

PHYLOGENETIC RELATIONSHIPS IN ZnT SUPERFAMILY OF ZINC ION TRANSPORTERS *IN SILICO*

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Abstract: The Zinc transporter (ZnT superfamily) facilitates Zn-transport into intracellular vesicles in life forms. Existing database has been enriched with the identification of new Zn-transporters important for therapeutic and intra-channel metal binding capacity. The present work uses a consensus sequence approach to identify extra-membrane metal-binding motif in Zn transporter protein family. Molecular phylogeny among members of ZnT superfamily proteins was analyzed. In all 166 representative sequences covering all known members of ZnT protein subfamilies (subgroups Znt1-Znt10) from bacteria, archaea and eukarya were selected and used to obtain ten consensus sequences, each representing a specific ZnT subgroup (1-10). Using bioinformatics tools a phylogenetic tree was constructed to explore the evolutionary link between the various ZnT subgroup considering members from lower and higher life forms. The unrooted phylogenetic tree obtained by Clustal W and NJ method revealed the existence of three clearly separated clades, which were segregated early in the evolution of this superfamily. Consensus sequence analysis using Weblogo revealed motifs of (HX)_n type that evolved to bind the Zn ions effectively, enabling Zn transport. No significant similarity among members of Znt1 and Znt10 or Znt2 and Znt10 suggest that gene duplication event in the ZnT superfamily gene probably occurred in the later phase of evolution when individual specificities of the distinguished ZnT subgroups were already formed.

Keywords: Bioinformatics; Consensus sequence; Motif; Phylogeny; Zinc-transporter.

Introduction

Zinc is an essential trace element required for the structural stability of a variety of proteins involved in transcription and protein trafficking as well as for the catalytic activity of metalloenzymes. More than 300 metalloenzymes of six major functional classes require zinc as a key structural component or as a cofactor (Vallee and Auld, 1990; Vallee and Falchuk, 1993; Berg and Shi, 1996), which include pancreatic carboxypeptidases, alkaline phosphatase, various dehydrogenases and superoxide dismutase (Frausto da Silva, 1991). In mammals, zinc is absorbed through the brush border of small intestinal mucosa from diet and transported

through blood to the tissues and cells where zinc is needed (Reyes, 1996). Moreover, zinc is an element requisite for zinc containing DNA binding proteins. Human genes encoding C2H2 zinc-finger proteins amount to more than 3-4% of the total (Kowalski *et al.*, 2002; Mackay *et al.*, 1998). Zinc can potentially modulate cellular signal recognition, second messenger metabolism and the function of protein kinase and protein phosphatase (Reyes, 1996). Consequently, the disturbance of zinc homeostasis causes a variety of severe detrimental effects on animals, including humans.

The understanding of zinc transport and its regulation in plants has increased with the identification of both Zrt-Irt-like proteins and Cation Diffusion Facilitator family genes in many plant species. Recently, 86 ZIP members were

reported in nonredundant protein database at NCBI (Gaither and Eide, 2001). The high number of potential metal ion transport proteins in plants and animals no doubt stems from the greater diversity of tissue-specific roles to be played by these proteins in multicellular organisms. Irt1 was the first ZIP protein to be identified in any organism (Eide *et al.*, 1996). Similar to that of any multicellular organism, zinc transporters are required for both cellular zinc uptake as well as efflux to allow the utilization of the metal. In plants, for example, a zinc efflux transporter is required to pass zinc from the root tissue into the xylem for distribution to aerial portions of the plant.

In animals, zinc balance is primarily maintained through a regulated rate of intestinal uptake, fecal elimination of excess zinc, renal reabsorption and distribution to cells, including intracellular storage (Krebs, 2000). Most, if not all, of these zinc movement pathways are mediated by membrane proteins or zinc transporters (Vallee and Falchuk, 1993; Krebs, 2000).

Transporter-mediated movement of zinc has been suggested by a number of classical kinetic studies of zinc transfer across the membrane, because its rate is time and temperature-dependent and saturable (Reyes, 1996; Nagao *et al.*, 1999). The CDF and ZIP family transporters have been shown to have high sequence similarity and structural topology (Nies, 1992; Conklin *et al.*, 1992; Nies and Silver, 1995). Both ZIP and CDF members have a His-rich loop exposed to cytoplasm (Paulsen and Saier, 1997; Gaither and Eide, 2001). During this decade, a number of zinc transporters have been cloned from a variety of organisms, and the number of zinc transporter candidates found from the sequence homology is rapidly growing. Some zinc transporters have been investigated at molecular levels, and there has been an increasing body of evidence demonstrating that functional analyses of zinc transporters, including their gene expression, are very important for understanding the mechanism by which zinc homeostasis is achieved.

The present work therefore, aims at the phylogenetic and sequence analysis of the ZnT transporter protein superfamily consisting of Znt1 to Znt10 subfamilies reported in the database.

Based upon the representative consensus sequence derived for each Znt subfamily the structural and computational analysis of zinc transporters is done to identify motifs responsible for structure-function relationships of the members of ZIP-family protein, that can be targeted for enhanced Zn uptake in cells using biotechnological interventions.

Methodology

Computational Resources and Sequence Data

For the phylogenetic and sequence analysis of ZnT family of zinc transporters, the protein sequences were retrieved from the protein database at NCBI (<http://www.ncbi.nlm.nih.gov>). The members of ZnT family that have been grouped into 10 subfamilies from Znt 1-10 were selected. The retrieved protein sequences with their gi no., accession no., and their source organism are listed in Tables 1-10. They include all the protein sequences that were selected for present study and the retrieved protein sequences were stored family wise in fasta format for further analysis. Owing to the currently unequal availability of the sequences of the proposed ZnT family, only representative sequences spreading over all kingdoms were selected for a statistically equilibrated phylogenetic analysis (Zamocky, 2004).

Multiple Sequence Alignment and Consensus Sequence

To study the similarity/differences and to look at the phylogenetic relationship among the subfamilies of the ZnT family, all the 166 member proteins of Znt1-Znt10 families listed in tables 1-10, were subjected to multiple sequence alignment using ClustalW program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). The sequences were noted in jalview (<http://www.jalview.org>) and the consensus sequence for each group of proteins belonging to Znt (1-10) families were obtained in a family specific manner shown in table(s) 1-10.

