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Phylogenetic Analysis of NHX1 Gene in Different Species of Plants Based on Coding Sequences

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The world population is anticipated to be about six billion by the end of the year 2050 which is very alarming (Najafi et al. 2019). On the other hand, food productivity is reducing due to various abiotic stresses; therefore, minimizing this situation is a major concern for all nations to cope with the increasing food requirements. Soil salinity is a serious environmental stress limiting plant productivity (Shrivastava et al. 2015). Sodium ions (Na^+) abundant in saline soil, are cytotoxic in plants when they accumulate to high concentrations (Hasegawa et al. 2013). Over accumulation of Na^+ in the cytosol causes protein synthesis inhibition, many enzymatic reactions, and photosynthetic processes (Yamaguchi et al. 2013). Therefore, it is crucial to exclude Na^+ from photosynthetic organs for adequate metabolism and efficient carbon fixation.

Until now, several classes of Na^+ transporters have shown to play significant roles in Na^+ homeostasis during salinity stress (Uozumi et al. 2010). The NHX transporter has drawn particular attention because of its capability to transport Na^+ across cellular membranes. To date, most of NHX proteins studied facilitate electroneutral Na^+/H^+ and K^+/H^+ antiport through the membrane by using the H^+ gradient as a driving force (Uozumi et al. 2010). These were observed to be responsible for Na^+ reclusion into intracellular compartments under salinity stress. Overexpression of vacuolar NHX gene has conferred salt tolerance to a range of plant species such as wheat, rice, and tomato with a concomitant increase in tissue Na^+ (Sahoo et al. 2016, Zhang et al. 2017). Moreover, the role of vacuolar NHXs in Na^+ compartmentation under salinity stress is strongly supported by the findings of the Arabidopsis AtNHX1 mutant exhibited Na^+ sensitivity and significantly less vacuolar Na^+/H^+ antiport activity (Apse et al. 2003). Besides an essential role in salt tolerance, pH regulation and organ developments have been demonstrated for vacuolar NHXs (Yamaguchi et al. 2001). Under normal conditions, the

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Arabidopsis AtNHX1/AtNHX2 double knockout plant showed severe plant growth defects, leaf cell expansion, and reproductive organ (Barragán et al. 2012, Bassil et al. 2011).

Plant genomes often contain several intracellular isoforms of the NHX gene (Bassilet al. 2012). These NHX transporters are classified into the IC-NHE/NHX family, a part of the large cation/proton antiporter 1 (CPA1) family (Yamaguchi et al. 2013). IC-NHE/NHX family is further classified into vacuolar (Class I) or endosomal (Class II) NHXs, according to their sequence similarity and subcellular localizations of representative members (Bassil et al. 2012, Rodríguez-Rosales et al. 2009). Until now most of the plant species sequenced contain both types of NHXs (Bassil et al. 2012, Rodríguez-Rosales et al. 2009), and functional redundancies of vacuolar or endosomal NHXs had been recently reported in Arabidopsis (Bassil et al. 2011).

Recently, two distinct topology models have been suggested for AtNHX1. The protease protection analysis had been used to propose the first model in vacuoles of yeast expressing full-length AtNHX1 containing epitope tags inserted into each hydrophilic loop (Yamaguchi et al. 2003). This model is consist of nine transmembrane segments with a C-terminal hydrophilic tail facing toward the vacuolar lumen. There was another topology model was proposed on the basis of in vitro translation experiments using truncated AtNHX1 genes. The second model suggested that several transmembrane segments of AtNHX1 retain similar to pogenic properties as human NHE1 (Sato et al. 2005).

Phylogenetic analysis is a powerful tool for addressing many different evolutionary questions (Mansouri et al. 2013). Identifying of one gene and its homologs in several species indicates that these genes divergence from their common ancestor. The primary objective is to infer the pattern of the evolutionary relationships between the protein sequences of NHX1 gene that are being compared. These relationships will be revealed by the topology of the tree that will be constructed. This phylogeny will provide another perspective on biodiversity that allows an objective way to compare uniqueness and diversity of mentioned taxa.It is expected that our findings will shed light on functional characterization of mentioned NHX1 genes.

All sequences of NHX1 gene of different sources of organisms available in GenBank were downloaded from the NCBI database (www.ncbi.nlm.nih.gov/genbank), as showed in Table 1.

