most closely related to the proximate ancestor of vertebrates in phylogeny [16,17]. Liu *et al.* [18,19] recently

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reported the cloning of ribosomal proteins S15a, L19, S20 and L10 cDNAs from amphioxus *Branchiostoma belcheri tsingtauense*. However, no information has been available so far for S23 and L35 in this evolutionarily important organism.

Gene/genome duplication has been an interesting topic for biologists for decades [20–22]. It is proposed that two rounds of large-scale gene duplication took place during early chordate evolution: one occurred close to the origin of vertebrates, the other close to the origin of jawed vertebrates [21,23–25]. Comparison of the numbers of luxury protein genes such as *Hox* [26], *Otx* [27], *Msx* [28] and *hedgehog* [29] provides substantial evidence for this hypothesis. Evolutionarily, it remains uncertain whether the housekeeping genes like S23 and L35 also follow the two-round duplication rule, and data comparing housekeeping gene copy numbers in different species are still lacking.

The aims of the present study were to characterize S23 and L35 cDNAs from amphioxus *B. belcheri tsingtauense* and to determine these gene copy numbers in its genome.

Experimental Procedures

The cDNA library was constructed using the SMART cDNA library construction kit (Clontech, Palo Alto, USA) according to the method described previously [30]. cDNA clones were randomly selected for sequencing. Both strands of all selected clones were sequenced with the ABI PRISM 377XL DNA sequencer (PE company, Foster City, California, USA) and all sequences were then analyzed for coding probability with the DNATools program developed by Rehm [31].

Initial comparison against the GenBank protein database was performed using the BLAST network server at the National Center for Biotechnology Information [32]. Multiple protein sequences were aligned by the Clustal method, using the MegAlign program in the DNAStar software package developed by Burland [33]. Accession numbers of the ribosomal protein sequences in the GenBank database used for comparison are listed in **Table 1** and **Table 2**.

Genomic DNAs for Southern blotting analysis were isolated from adult amphioxus. A total of 30 amphioxus were ground in liquid nitrogen, and the powder was suspended in 15 ml of lysis buffer (pH 8.0) containing 10 mM Tris-HCl, 100 mM EDTA and 0.5% SDS. After treatment with proteinase K (100 mg/ml, final concentration) at 55 °C for 3 h, it was cooled to room temperature and mixed with an equal volume of saturated phenol (pH 8.0). The mixture was centrifuged at 5000 *g* at 4 °C for 20 min, and the supernatant was pooled and mixed with an equal volume of phenol:chloroform (1:1, *V*/*V*). The mixture was centrifuged as above and the supernatant was collected. DNA was precipitated by ethanol and digested with various restriction enzymes at 37 °C for 20 h: *Eco*RV, *Pst*I, *Hin*dIII, *Bst*XI and *Bgl*II (one unit per microgram DNA) for genomic DNA to be hybridized with digoxigenin (DIG)-labeled cDNA probes of AmphiS23; and *Eco*RI, *Pst*I, *Hin*dIII, *Eco*RV and *Bst*XI (one unit per microgram

Table 1 Representative members of the ribosomal protein S23 family

Protein	Organism (abbreviation)	Accession No.	Amino acids	Source
S23Hs	*Homo sapiens* (Hs)	AAH70221	143	GenBank
S23Ss	*Sus scrofa* (Ss)	AAR22386	143	GenBank
S23Rn	*Rattus norvegicus* (Rn)	CAA54584	143	EMBL
S23Cl	*Chinchilla lanigera* (Cl)	AAS59430	143	GenBank
S23Xl	*Xenopus laevis* (Xl)	AAH77634	142	GenBank
S23Gm	*Gillichthys mirabilis* (Gm)	AAG13288	143	GenBank
S23Ip	*Ictalurus punctatus* (Ip)	AAK95205	143	GenBank
S23Bb	*Branchiostoma belcheri tsingtaunese* (Bb)	AAN86978	143	GenBank
S23Dm	*Drosophila melanogaster* (Dm)	Q8T3U2	143	SwissProt
S23Bm	*Bombyx mori* (Bm)	CAH04343	143	EMBL
S23Lr	*Lumbricus rubellus* (Lr)	CAC14789	143	EMBL
S23Ce	*Caenorhabditis elegans* (Ce)	NP_502365	143	GenBank