Phylogenetic Analysis

To study the phylogenetic relation between the three kingdoms of life (bacteria, archaea and

Table 1
List of Proteins of Znt1 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1	41053720	NP_957173	<i>Danio rerio</i>	
2	7381054	AAF61374, AF133267	<i>Thlaspi caerulescens</i>	
3	6678017	NP_033605	<i>Mus musculus</i>	MGFKITRSQRLSAVIGIMIVFDVAEM
4	52352803	NP_067017	<i>Homo sapiens</i>	GCWGRNRGRLLCMLALTFMFFVLEV
5	1395831	AAK50854, AF364518	<i>Homo sapiens</i>	VVSRVTKSLAMLSDSFHMLSDVLALV
6	12382779	AAG53405	<i>Homo sapiens</i>	VALVAVRFARLAGAAGVAIPLRTHAT
7	4808520	AAD29840	<i>Homo sapiens</i>	QKNTFGWIRAEVMGALVNGVFLTAL
8	75076404	Q4R6K2	<i>Macaca fascicularis</i>	CFAILLEAIERFIEPHEMQQPLVVLGVG
9	23503107	Q9Y6M5	<i>Homo sapiens</i>	AAGLLVNVGLGLCLFHHHSGFGQDSGHG
10	105671481	ABF74685	<i>Hebeloma cylindrosporum</i>	HSHGHHGHGHGLAKGPRVEQGYISHGG
11	161513446	CAJ44263	<i>Thlaspi caerulescens</i>	SDPARGIAVRSTLHNCVINDRILIYSMQ
12	56118710	NP_001007940	<i>Xenopus tropicalis</i>	DECTLSSPNTRRRRTLDSQHRGSPRYSN
13	8134846	Q60738	<i>Mus musculus</i>	NVEDIQVHPATMRQKSTRAGSSDINEAP
14	8134838	Q62720	<i>Rattus norvegicus</i>	GEQGPDQENLAETSERALLSETNTLVA
15	108742984	CAG34108	<i>Thlaspi caerulescens</i>	NTSNSNGLVLPVADPEKDRSKDHNEVQ
16	56406615	AAV87660	<i>Cyprinus carpio</i>	VNGNLIVEIDHLEAEHDRAGQLNMRG
17	56406617	AAV87661	<i>Oncorhynchus mykiss</i>	VFLHVLGDALGSVIVVVALVFYFSW
18	58577265	CAE00445	<i>Glomus intraradices</i>	KGCSEGRFCVNPFCFDPCKIFVEIINSPL
19	169784289	XP_001826606	<i>Aspergillus oryzae</i>	ALGS——VYEAGPCWVLYLDPTLCIIM
20	169776045	XP_001822489	<i>Aspergillus oryzae</i>	VCI
21	169765438	XP_001817190	<i>Aspergillus oryzae</i>	LLYTTYPLLKESALILLQTVPKQIDIHD
22	21361112	NP_003450	<i>Homo sapiens</i>	LKE
23	30682009	NP_172566	<i>Arabidopsis thaliana</i>	DLRLGCSHPDIPAHGAISRLFHARNFYPCIE
24	83775351	BAE65473	<i>Aspergillus oryzae</i>	AALVPGVEAVHELHVWQLAGSRIIATA
25	83771224	BAE61356	<i>Aspergillus oryzae</i>	HIK
26	83765045	BAE55188	<i>Aspergillus oryzae</i>	CEDPTSGEESERYMEVAKTIKDCFHNHGIHAT
27	14582255	AAK69429	<i>Thlaspi caerulescens</i>	TIQPEFASVSGSKSSVVPCELACRTQCALKQ
28	124265492	YP_001019496	<i>Methylibium petroleiphilum PM1</i>	CCG
29	120404536	YP_954365	<i>Mycobacterium vanbaalenii</i> PYR1	TEPQAHSG— GKDACKMPCGSISCLELSECCEKKP—— KTRRTKAENSIPAVVIEIKNMPNKPPESSL
30	26518515	AAN79725	<i>Micromonospora chersina</i>	
31	27883833	AAO25899	<i>Micromonospora chersina</i>	
32	108760189	YP_630586	<i>Myxococcus xanthus DK 1622</i>	

Table 2
List of Proteins of Znt2 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1	113677287	NP_001038485	<i>Danio rerio</i>	ALWKSEASRIPPRPGVDLPSEL
2	52352805	NP_001004434	<i>Homo sapiens</i>	AVQSNHYCHAQKDSGSHPDKEK
3	14210520	NP_115902	<i>Homo sapiens</i>	QKARRKLYVASAICLVFMIGEIIIG
4	14582257	AAK69430, AF275752	<i>Thlaspi caerulescens</i>	GYLAQSLAIMTDAAHLLTDFASM LISLFSLVVSSRPATKTMNFGWQR
5	60390858	Q9BRI3	<i>Homo sapiens</i>	AEILGALLSVLSIWVVTGVLVYLA
6	133908626	NP_001076591	<i>Rattus norvegicus</i>	VQRLISGDYEIKGDTMLITSGCAV
7	6981714	NP_037022	<i>Rattus norvegicus</i>	AVNIIMGLALHQSNDGFGFEEDK
8	89179312	NP_001034766	<i>Mus musculus</i>	CGDHIHETSCHDNSSSLILSDHKN
9	8134839	Q62941	<i>Rattus norvegicus</i>	SHNMENGSVKKNFSTKRYKNINS
10	38304040	AAH61997	<i>Rattus norvegicus</i>	IIIDENDIRDNKNNSGNKIDGTSCN
11	28070974	CAD61960	<i>Thlaspi caerulescens</i>	YVTFDYDNMEEDDKNVGHVND
12	124511858	XP_001349062	<i>Plasmodium falciparum</i> 3D7	MSEQNNNFNMNKEYMSKNVYNST NKNSNNSSSKGKASNIQNNLNN
13	23498830	CAD50907	<i>Plasmodium falciparum</i> 3D7]	—MEAKEKQHLLDARPAIRSFFG NNNINNNINHNNTYNQINDEKT
14	9437324	AAF87311	<i>Mus musculus</i>	KRKKKSCCDVEKVEEDDLQKDH AYIIESEYCDHDAHGHSHGHSHS NHEDSSQQQQNPVRAAFIHVIG DLLQGLSVGLVAAYIYFKPEY KYVDPICITFLFSILVLGTTLTILRD VILVLMGTPKGVDFTTVKNLLL SVDGVEALHSLHIWALTVAQPVL SVHIAIAQNVDAQAVLKTARDRL QGKFNHFTMTIQIESYSARSNCET EDMKSCQACQGPSE—