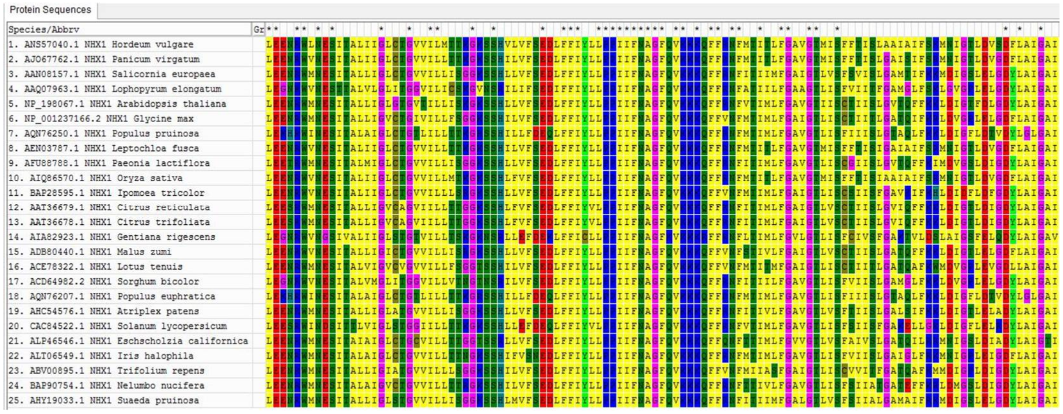
The program MEGA6 was used for multiple sequence alignment and phylogenetic tree construction. Multiple sequence alignment was done by the Clustal W method (http://www.genome.jp/tools/clustalw). The phylogenetic tree was drawn based on aligned protein sequences using the Maximum likelihood method based on the Poisson correction model (Tamura et al. 2013). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Table 1. Descriptions of NHX1 gene sequences from different species.

Accession No.	cDNA length (bp)	mRNA length (bp)	Protein Id	Family	Species	No. of sequence
KX268353.1	1617	1617	ANS57040.1	Gramineae	*Hordeum vulgare voucher*	1
KJ739865.1	1666	1666	AJO67762.1	Gramineae	*Panicum virgatum*	2
AY131235.1	2668	2668	AAN08157.1	Amaranthaceae	*Salicornia europaea*	3
AF507044.1	1955	1955	AAQ07963.1	Gramineae	*Lophopyrum elongatum*	4
NM_122597.3	2553	2553	NP_198067.1	Brassicaceae	*Arabidopsis thaliana*	5
NM_001250237.2	2732	2732	NP_001237166.2	Fabaceae	*Glycine max*	6
KX132347.1	4370	4370	AQN76250.1	Salicaceae Mirb.	*Populus pruinosa*	7
JF933902.1	1623	1623	AEN03787.1	Gramineae	*Leptochloa fusca*	8
JX524227.1	2104	2104	AFU88788.1	Gramineae	*Paeonia lactiflora*	9
KM083065.1	2313	2313	AIQ86570.1	Gramineae	*Oryza sativa Indica*	10
AB982130.1	1629	1629	BAP28595.1	Convolvulaceae	*Ipomoea tricolor*	11
AY607026.1	4920	4920	AAT36679.1	Rutaceae	*Citrus Reticulata*	12
AY607025.1	4939	4939	AAT36678.1	Rutaceae	*Citrus trifoliata*	13
KF941190.1	1602	1602	AIA82923.1	Gentianaceae	*Gentiana rigescens*	14
GQ503257.1	2480	2480	ADB80440.1	Rosaceae	*Malus zumi*	15
EU727217.1	1632	1632	ACE78322.1	Fabaceae	*Lotus tenuis*	16
EU482408.2	1473	1473	ACD64982.2	Poaceae	*Sorghum bicolor*	17
KX132304.1	4376	4376	AQN76207.1	Salicaceae	*Populus euphratica*	18
KC608048.1	1668	1668	AHC54576.1	Amaranthaceae	*Atriplex patens*	19
AJ306630.1	2122	2122	CAC84522.1	Solanaceae	*Lycopersicon esculentum*	20
KU156822.1	1587	1587	ALP46546.1	Papaveraceae	*Eschscholzia californica*	21
KT306824.1	1946	1946	ALT06549.1	Iridaceae	*Iris halophila*	22
EU109427.1	2394	2394	ABV00895.1	Fabaceae	*Trifolium repens*	23
AB501188.1	2237	2237	BAP90754.1	Nelumbonaceae	*Nelumbo nucifera*	24
KJ452342.1	1662	1662	AHY19033.1	Amaranthaceae	*Suaeda pruinosa*	25