Table 2 Representative members of the ribosomal protein L35 family

Protein	Organism (abbreviation)	Accession No.	Amino acids	Source
L35Hs	*Homo sapiens* (Hs)	AAH71915	123	GenBank
L35Ss	*Sus scrofa* (Ss)	AAS55902	123	GenBank
L35Gg	*Gallus gallus* (Gg)	BAB21248	123	GenBank
L35Oh	*Ophiophagus hannah* (Oh)	AAR10441	123	GenBank
L35Xt	*Xenopus tropicalis* (Xt)	AAH77011	123	GenBank
L35Dr	*Danio rerio* (Dr)	AAM34649	123	GenBank
L35Ip	*Ictalurus punctatus* (Ip)	AAK95161	123	GenBank
L35Hc	*Hippocampus comes* (Hc)	AAQ63320	123	GenBank
L35Bb	*Branchiostoma belcheri tsingtaunese* (Bb)	AAN52380	123	GenBank
L35Dm	*Drosophila melanogaster* (Dm)	AAM48393	123	GenBank
L35Bm	*Bombyx mori* (Bm)	AAV34846	123	GenBank
L35Ce	*Caenorhabditis elegans* (Ce)	AAA28216	123	GenBank

DNA) for genomic DNA to be hybridized with DIG-labeled cDNA probes of AmphiL35. The digested DNAs were separated on a 1% agarose gel using 1×TBE (89 mM Tris-borate and 2 mM EDTA) and transferred onto nylon membranes (Osmonics Inc., Minnesota, USA). The membranes were hybridized with the DIG-labeled DNA probes produced with a DIG DNA labeling kit (Roche, Basel, Switzerland). Hybridized bands were visualized according to the instructions of the detection kit.

Results and Discussion

The first cDNA encoding amphioxus ribosomal protein S23, AmphiS23, was identified from the gut cDNA library as revealed by BLAST search. **Fig. 1** shows the nucleotide and deduced amino acid sequences of AmphiS23 cDNA (GenBank accession No. AY168453). It was 546 bp long and consisted of a 26 bp 5′ untranslated region

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GCTCTTTCCTGAACCCAAAATCCAAGATGGGTAAGCCCCGTGGTCTGCGTTCTGCTCGT     59
                            M  G  K  P  R  G  L  R  S  A  R      11
AAGCTGAAGGATCACCGCCGGCAGCAGCGCTGGCACGACAAGTCCTTCAAGAAGGCCCAT   119
 K  L  K  D  H  R  R  Q  Q  R  W  H  D  K  S  F  K  K  A  H     31
CTGGGTACAGCCGTGAAGGCCAGCCCCTTCGGAGGAGCCTCTCATGCCAAGGGCATCGTG   179
 L  G  T  A  V  K  A  S  P  F  G  G  A  S  H  A  K  G  I  V     51
CTCGAGAAAATTGGCGTTGAGGCCAAGCAGCCAAACTCTGCCATCAGGAAGTGCGTCCGT   239
 L  E  K  I  G  V  E  A  K  Q  P  N  S  A  I  R  K  C  V  R     71
GTCCAGCTCATCAAGAACGGCAAGAAGATCACAGCCTTCGTACCCAACGACGGCTGCCTC   299
 V  Q  L  I  K  N  G  K  K  I  T  A  F  V  P  N  D  G  C  L     91
AACTACATCGAGGAAAACGACGAGGTTCTGGTCTCTGGATTTGGTCGTAAGGGCCGTGCC   359
 N  Y  I  E  E  N  D  E  V  L  V  S  G  F  G  R  K  G  R  A    111
GTCGGTGACATCCCCGGCGTCAGGTTCAAGGTCGTGAAGGTCGCCAACGTGTCACTCCTC   419
 V  G  D  I  P  G  V  R  F  K  V  V  K  V  A  N  V  S  L  L    131
GCCCTGTTCAAGGAGAAGAAGGAGCGGCCAAGATCGTAGGACTACCACGTCTGCTTCTTT   479
 A  L  F  K  E  K  K  E  R  P  R  S  *                         142
GTGAAAATTCCAAAAAATAAAGGGAAGATGTACTCCCGAAAAAAAAAAAAAAAAAAAAAA   539
AAAAAAA                                                        546
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Fig. 1 Nucleotide and deduced amino acid sequences of amphioxus S23 gene (GenBank accession No. AY168453)

The presumed translational start and terminal sites are underlined, and the asterisk represents the stop codon. The potential polyadenylation signal upstream with respect to the poly(A) tail is boxed and the oligopyrimidine tract within the 5′ UTR is double underlined.