Table 3
List of Proteins of Znt3 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1.	4502861	NP_001275	<i>Homo sapiens</i>	-----MEPSPATGGSETTRLVSPRDRGGAGGGLR
2.	52352803	NP_067017	<i>Homo sapiens</i>	LKSLFTEPSEPLPEEPKLEEMAFHHCHKDPVPQSG
3.	19527368	NP_598896	<i>Mus musculus</i>	—LSPERAQARRQLYAACAVCFIFMAGEVVGGYL
4.	20429146	CAD30327	<i>Rattus norvegicus</i>	AHSLAIMTDAAHLLADIGSMASLFSVLWLTSTRPATRTM
5.	55778706	AAH86513	<i>Rattus norvegicus</i>	TFGWHRSETLGALASVSLWIVTGILLYLAFLRL
6.	122132444	Q08E25	<i>Bos taurus</i>	HSDYHIEAGAMLLTASIAVCANLLMAFVLHQAG
7.	8134844	P97441	<i>Mus musculus</i>	PPHSHGSRGAEYAPLEEKGEP—LSLGNTSVR
8.	8134847	Q99726	<i>Homo sapiens</i>	AAFVHVLGDLLQSFGVLAASILYFKPQ—
9.	21361112	NP_003450	<i>Homo sapiens</i>	—————YKVADPISTFLFSICALGSTAPTLR
10.	61557417	NP_001013261	<i>Rattus norvegicus</i>	DVLLVLMEGA
11.	6755552	NP_035903	<i>Mus musculus</i>	PRSVFEPVRDTLLSVPGVRATHDLHLWALTLY HVASAHLAIDSTADPEAVLAEASSRLY—G-----SRF GFSSCTLQVEQYQPEMAQCLRCQEPQA----- -----

Table 4
List of Proteins of Znt4 Family

S.no.	gi no.	Accession no.	Source organism	Consensus sequence
1.	2582990	AAB82593	<i>Mus musculus</i>	MAGSGAWKRLKSMRLRKDDAPL
2.	2580603	AAB82414	<i>Mus musculus</i>	FLNDTSAFEFSDEAGDEGLSRFN
3.	2580602	AAB82413	<i>Mus musculus</i>	KLRVVVADDGSEAPERPVNGAH
4.	2580601	AAB82412	<i>Mus musculus</i>	PTLQAD
5.	2580600	AAB82411	<i>Mus musculus</i>	DDSLDQDLPLTNSQLSLKVDSC
6.	118108564	XP_001237005	<i>Gallus gallus</i>	DNCSKQREILKQRKVKARLTIAA
7.	41055299	NP_956937	<i>Danio rerio</i>	VLYLLFMIGELVGGYIANSIAIMT
8.	12006859	AAG44953	<i>Thlaspi caerulescens</i>	DALHMLTDLSAAILTLLALWLSSK
9.	21359890	NP_037441	<i>Homo sapiens</i>	SPTKRFTFGFHRLEVLSAMISVLLV
10.	52630421	NP_035904	<i>Mus musculus</i>	YILMGFLLYEAVQRTIHMNYEING
11.	8134848	Q9TTF3	<i>Bos taurus</i>	DIMLITAAVGVA NVIMGFLLNQS
12.	8134837	O55174	<i>Rattus norvegicus</i>	—GHAGAGSHSLKSLLRKDDGCLFL
13.	6561137	AAF16866	<i>Bos taurus</i>	NDQDALDFRAEFSDELGDRFNKVG
14.	8134841	O35149	<i>Mus musculus</i>	LVADDDSEAPERPVNGAHPALQAD
15.	8134840	O14863	<i>Homo sapiens</i>	DDSLADQDLPLTNSQLSLK-NPCLP-F
16.	2582415	AAB82561	<i>Homo sapiens</i>	PW-KFPFP-FFAM-MDPCD
17.	13936263	AAK40257	<i>Homo sapiens</i>	NCSKRRELLKQRKVKERLTIAAVLY
18.	25453384	NP_742063	<i>Rattus norvegicus</i>	LLFMIGELVGGYVANSIAIMTDALH
19.	18406954	NP_564766	<i>Arabidopsis thaliana</i>	MLTDLSAAILTLLALWLSSKSP
20.	19069676	CAD26061	<i>Encephalitozoon cuniculi</i> GB-M1	TRRFTFGFHRLEVLSAMISVMLVYV GHGH-HG——GL-LMGFLLYEAVQ
21.	2951686	CAA76372	<i>Rattus norvegicus</i>	RTIHMNYEINGDVMLITAAV GVAVNVIMGFLLNQSGHHHSHAH HGGCISQSLPSNPSMVSSGHNHG QDSLAVRAAFVHALGDLVQSVGV IAAYIIRFKPEYKIADPICTYIFSLVA FTTFRIIWDTVVIILEGVPSHLNVDYI KESLMKIEDVYSVEDLN IWSLTSGKATAIVHMQIPGSSSKWE EVQSKAKHLLLNTFGMYKCTIQLQS YRQEAIRTCANCQSST

Table 5
List of Proteins of Znt5 Family

S.no.	gi no.	Accession no.	Source organism	Consensus sequence
1.	50539704	NP_001002322	<i>Danio rerio</i>	MEEKYGGDVLAGPGGGGGLGPV
2.	67763826	NP_075023	<i>Mus musculus</i>	DVPSARLTKYIVLLCFTKFLKAVG
3.	72535154	NP_001026590	<i>Gallus gallus</i>	LFESYDLLKAVHIVQFIFILKLTAF
4.	56555151	AAV98201	<i>Gallus gallus</i>	FMVLFQKPFSSGKTITKHQWIKIFK
5.	74723898	Q8TAD4	<i>Homo sapiens</i>	HAVAGCIISLLWFFGLTLCGPLRTL
6.	20070323	NP_075053	<i>Homo sapiens</i>	LLFEHSDIVVISLLSVLFTSSG
7.	13443012	NP_076960	<i>Homo sapiens</i>	GGPAKTRGAFFIIAVICLLLFNDND
8.	75571395	Q5ZLF4	<i>Gallus gallus</i>	LMAKMAEHPEGHSDSALTHMLYT
9.	82182985	Q6DG36	<i>Danio rerio</i>	AIAFLGVADHKGGVLLLVLALCC
10.	62859575	NP_001015911	<i>Xenopus tropicalis</i>	KVGFHTASRKLSVDVGGAKRLQAL
11.	123892353	Q28CE7	<i>Xenopus tropicalis</i>	SHLVSVLLCPWVIVLSVTTESKVE
12.	81915062	Q8R4H9	<i>Mus musculus</i>	SWFSLIMPFATVIFVFMILDFYV
13.	151555505	AAI48651	<i>synthetic construct</i>	DSICSVKMEVSKCARYGSFPFISALL
14.	157169762	AAI53203	<i>synthetic construct</i>	FGNFWTHPITDQLRAMNKAHQES
15.	120660062	AAI30453	<i>Homo sapiens</i>	TEHVLSSGGVVVSAIFFILSAN
16.	120659882	AAI30455	<i>Homo sapiens</i>	ILSSPSKRGQKGTIGYSPEGTPLYN
17.	116283628	AAH29217	<i>Mus musculus</i>	FMGDAFQHSSQSIPRFIKDSLKQILEE
18.	114325423	AAH29033	<i>Mus musculus</i>	SDSRQIFYFLCLNLLFTFV
19.	23270961	AAH33452	<i>Mus musculus</i>	ELFYGVLTNSLGLISDGFHMLFDCS
20.	16924303	AAH17441	<i>Homo sapiens</i>	ALVMGLFAALMSRWKATRIFYGY
21.	12654017	AAH00808	<i>Homo sapiens</i>	GRIEILSGFINGLFLIVIAFFVF
22.	87248726	ABD36415	<i>Gallus gallus</i>	MESVARLIDPPELDTNMLTPVSVGGL
23.	62913979	AAH08198	<i>Homo sapiens</i>	IVNLIGICAFSHGAHSHGHGASQGS
24.	20135611	AAM09099	<i>Homo sapiens</i>	CHSHDHS SHHGHGHGDHGHHS
25.	19744306	AAL96438	<i>Mus musculus</i>	HEHKHGH THGHGH DHGHSHGQDY
26.	19744304	AAL96437	<i>Homo sapiens</i>	CHDDHCLESAGGGMNANMRGVFL
27.	12006851	AAG44949	<i>Thlaspi caerulescens</i>	HVLADTLGSIGVIVSTVLIEQFGWFI
28.	56961662	NP_001008788	<i>Gallus gallus</i>	ADPLCSLFI AVLIFLSVIPLIKDACQVL
29.	12963579	NP_075703	<i>Mus musculus</i>	LLRLPPEHEKELHIALEKIQKIEGLISY
30.	157822941	NP_001099874	<i>Rattus norvegicus</i>	RDPHFWRHSASIVAGTIHQVTSVDLE
31.	82126353	Q5MNV6	<i>Gallus gallus</i>	QRIVQQVTG—————ILKDAGVNNLTI
32.	57530327	NP_001006402.	<i>Gallus gallus</i>	QVEKEAYFQHMSGLSTGFHDVLAMT
33.	56555153	AAV98202	<i>Gallus gallus</i>	KQMESMKYCKDGTIYIMYKDGTYIM—————