A total of 25 cDNA sequences of NHX1 genes from different source organisms were considered in silico analysis. According to (Table 1). Lengths of cDNA in 8 species like *Hordeum vulgare voucher* (1617 bp), *Panicum virgatum* (1666 bp), *Leptochloa fusca* (1623 bp), *Ipomoea tricolor* (1629 bp*), Gentiana rigescens* (1602 bp), *Lotus tenuis* (1632 bp), *Atriplex patens* (1668 bp), *Suaeda pruinosa* (1662 bp) were nearly equal that are belonging to the family Gramineae, Convolvulaceae, Gentianaceae, Fabaceae and Amaranthaceae respectively. *Citrus Reticulata* (4920 bp) and *Citrus trifoliate* (4939 bp) was also nearly equal in size belonging to the Rutaceae family. This feature showed that cDNA sequences of NHX1 genes in these species remain conserved during divergence from their common ancestor. Moreover, in all species, the length of mRNA and length of cDNA was equal, representing the NHX1 gene sequence is a whole exon region in this species. In silico analysis of NHX1 gene sequences and its comparison with its homologs in the other species, it revealed the sequence-based similarity between different species and clustering in distinct groups based on similarity.

Alignment by Clustal W Method: Multiple sequence alignment of NHX1 gene sequences showing homology in different regions (Fig. 1).



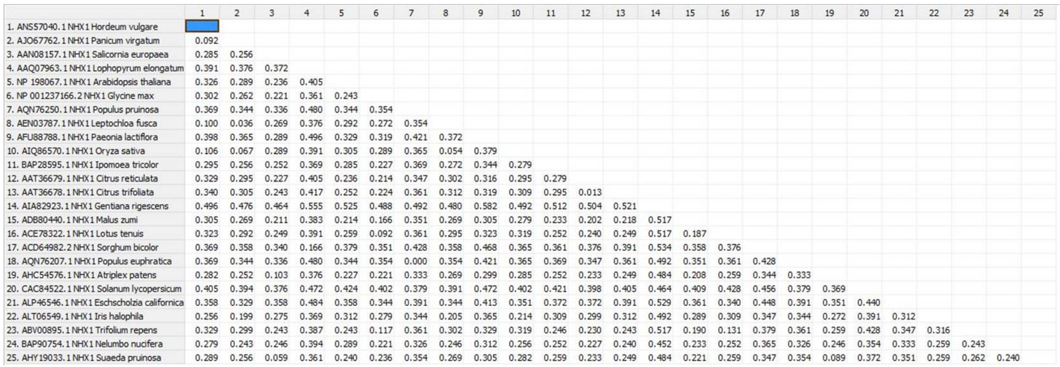
1. ANS57040.1 NHX1 Hordeum vulgare
2. AJO67762.1 NHX1 Panicum virgatum
3. AAN08157.1 NHX1 Salicornia europaea
4. AAQ07963.1 NHX1 Lophopyrum elongatum
5. NP_198067.1 NHX1 Arabidopsis thaliana
6. NP_001237166.2 NHX1 Glycine max
7. AQN76250.1 NHX1 Populus pruinosa
8. AEN03787.1 NHX1 Leptochloa fusca
9. AFU88788.1 NHX1 Paeonia lactiflora
10. AIQ86570.1 NHX1 Oryza sativa
11. BAP28595.1 NHX1 Ipomoea tricolor
12. AAT36679.1 NHX1 Citrus reticulata
13. AAT36678.1 NHX1 Citrus trifoliata
14. AIA82923.1 NHX1 Gentiana rigescens
15. ADB80440.1 NHX1 Malus zumi
16. ACE78322.1 NHX1 Lotus tenuis
17. ACD64982.2 NHX1 Sorghum bicolor
18. AQN76207.1 NHX1 Populus euphratica
19. AHC54576.1 NHX1 Atriplex patens
20. CAC84522.1 NHX1 Solanum lycopersicum
21. ALP46546.1 NHX1 Eschscholzia californica
22. ALT06549.1 NHX1 Iris halophila
23. ABV00895.1 NHX1 Trifolium repens
24. BAP90754.1 NHX1 Nelumbo nucifera
25. AHY19033.1 NHX1 Suaeda pruinosa

Fig. 1. Multiple sequence alignment of protein in a part of NHX1 gene sequence in the investigated species

The result of protein sequences alignment revealed that some sequences have high similarity for this gene and in some regions have a point different that can have an environmental effect such as mutation. Finally, the whole sequences' alignment showed the highly conserved region to primer design for PCR reaction to study genetic diversity and identification gene in plants.