(UTR), an open reading frame (ORF) of 432 bp and an 88 bp 3′ UTR. The ORF encoded a 143 amino acid protein with a calculated molecular mass of 15,851 Da and a pI of 10.706. The 5′ UTR had an in-frame stop codon TGA upstream of the first start codon ATG and a polypyrimidine sequence, CTTTC, which has been found at the 5′ end of many eukaryotic ribosomal protein mRNAs [34]. The 3′ UTR had a polyadenylation signal AATAA 18 bases upstream of the poly(A) site which is required for post-translational cleavage-polyadenylation of the 3′ end of the pre-mRNA [35].

The deduced protein sequence of AmphiS23 was compared with those of the other known S23 proteins from various organisms in the GenBank database (**Table 1**). AmphiS23 shares more than 83% identity with its homologues in the vertebrates such as humans, rats, pigs, frogs and teleosts, and more than 84% identity with those in the invertebrates like insects, annelids and nematodes (**Fig. 2**).

AmphiS23 is a rather hydrophobic protein with 50 hydrophobic amino acids out of 143 residues. It has a high percentage of basic amino acids (20 lysines and 13 arginines) mostly located in the N-terminal half of the deduced amino acid sequence, and a low percentage of acidic amino acids (5 aspartic acids and 7 glutamic acids) mostly situated in the C-terminal half. The strong basic character of S23 including AmphiS23 may be instrumental for its binding to rRNA in the 40S subunit of eukaryotic ribosomes [36–38].

The second identified cDNA clone encoded amphioxus ribosomal protein L35, AmphiL35. **Fig. 3** shows the nucleotide and deduced amino acid sequences of AmphiL35 cDNA (GenBank accession No. AY168767). The cDNA comprised 473 bp and included a 5′ UTR of 27 bp, an ORF of 372 bp and a 3′ UTR of 74 bp. The ORF encoded a 123 amino acid protein with a calculated molecular mass