Table 6
List of Proteins of Znt6 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1.	45387725	NP_991214	<i>Danio rerio</i>	
2.	31559966	NP_659047	<i>Mus musculus</i>	-----
3.	20563194	AAM27917, AF395840	<i>Mus musculus</i>	----- M
4.	21687224	NP_060434	<i>Homo sapiens</i>	VALDVLGITDSDAPVYRQKQEAD
5.	81873983	Q8BJM5	<i>Mus musculus</i>	TLVMGTIHLFRKPQRSFFGKLLQE
6.	57530327	NP_001006402	<i>Gallus gallus</i>	FRLVAADRRSWKILLFGAINVICTG
7.	58332738	NP_001011444	<i>Xenopus tropicalis</i>	FLL-----MWCSSTNSIALTAY
8.	162416263	Q6NXT4	<i>Homo sapiens</i>	TYLTIFDLF-----S-L-----
9.	122142394	Q0VC54	<i>Bos taurus</i>	-----P-SLI
10.	82186094	Q6P0D1	<i>Danio rerio</i>	TCLISYWVMMRKPSPVYSFGFERL
11.	82179289	Q5I0B2	<i>Xenopus tropicalis</i>	EVLAVFASTVLAQ-----LGALFILK
12.	75571226	Q5ZIH3	<i>Gallus gallus</i>	ESAERFLEQPEIHTGRLL
13.	45829813	AAH68169	<i>Mus musculus</i>	VGTFVALSFNLTMLSIRNKPFAFV
14.	42542781	AAH66162	<i>Mus musculus</i>	SEAASTSWLQEHVADLSRSLCGIIP
15.	45219914	AAH66903	<i>Homo sapiens</i>	GLSSIFLPRMNP-----FVLIDLA
16.	21619123	AAH32525	<i>Homo sapiens</i>	GAFALCITYMLIEINN-YFAVD TAS
17.	13543167	AAH05753	<i>Mus musculus</i>	AIAIALMT-----FGTMYPMSV
18.	56387491	AAV86135	<i>Oncorhynchus mykiss</i>	YSGKVLLQTTPPHVI
19.	74723898	Q8TAD4	<i>Homo sapiens</i>	GQLDKLIREVSTLDGVLEVRN-----
20.	66735073	AAY53770	<i>Gallus gallus</i>	-----EHFWTLGFGSLAGSVHVRIR RDANEQM-----VLA HVTNRLYTLVSTLTVQIFKDDWI RPALSSGPVAPNVLNFS DHHTAAAVIP MPLLKNQVDELNPVTSTPAKPSSPPP EFSFNTP— GKNVNPVILLNTQTRPYGLGLNHGH TPYSSVFNQGLGVPGI----- GATQGLRTGFTNIPSRYGTTNNPGRM GQPRP—