Evolutionary divergence between sequences: The number of amino acid differences per site from between sequences were shown (Fig. 2). The analysis involved 25 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 399 positions in the final dataset.

Phylogenetic tree: This analysis showed evolutionary relationships predicted from the multiple sequence alignment. Each pair branches length's represents the distance between sequence pairs, and the units at the bottom of the tree show the number of substitution events. Results of the phylogenetic tree (Fig. 3) showed that species classified into four major groups, group 1 consists of 2 plant (*Gentiana rigescens*, *Solanum lycopersicum*); group 2 includes 2 plant (*Populus pruinosa, Populus euphratica*); group 3 consists of 1 plant (*Eschscholzia californica*); group 4 consists of 20 plants (*Hordeum vulgare* *voucher*, *Panicum virgatum*, *Salicornia europaea*, *Lophopyrum elongatum*, *Arabidopsis thaliana*, *Glycine max*, *Leptochlo afusca*, *Paeoniala ctiflora*, *Oryza sativa Indica*, *Ipomoea tricolor*, *Citrus Reticulata*, *Citrus trifoliate*, *Maluszumi*, *Lotus tenuis*, *Sorghum bicolor*, *Atriplex patens*, *Iris halophile*, *Trifolium repens*, *Nelumbo nucifera*, *Suaeda pruinosa*). The NHX1 sequence from *Gentiana rigescens* and *Solanulym copersicum* was assigned into the most distal clade.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. ANS57040.1 NHX1 Hordeum vulgare																									
2. AJO67762.1 NHX1 Panicum virgatum	0.092																								
3. AAN08157.1 NHX1 Salicornia europaea	0.285	0.256																							
4. AAQ07963.1 NHX1 Lophopyrum elongatum	0.391	0.376	0.372																						
5. NP 198067.1 NHX1 Arabidopsis thaliana	0.326	0.289	0.236	0.405																					
6. NP 001237166.2 NHX1 Glycine max	0.302	0.262	0.221	0.361	0.243																				
7. AQN76250.1 NHX1 Populus pruinosa	0.369	0.344	0.336	0.480	0.344	0.354																			
8. AEN03787.1 NHX1 Leptochloa fusca	0.100	0.036	0.269	0.376	0.292	0.272	0.354																		
9. AFU88788.1 NHX1 Paeonia lactiflora	0.398	0.365	0.289	0.496	0.329	0.319	0.421	0.372																	
10. AIQ86570.1 NHX1 Oryza sativa	0.106	0.067	0.289	0.391	0.305	0.289	0.365	0.054	0.379																
11. BAP28595.1 NHX1 Ipomoea tricolor	0.295	0.256	0.252	0.369	0.285	0.227	0.369	0.272	0.344	0.279															
12. AAT36679.1 NHX1 Citrus reticulata	0.329	0.295	0.227	0.405	0.236	0.214	0.347	0.302	0.316	0.295	0.279														
13. AAT36678.1 NHX1 Citrus trifoliata	0.340	0.305	0.243	0.417	0.252	0.224	0.361	0.312	0.319	0.309	0.295	0.013													
14. AIA82923.1 NHX1 Gentiana rigescens	0.496	0.476	0.464	0.555	0.525	0.488	0.492	0.480	0.582	0.492	0.512	0.504	0.521												
15. ADB80440.1 NHX1 Malus zumi	0.305	0.269	0.211	0.383	0.214	0.166	0.351	0.269	0.305	0.279	0.233	0.202	0.218	0.517											
16. ACE78322.1 NHX1 Lotus tenuis	0.323	0.292	0.249	0.391	0.259	0.092	0.361	0.295	0.323	0.319	0.252	0.240	0.249	0.517	0.187										
17. ACD64982.2 NHX1 Sorghum bicolor	0.369	0.358	0.340	0.166	0.379	0.351	0.428	0.358	0.468	0.365	0.361	0.376	0.391	0.534	0.358	0.376									
18. AQN76207.1 NHX1 Populus euphratica	0.369	0.344	0.336	0.480	0.344	0.354	0.000	0.354	0.421	0.365	0.369	0.347	0.361	0.492	0.351	0.361	0.428								
19. AHC54576.1 NHX1 Atriplex patens	0.282	0.252	0.103	0.376	0.227	0.221	0.333	0.269	0.299	0.285	0.252	0.233	0.249	0.484	0.208	0.259	0.344	0.333							
20. CAC84522.1 NHX1 Solanum lycopersicum	0.405	0.394	0.376	0.472	0.424	0.402	0.379	0.391	0.472	0.402	0.421	0.398	0.405	0.464	0.409	0.428	0.456	0.379	0.369						
21. ALP46546.1 NHX1 Eschscholzia californica	0.358	0.329	0.358	0.484	0.358	0.344	0.391	0.344	0.413	0.351	0.372	0.372	0.391	0.529	0.361	0.340	0.448	0.391	0.351	0.440					
22. ALT06549.1 NHX1 Iris halophila	0.256	0.199	0.275	0.369	0.312	0.279	0.344	0.205	0.365	0.214	0.309	0.299	0.312	0.492	0.289	0.309	0.347	0.344	0.272	0.391	0.312				
23. ABV00895.1 NHX1 Trifolium repens	0.329	0.299	0.243	0.387	0.243	0.117	0.361	0.302	0.329	0.319	0.246	0.230	0.243	0.517	0.190	0.131	0.379	0.361	0.259	0.428	0.347	0.316			
24. BAP90754.1 NHX1 Nelumbo nucifera	0.279	0.243	0.246	0.394	0.289	0.221	0.326	0.246	0.312	0.256	0.252	0.227	0.240	0.452	0.233	0.252	0.365	0.326	0.246	0.354	0.333	0.259	0.243		
25. AHY19033.1 NHX1 Suaeda pruinosa	0.289	0.256	0.059	0.361	0.240	0.236	0.354	0.269	0.305	0.282	0.259	0.233	0.249	0.484	0.221	0.259	0.347	0.354	0.089	0.372	0.351	0.259	0.262	0.240	