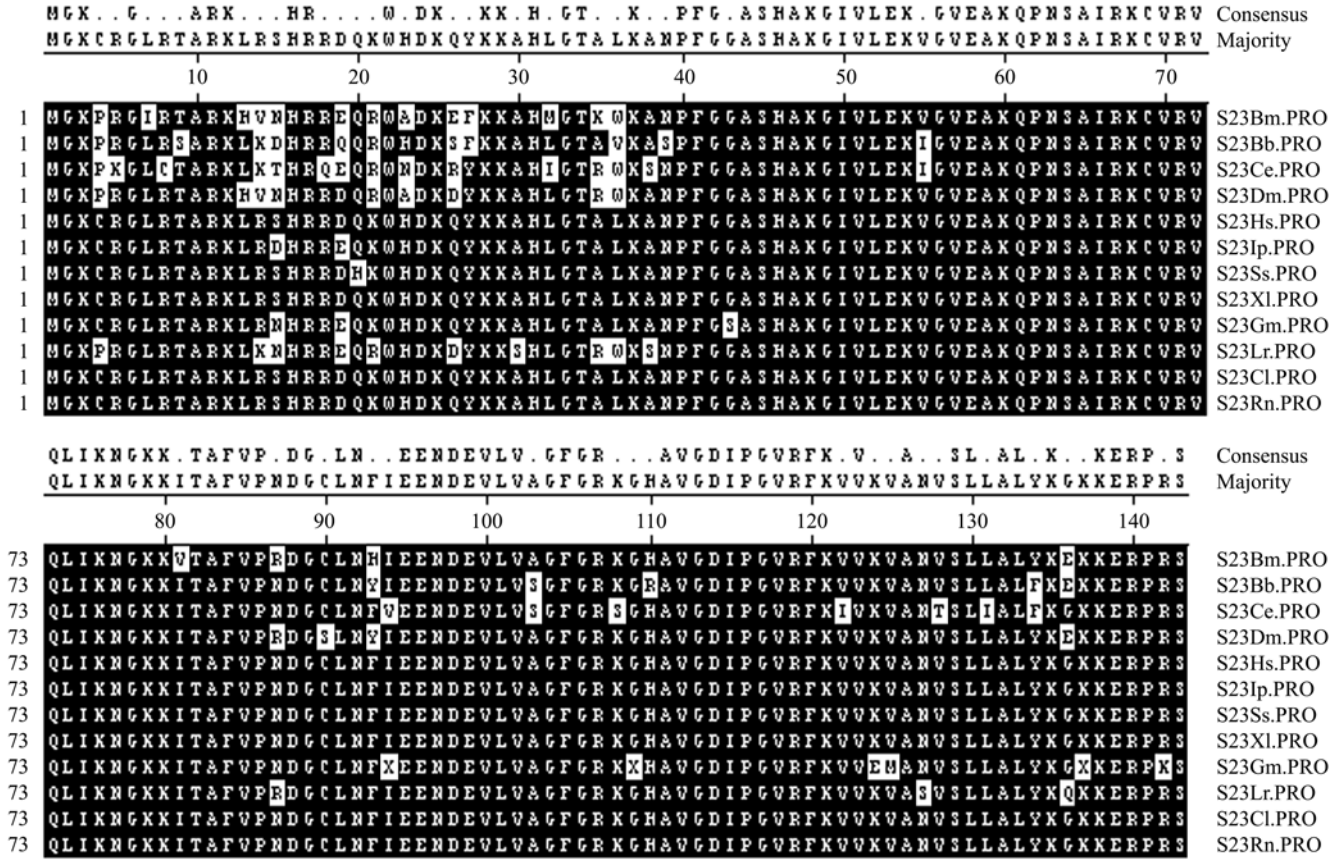

Fig. 2 Amino acid sequence alignment of representative S23 proteins using the MegAlign program (DNAStar) by the Clustal method

Shaded (with solid black) residues are the amino acids that match the consensus. 'Consensus': when all match the residue of the Consensus show the residue of the Consensus, otherwise show '·'. See **Table 1** for sequence reference.

of 14,543 Da and a pI of 10.8. The 5′ UTR of AmphiL35 had an oligopyrimidine tract CTTTTTCC upstream of the start codon ATG, which consists of a C residue at the cap site, followed by an uninterrupted sequence of up to 13 pyrimidines [39–43], and possibly plays a critical role in the translational control mechanism [44]. The 3′ UTR of AmphiL35 had a polyadenylation signal ATTAA, which is required for post-translational cleavage-polyadenylation of the 3′ end of the pre-mRNA.

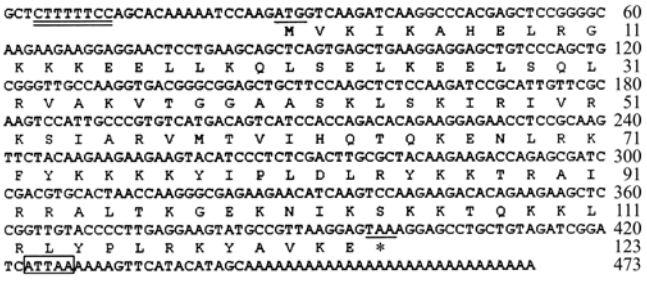

Fig. 3 Nucleotide and deduced amino acid sequences of amphioxus L35 gene (GenBank accession No. AY168767)

The presumed translational start and terminal sites are underlined, and the asterisk represents the stop codon. The potential polyadenylation signal upstream with respect to the poly(A) tail is boxed and the oligopyrimidine tract within the 5′ UTR is double underlined.

Comparison of the deduced AmphiL35 amino acid sequence with that of its counterparts in the GenBank database (**Table 2**) showed that AmphiL35 possesses more than 63% identity with its homologues in the vertebrates including human beings, pigs, birds, snakes, frogs, catfish, zebrafish and seahorses, and more than 52% identity to those in the invertebrates like insects and nematodes. As **Fig. 4** shows, like the other known L35 proteins, AmphiL35 also has an excess of basic residues over acidic ones (4:1), which is the same as the proportion of basic residues versus acidic ones in the amino acid sequence of L35 from mammals, birds, reptiles, amphibians, teleosts, insects and nematodes.