Table 7
List of Proteins of Znt7 Family

S.No.	gi no.	Accession no.	Source organism	Consensus sequence
1.	41054213	NP_956100.1	<i>Danio rerio</i>	MEEKYSSQALAGGGV—GL
2.	27497128	AAO17324	<i>Homo sapiens</i>	GPVDVPSARLTRYIVLLCFA
3.	27497126	AAO17323	<i>Mus musculus</i>	KFLKAVGLFESYDLLKAVHL
4.	12963579	NP_075703	<i>Mus musculus</i>	VQFIFIVKLGSFAF
5.	81881944	Q9JKN1	<i>Mus musculus</i>	FMVLFQKPFSSGKVVTKHQW
6.	56961662	NP_001008788	<i>Gallus gallus</i>	IKIFKHAVVGCIIISLLWFFGLTL
7.	56555153	AAV98202	<i>Gallus gallus</i>	CGPLRTLLEHSDVVLVLSLS
8.	40255009	NP_598003	<i>Homo sapiens</i>	VLFTSSG
9.	74751261	Q8NEW0	<i>Homo sapiens</i>	GGPAKTRGAFFIIAVICLLLF
10.	82126353	Q5MNV6	<i>Gallus gallus</i>	NDDLMAKIAEHPEGHHSALHT
11.	166228732	Q5BJM8	<i>Rattus norvegicus</i>	VLYTVIAFLGVADHKGGLVLL
12.	166228724	A5PMX1	<i>Danio rerio</i>	VLALCC
13.	45361357	NP_989256	<i>Xenopus tropicalis</i>	KVGFHMASRKLSVDVGGAKRL
14.	166228723	A4IFD7	<i>Bos taurus</i>	QALSHLVSVLLCPWVIVLSLT
15.	82186333	Q6P3N9	<i>Xenopus tropicalis</i>	ESKVESWSSLIMPFITVIFVVID
16.	18859139	NP_571139	<i>Danio rerio</i>	FYV
17.	40353020	AAH64692	<i>Homo sapiens</i>	ESICSVKMESKSCARYGSFLIFIS
18.	29504753	AAH50193	<i>Mus musculus</i>	ALLFGNFWTHPITDQLRAMNKPA
19.	16877804	AAH17136	<i>Mus musculus</i>	HHESTEHLVSGGVVSAVFFMLPLS
20.	21280083	AAM21969	<i>Homo sapiens</i>	IKDSPSRKGQKGTIGYSPEGTPLY
21.	31559966	NP_659047	<i>Mus musculus</i>	NFMGDEYKPPKFNLFKGISGWFR
22.	72535154	NP_001026590	<i>Gallus gallus</i>	SILSDKTSRNLFFFLCLNLSFAFV
23.	74723898	Q8TAD4	<i>Homo sapiens</i>	ELLYGIWSNSLGLISDSFHMFFDS
24.	67763826	NP_075023	<i>Mus musculus</i>	TALLAGLAASVSKWRDNDASFG
25.	75571395	Q5ZLF4	<i>Gallus gallus</i>	YVRAEVLAFVNG-LFLIFTAFFI
26.	57530327	NP_001006402	<i>Gallus gallus</i>	FSEGERALAPPDVHHERLLLVSL
27.	56555151	AAV98201	<i>Gallus gallus</i>	GFVNVNIGIFVFQHGGS-HGHSHG
				GSGHGHSHSLFNGSAALDHS
				GHHGHSHEDHCHSHEAKHGAHS
				HDHDEHAHGHGHSHSHGHGYGS
				SCDGPSLKETTGPSRQILQGVFLHIL
				AD
				TLGSIGVIASAIMMQNFGMLIADPI
				CSILIAILIVSVIPLLRVGLMQRT
				PPSLENALPQCYQVRVQQLQ
				GVYSLQEPHFWTLCSDVYVGTCLKV
				VAPDADARWILSQTHNRL-TLVSTLT
				VQIFTQAGVRQLYVQIDFAAM
				FQHMSGLSTGFQDVLAMTQHLESMK
				YYKDGTIIMPPEFSFNTPGKNV-
				PVILLNTQTRPY-LGLN-G-TPYS
				SV—QG—PG-GA-QG-R—F—PSRYG-N
				—GQ-RP

Table 8
List of Proteins of Znt8 Family

S.no.	gi no.	Accession no.	Source organism	Consensus sequence
1.	64762489	NP_776250	<i>Homo sapiens</i>	MGRYSGKTCRLLFMLVLTVAFFVAELVSGY
2.	74723654	Q8IWU4	<i>Homo sapiens</i>	LGNSIALLSDSFNMESDLISLCVGLSAGYIAR
3.	81873697	Q8BGG0	<i>Mus musculus</i>	RPTRGFSATYGYARAEEVVGALSNAVFLTAL
4.	37785938	AAP44332	<i>Homo sapiens</i>	CFTIFVEAVLRGHLARPERIDDPPELVIVGV
5.	52351208	NP_061183	<i>Homo sapiens</i>	GLLVNVVGLLIFQDCAAWFACCLRGRSRR
6.	148921810	AAI46292	synthetic construct	QQRQQLAEGCVPGAFFGGPQGAEDPRRAAD
7.	74722882	Q6XR72	<i>Homo sapiens</i>	PTAPGSDSAVTLRGTSVERKREKATVFAN
8.	92058723	AAI14620	<i>Homo sapiens</i>	VAGDSFNTQNEPEDMMKKEKSEALNIRGV
9.	71297457	AAH36078	<i>Homo sapiens</i>	LLHVMGDALGSVVVVITAIIFYVLPKSEDP
				CNWQCYIDPSLTVLMVIIISSAFPLIKETAAI
				LLQMVPKGVNMEELMSKLSAVPGISSVHEV
				HIWELVSGKIIATLHIKYPKDRGYQDASTKIR
				EIFHHAGIHNVTIQFENVLDKEPLEQKDLLL
				CNSPCISKGCAKQLCCPPGALPLAHVNGCAE
				HFLCHVNGCAEHNGGPLSDTYGSDGLSRRD
				AREVAIEVSLDSCSLDHGQSLNKTQEDQCY
				VNRTHF

Table 9
List of Proteins of Znt9 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1.	57164948	NP_006336	<i>Homo sapiens</i>	MGRYSGKTCRLLFMLVLTVAFFVAELVSGYLGNSIALLSDSFNMESDLISLCVGLSAGYIARRPTRGFSATYGYARAEEVVGALSNAVFLTALCFTIFVEAVLRGHLAR
2.	56693221	NP_001008575	<i>Danio rerio</i>	PERIDDPPELVIVGVLLLVNVVGLLIFQDCAAWFACCLRGRSRRLLQQRQQLAEGCVPGAFFGGPQGAEDPRRAADPTAPGSDSAVTLRGTSVERKREKATVFANVAGDSFNTQNEPEDMMKKEKSEALNIRGVLLHVMGDALGSVVVVITAIIFYVLPKSEDP
3.	16877404	AAH16949	<i>Homo sapiens</i>	CNWQCYIDPSLTVLMVIIISSAFPLIKETAAIILQMVPKGVNMEELMSKLSAVPGISSVHEVHIWELVSGKIIATLHIKYPKDRGYQDASTKIREIFHHAGIHNVTIQFENVLDKEPLEQKDLLL
4.	14043490	AAH07732	<i>Homo sapiens</i>	CNSPCISKGCAKQLCCPPGALPLAHVNGCAEHFLCHVNGCAEHNGGPLSDTYGSDGLSRRDAREVAIEVSLDSCSLDHGQSLNKTQEDQCYVNRTHF
5.	18606169	AAH22981	<i>Homo sapiens</i>	YVNRTHF
6.	46850114	AAT02479	<i>Homo sapiens</i>	