Fig. 2. Estimates of evolutionary divergence between sequences.

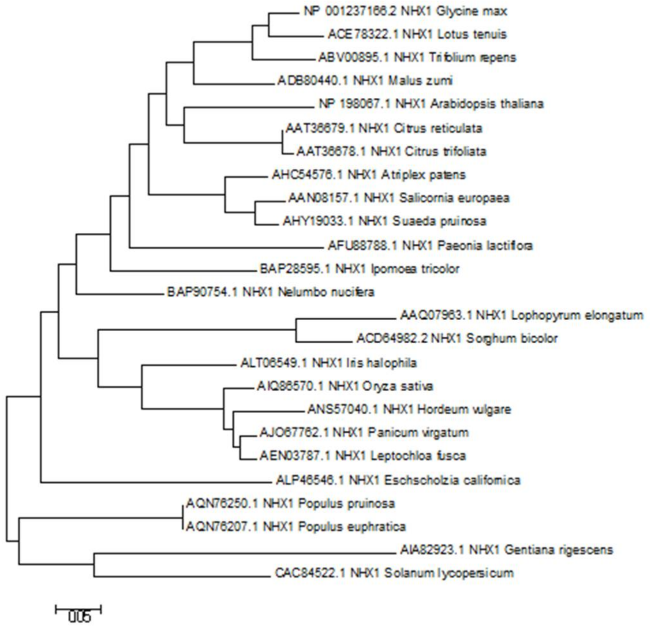


Fig. 3. Phylogenetic analysis of NHX1 gene identified in different source organisms.

The branching pattern in the phylogenetic tree (Fig. 3) reflects how species or other groups evolved from a series of common ancestors. This analysis suggested that plants' ancestor contained several NHX1 genes, which fashioned many regulatory proteins to control salt during abiotic stress. In recent studies, it has been found that compositional heterogeneity had an effect on the phylogenetic method (Sheffield 2012). Non stationary evolution (when the substitution pattern in nucleotide sequence is not uniform across an evolutionary tree) produces compositional heterogeneity (Sheffield 2012). Compositional heterogeneity was also observed in this study (Fig. 3). The Phylogenetic tree was constructed with NHX1 gene sequences from all species clarified the heterogeneous evolutionary lineages between these genes.

This study provides perspectives for future research on the NHX1 gene in diverse organisms, including substrate identities, physiological functions, and coevolution with NHX1 in their interacting partner organisms.

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