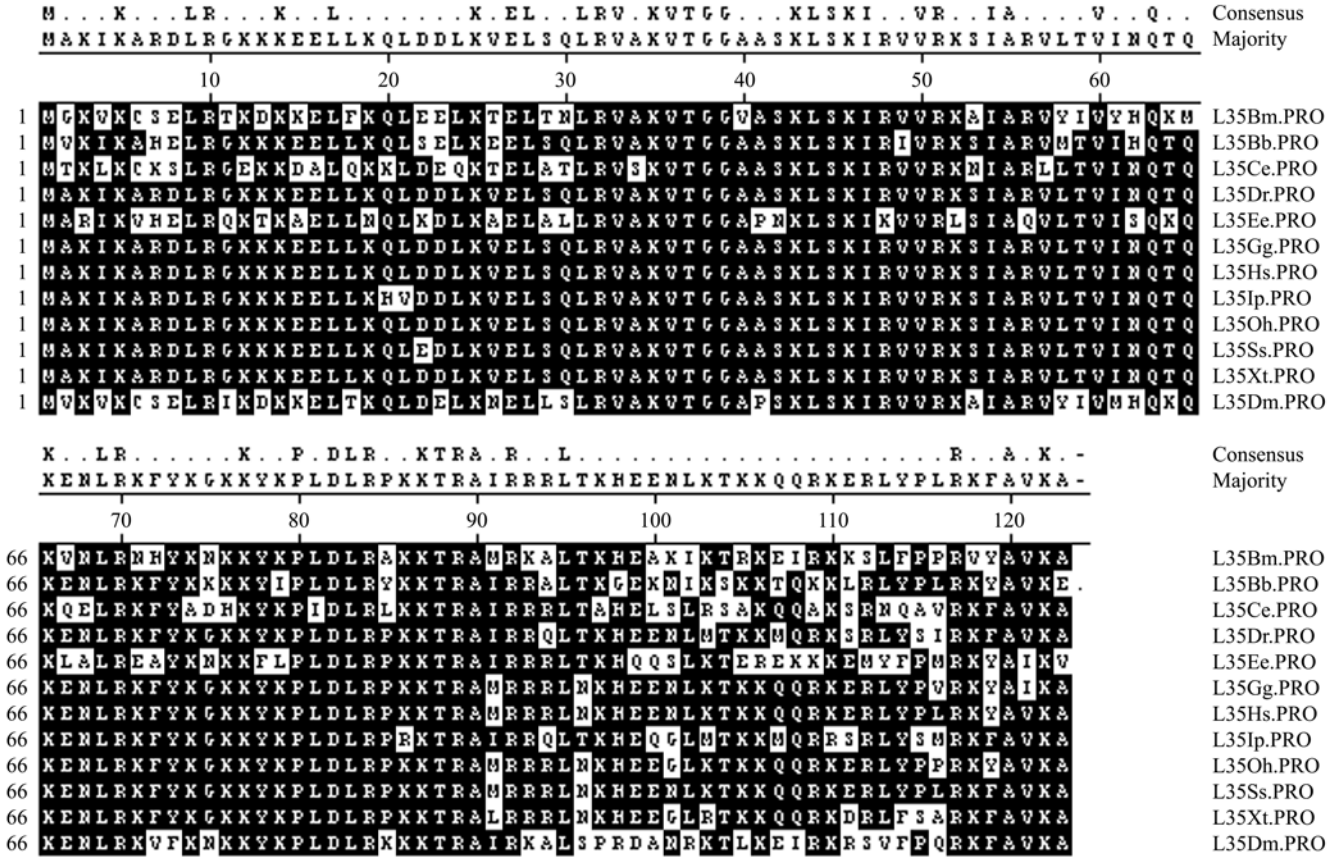

Fig. 4 Amino acid sequence alignment of representative L35 proteins using the MegAlign program (DNAStar) by the Clustal method

Shaded (with solid black) residues are the amino acids that match the consensus. 'Consensus', when all match the residue of the Consensus show the residue of the Consensus, otherwise show '·'. See **Table 2** for sequence reference.