Table 10
List of Proteins of Znt10 Family

S. No.	gi no.	Accession no.	Source organism	Consensus sequence
1.	52351208	NP_061183	<i>Homo sapiens</i>	MLPGLAAAAHRCSSWSSLCRLRLRCRAAACNPSDRQ—EWQNLVTFGSFSN
2.	123788615	Q3UVU3	<i>Mus musculus</i>	MVPCSHPYIGTSLSQVKLY
3.	74722882	Q6XR72	<i>Homo sapiens</i>	STN-VQKEGQGS—QTLRVEKVPSFE-TAEGIGAEKAPLKQEPLQVRVKAVLKKREY
				GSKYTQNNFITGVRAINEFCL
				KSSDLEQLRKIRRRSPHEDTESFTVYLRSDVEAKSLEVWGSPEALAREKKLRKEAEIE
				YRERLFRNQKILREYRDFLGNTKPRSRTASVFFKGPVKVVMVAICINGLNCFFKFLA
				WIYTGASAMFSEAIHSLSDTCNQGLLALGISKSVQTPDPSPHYGFSNMRYISSLSISGV
				GIFMMGAGLSWYHGVMLLHPQPIESLLWAYCILAGSLVSEGATLLVAVNELRRNA
				RAKGMSFYKYVMESRDPSTNVILLEDTAAVLGVIIAATCMGLTSITGNPLYDSLGL
				GVGTLGTMVSAFLIYTNTEALLGRSIQPEQVQRLTELENDPSVRAIHDVKATDLGL
				GKVRFKAEDVDFDGRVVTSSYLEKQDFDQMLQEIQEVKTPEELETFMLKHGENIIDTL
				GAEVDRLEKELKKRNPEVRHVDLEIL

eukarya) for the Znt1 and Znt2 protein families, a Blast-p (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) were performed for all the proteins of Znt1 and Znt2 listed in table 1 and 2. The 32 member homologs of Znt1 subfamily (Table 1) with identity greater than 80% were obtained which included bacterial and archaeal members as well. These were multiple aligned and were used for phylogenetic reconstruction of Znt1 family by using Clustal W program (clustering algorithm option was set at UPGMA for fast tree construction and the bootstrap value was 100 replicates). Phylogenetic reconstruction was performed for the 14 protein members of Znt2 subfamily (no bacterial or archaeal hits was observed after blastp run for Znt2 proteins). Blastp was also carried out for the ten consensus sequences of Znt 1-10 subfamily in the similar manner as that for Znt1 or Znt2.

ZnT Signature Motif Analysis

Sequence logo representation of the Znt1, Znt2 and the entire 10 consensus sequences of Znt1-10 were constructed with the web interface program WebLogo (<http://weblogo.berkeley.edu/logo.cgi>). The logo representations were analyzed for motif signature sequences characteristic of Znt proteins.

Results

Phylogeny, Sequence Analysis and Signature Motifs of Znt1 Subfamily

All the 32 members of Znt1 family (Table 1) clustered into three groups where Group I included only animal Znt1 proteins, Group II included plants, bacterial, fungal and animal members and Group III included only fungal members as shown in Fig. 1. These groups were further subdivided into various subgroups.

Group I of animal Znt1 proteins bifurcated further in two subgroups A and B, where subgroup A contained all vertebrates other than mammals and subgroup B included only mammals. Subgroup B further included two sub subgroups, B1 included all the representatives from mammals other than primates and B2 with all primates alone. In B2 all the human Znt1 proteins clustered together however only Znt1 protein from human (gi no. 13958315, accession

no. AAK50854, AF364518) clustered separately with *Macaca fascicularis* suggesting its more similarity with that of primates.

Group II included plants, bacterial, fungal and animal Znt1 proteins. This group also got subdivided into two subgroups C and D, the former included plant, bacterial and animal members and latter included only fungal, respectively. The former (C) subdivided into two sub subgroups, C1 with plant members only and C2 with bacterial members alone. A human protein (gi no. 21361112, accession no. NP_003450) which would normally cluster with that of Group B2, was present in between the two subgroups C and D in this study and was found related to plant, bacteria and fungi (Fig. 1). The C1 group showed the presence of all the Znt1 proteins from *Thlaspi caerulescens* and *Arabidopsis thaliana* except one of the *T. caerulescens* protein (gi no.161513446, accession no. CAJ44263), which was more closely situated to the Znt1 protein of *Arabidopsis thaliana* in the phylogram (Fig. 1). The protein is a very small one consisting of 21 amino acids only.

Group III exclusively contained only fungal members and was very distantly related to Group I and Group II, especially subgroup C (Fig. 1).

The sequence analysis of the 32 protein sequences for Znt1 subfamily (Table 1) revealed the presence of short conserved regions upon multiple alignment at residues 144-154, 201-216, 226-233, 240-253, 327-344, 357-378, 386-397, 400-431, 738-749.

Weblogo analysis located the presence of one signature sequence (HXHXHX) at the residue position 211-216 (where H=3, X=3, n = 3 and X is either G or S) for Znt1 (Fig. 2a).

Phylogeny, Sequence Analysis and Signature Motifs of Znt2 Subfamily

Fig. 3 shows the phylogram of Znt2 family generated using 14 proteins retrieved from the protein database at NCBI with clustering of the two proteins from *T. caerulescens* (gi no.14582257, 28070974) to form a single cluster. This cluster also included a protein of *Mus musculus* (gi no. 9437324, accession no. AAF87311). The two proteins of *Plasmodium falciparum* (gi

no.124511858, accession no. XP_001349062 and gi no. 23498830, accession no. CAD50907) formed a single cluster with a close linkage to the protein of *T. caerulescens* and *Mus musculus*. All these proteins formed a single cluster and were linked to the protein of the mammal *Danio rerio* (gi no.113677287, accession no. NP_001038485). This suggests that *P. falciparum* protein for Znt2 is more closely related to *T. caerulescens*, which in turn is closer to *Mus musculus*. The Znt2 proteins from *Homo sapiens* clustered together and showed close relationship with plants and with the protein of *Mus musculus* (gi no. 89179312, accession no. NP_001034766). All the four proteins of *Rattus norvegicus* clustered together and were closely related to *Homo sapiens* (Fig. 3) suggesting that all the proteins from *Rattus*, *Mus* and *Homo sapiens* originated from a common mammalian ancestor.

The sequence analysis of the 14 members of Znt2 proteins (Table 2) revealed both short and long conserved regions at residues 43-202 and 553-596. Very large patches of well conserved regions were present at the sequence position 417 to 551. These results also suggest that there is very significant similarity between the various Znt2 proteins from different organisms and therefore they exist together as a single cluster in the phylogram (Fig. 3).

Two signature motifs of the type (HX)_n were observed in consensus sequence for Znt2 one of type HXHXHXHX at the sequence position 418-425 (where H=4, X=4, n = 4 and X is either G or S) and other of type HXHXHXHXHX (where H=5, X=5, n = 5 and X is either G or S) at residue 441-447 (Fig. 2b) on the Weblogo graph.

Phylogeny, Sequence Analysis of Znt1-10 Subfamily and Signature/marker Motifs of ZnT Superfamily

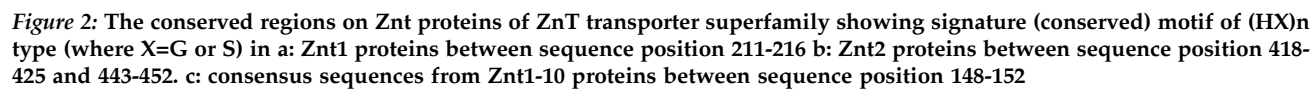
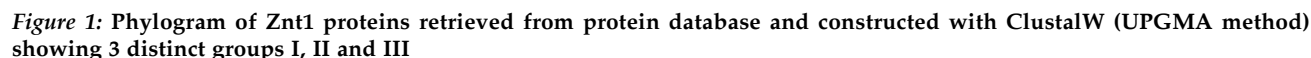
The consensus sequences obtained for each Znt family 1-10 using the representative members displayed in Tables 1-10 when used to construct a phylogram, clustered largely into 3 major groups namely, group - a, b and c (Fig. 4). Group a further subdivided in two groups a1 and a2. Subgroup a1 included Znt8 and Znt9 as a single cluster and was more close to Znt1. Subgroup a2 consisted of Znt2, Znt3 and Znt4 clustered together. Group b also formed two subgroups: b1

having Znt5 and Znt7 and b2 with Znt6 alone. Group c consisted of the members from Znt10 protein subfamily alone, suggesting thereby that it is distantly related to all the other members of the Znt family. Blast 2 results between Znt10 and Znt1/Znt2 did not show any significant similarity with Znt1 and Znt2, strengthening the view that Znt10 has very little similarity with other Znt families. The ten representative consensus sequences when analyzed for the signature motif for ZnT superfamily showed presence of only short conserved regions, (HX)_n motif of the type (HXHXH) (where H=3, X=2, n = variable but X is either G or S) at residues 148-152 (Fig. 2c).

To further verify the phylogram, the consensus sequences for either Znt1 or Znt2 were taken as query sequence and a blast2 search was performed with the consensus sequence of other members of the Znt family. No significant similarity could be obtained between Znt1 and Znt2/Znt3/Znt4/Znt6/Znt10. One region of significant similarity was found between Znt1 and Znt7 with 30% identity, whereas two regions of significant similarities were found between Znt1 and Znt5 (31% and 20%), Znt1 and Znt8 (32% and 42%) and Znt1 and Znt9 (32% and 42%), respectively. No significant similarity was found between Znt2 and Znt10.

With Znt2 as query, only one region of significant similarity was observed between Znt2 and Znt1 (24% identity) and between Znt2 and Znt6 (26% identity). Two regions of significant similarities were observed between Znt2 and Znt3 having 51% and 53% identities. Between Znt2 and Znt5 a 26% and 22% identity was noted whereas between Znt2 and Znt7 only 27% and 22% identities were observed. Znt2 and Znt8/Znt9 had almost similar identities of 26% and 24%. Three regions of significant similarity were found between Znt2 and Znt4 with 46%, 41% and 39% identities.

When 10 consensus sequences for Znt subfamilies 1-10 were taken as query sequence with respect to Znt1 alone, the results obtained showed no significant similarity between Znt6 and Znt1 or Znt10 and Znt1 as before, however, a single region of significant similarity was found between Znt3 and Znt1 (26% identities) and between Znt4 and Znt1 (28% identity). Two



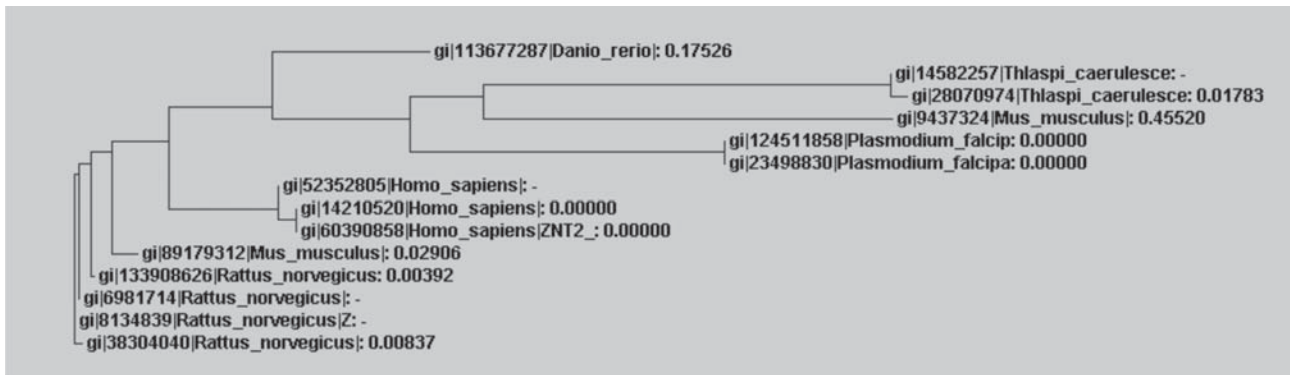


Figure 3: Phylogram of Znt2 proteins retrieved from protein database and constructed with ClustalW (UPGMA method)