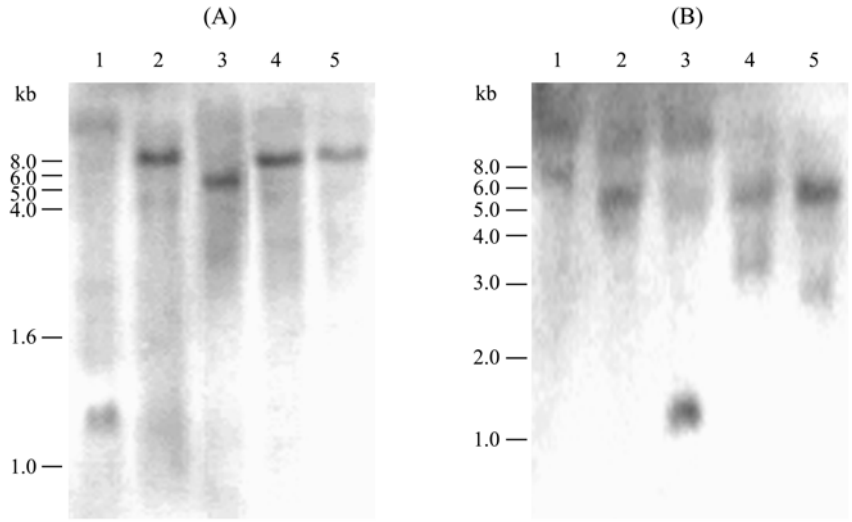

Fig. 5 **Southern blotting analysis of genomic DNAs extracted from adult amphioxus *B. belcheri tsingtauense*.**
(A) Southern blotting analysis using DIG-labeled cDNA probe of AmphiS23. 1, *Eco*RV; 2, *Pst*I; 3, *Hin*dIII; 4, *Bst*XI; 5, *Bgl*II. (B) Southern blotting analysis using DIG-labeled cDNA probe of AmphiL35. 1, *Eco*RI; 2, *Pst*I; 3, *Hin*dIII; 4, *Eco*RV; 5, *Bst*XI.

The homology of AmphiS23 and AmphiL35 to their known counterparts extends the range of species in which these proteins are highly conserved. This high conservation of S23 and L35 amino acid sequences in various organisms including the vertebrates and invertebrates suggests they have been subjected to strong selective pressure during evolution.

To analyze the copy number of AmphiS23 and AmphiL35 genes, the DIG-labeled cDNA probes of AmphiS23 and AmphiL35 were used to hybridize digests made from amphioxus genomic DNA with either the restriction enzymes *Eco*RV, *Pst*I, *Hin*dIII, *Bst*XI and *Bgl*II or the enzymes *Eco*RI, *Pst*I, *Hin*dIII, *Eco*RV and *Bst*XI. The enzymes used do not digest AmphiS23 or AmphiL35 cDNA strings. For AmphiS23, there is a single hybridization band for the genomic DNA digested with each of the enzymes *Pst*I, *Hin*dIII, *Bst*XI and *Bgl*II, and two hybridization bands were observed for the genomic DNA digested with *Eco*RV [**Fig. 5(A)**]. For AmphiL35, two hybridization bands for the genomic DNA digested with each of the enzymes *Eco*RI, *Pst*I, *Eco*RV and *Bst*XI, and three bands for the genomic DNA digested with the enzyme *Hin*dIII were revealed [**Fig. 5(B)**]. These suggest the presence of 1–2 copies of the S23 gene and 2–3 copies of the L35 gene in the genome of amphioxus *B. belcheri tsingtauense*. In contrast, there exist 6–13 copies of the S23 gene and 15–17 copies of the L35 gene in the rat genome [4,45]. From the comparison of the number of AmphiS23 and AmphiL35 genes with that of rat S23 and L35 genes, it is clear that S23 and L35 genes had undergone extensive duplication in the vertebrate like rat. It therefore appears that the divergence of the vertebrate from the common ancestor of cephalochordate/vertebrate is accompanied by a large-scale duplication of the housekeeping protein genes such as S23 and L35.

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