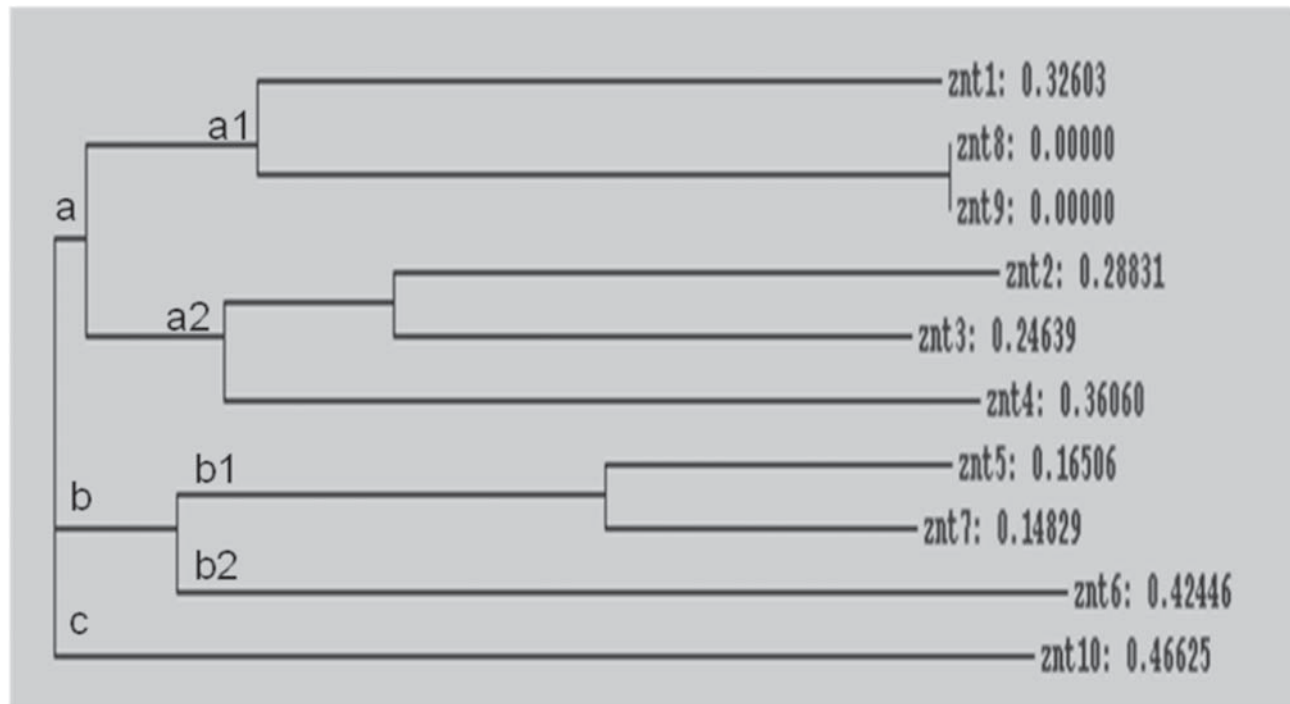


Figure 4: Phylogram of consensus sequences obtained from Jalview of Znt 1-10 aligned with ClustalW

regions of significant similarity were found between Znt5 and Znt1 with 32% and 21% identities, between Znt8 and Znt1 with 32% and 44% identities and between Znt9 and Znt1 with 32% and 44% identities. The similarity and % identity between Znt1 and Znt5/Znt7/Znt8/Znt9 remained same as before.

Upon taking consensus sequence from Znt1-10 as query against Znt2 no significant similarity could be observed between Znt1 and Znt2, between Znt10 and Znt2. One region of significant similarity was found between Znt6 and Znt2 with 26% identity while between Znt7 and Znt2 a 22% identity was noted. Two regions of significant similarity were present between Znt3 and Znt2

having 55% and 48% identities, between Znt5 and Znt2 with 27% and 22% identities and between Znt8/Znt9 and Znt2 25% identities for each region were observed. Three regions of significant similarity were found between Znt4 and Znt2 consisting of 46%, 38% and 46% identities, respectively.

Discussion

In the phylogenetic analysis of Znt1 proteins in this study, the proteins of same organism came closer in comparison to those from other organisms and the proteins of evolutionarily closely related organisms also had close proximity suggesting that there is more divergence during

the evolution of Znt1 proteins from bacteria to metazoans. Evidence from an evolutionary tree based on 18s rRNA sequences places the origin of the kingdom Plantae among the phyla of the ancestral kingdom Protozoa (Cavalier-Smith and Chou, 2003). Hence it is reasonable to suppose a common origin of extant Znt in the ancient line of protists. The sequence analysis of multiple aligned Znt1 proteins show very short patches of conserved regions among the members of Znt1 proteins.

When the blastp were performed it has been observed that most of the bacterial and archaeal hits were synthetics and are not directly involved in Zn transportation but indirectly they may be involved in regulating the transport of Zn by regulating the transcription and translation of Zn transporting proteins.

Phylogenetic analysis of Znt2 protein revealed more closeness among the members from both closely and distantly related organisms indicating that there is less divergence during the evolution of Znt2 proteins from lower to higher organism. It can also be seen at sequence level where large patches of well conserved region were found among the members of Znt2 proteins. We noted that no bacterial or archaeal member was found for the Znt2 proteins after performing blastp search against the non-redundant protein database. An important observation is the occurrence of two different Znt2 proteins from the same organism (*Mus musculus*) on different evolutionary branches (Fig. 3). This could possibly be due to early segregation in the diversification of this clade or due to presence of multiple forms of genes coding for Znt2 proteins, indicating a high level of sequence identity (which was true in our case also) but difference at protein level (Jespersen *et al.*, 1997).

The phylogeny of Znt subfamilies in this study showed in Znt5 and Znt7 to be more closer to Znt6 in comparison to Znt1. However, these results are different from that reported earlier (Kirschke and Huang, 2003) where the authors show more similarity between Znt5 and Znt7 with Znt1. Our results show Znt2, Znt3 and Znt4 to be closely related which is in accordance with the result reported by the same group (Kirschke and Huang, 2003). Like most of the transporter

protein families, Znt family proteins vary greatly in length from few amino acid residues to several hundred amino acid residues (Shah *et al.*, 2011). Histidine (H), glycine (G), serine (S), aspartate (D), leucine (L), valine (V), are the most frequent amino acid residues among the members of Znt family (Kirschke and Huang, 2003). These amino acids are present at the signature residues and can be used as a potential marker for Znt family (Montanini *et al.*, 2007). In the Znt family members six predicted transmembrane domains and a histidine (H) rich loop between transmembrane domain IV and V is present. The H-rich loop has been proposed to bind Zn, because histidine (H) is a common ligand for Zn (Kirschke and Huang, 2003). Here we found short as well as long conserved regions with (HX)*n* motifs, using the consensus sequence. Such motifs have been reported in members of ZnT family where *n* ranges from 3-6 and X is often G or C (Gaither and Eide, 2001). Therefore, consensus sequence approach seems to be a reliable tool for searching signature motifs in large protein families. The complete protein sequences are known for Znt from various prokaryotes, however, the distribution of Znt protein subfamilies are unequal among closely related organisms as seen in this study. This unequal distribution of Znt families can be attributed to a lateral gene transfer between otherwise phylogenetically unrelated organisms (Ragan, 2001). No significant similarity among members of Znt1 and Znt10 or Znt2 and Znt10 suggest that gene duplication event in the ZnT superfamily gene probably occurred in the later phase of evolution when individual specificities of the distinguished ZnT subgroups were already formed.

Conclusion

It is concluded from this study that members of Zn transporter families are present in all life forms. The Zn transporter protein contains both short and long signature motifs of (HX)*n* type that can be targeted and used as a marker for the study of Zn uptake and transport. A consensus sequence approach used in this study seems a useful computational tool that can be applied to large protein families for quick and reliable short-listing of protein signature motifs and for identification and annotation purposes. Of the ten

Znt subfamilies and 166 member representatives of ZnT superfamily included in this work, the Znt1 and Znt2 are large protein families and Znt10 seem to have very few proteins. There is almost no similarity between Znt1 and Znt10 or Znt2 and Znt10. Unlike Znt2, there occurs little similarity between the various Znt1 proteins from different organisms as revealed by the phylogram obtained suggesting more diversity among members of Znt1 subfamily than Znt2 subfamily. Znt2 protein of plants and protozoa probably diverged from the mammalian Znt2 proteins during evolution. Unequal distribution of Znt subfamilies can possibly be attributed to lateral gene transfer between unrelated organisms; however, this can be ascertained only when more members for each family are reported from all taxa.

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Abbreviations

ZnT, zinc transporter; ZIP, Zrt-Irt-like proteins; CDF, cation diffusion facilitator; Irt1, iron regulated transporter.

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