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ISOLATION AND SEQUENCE ANALYSIS OF A NOVEL PARTIAL VACUOLAR Na^+/H^+ ANTIPORTER cDNA FROM *Capparis orientalis, Lycium shawii* AND *Zygophyllum album*

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In Egypt, as in the majority of the arid and semi arid regions, drought and salinity are responsible of substantial losses of culture yield, deterioration of plant cover and erosion of soils. Water is one of the most important constraints of agriculture production with faces salinity and drought stress.

In plants, the Na^+/H^+ antiporter located at the plasma and vacuolar membrane can actively exclude excessive Na^+ from the cytosol or compartmentalize it into tonoplast to remove Na^+ toxicity (Apse *et al*., 1999; Hasegawa *et al*., 2000; Shi *et al*., 2002). In particular, the vacuolar Na^+/H^+ antiporter had been demonstrated to play a key role in salt tolerance of plants (Blumwald *et al*., 2000). The vacuolar Na^+/H^+ antiporter is a transmembrane protein which can catalyze the exchange of Na^+ and H^+ across the vacuolar membrane and maintains cellular pH and Na^+ homeostasis in plants (Apse *et*

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al., 1999; Shi and Zhu, 2002). Recently many genes encoding vacuolar Na^+/H^+ antiporters (NHX1) have been characterized and isolated from several plant species such as: *Arabidopsis thaliana* (Apse *et al*., 1999; Gaxiola *et al*., 1999), tomato (Zhang and Blumwald, 2001), *Brassica napus* (Wang *et al*., 2003), *Atriplex dimorphostegia* (Li *et al*., 2003), *Suaeda salsa* (Ma *et al*., 2004), *Zea mays* (Zorb *et al*., 2005), *Chenopodium glaucum* (Li *et al*., 2008), *Thellungiella halophila* (Wu *et al*., 2009), *Trifolium repens* (L.) (Tang *et al*., 2010), *Zygophyllum xanthoxylum* (Wu *et al*., 2011), *Halostachys caspica* (Guan *et al*., 2011), *Karelinia caspica* (Liu *et al*., 2012) and *Leptochloa fusca* (Rauf *et al*., 2014).

In the North coast of Marsa Matruh Governorate - Egypt, there are many desert plant species that tolerant drought and salinity stresses. Among these species, *Capparis orientalis* (LASAF/KABBAR): a leaf succulent obligate halophyte and a species of plant belong to family *Capparaceae*. Ahmed *et al*. (1972) reported that some Egyptian *Capparis* species contain of glucosinolates, glucoiberin, glucocapparin, sinigrin, glucocleomin, glucobrassicin and glucocapangulin were isolated, characterized and identified. In the Arabian folk medicine, several *Capparis* species have many uses (Shahina, 1994).

Lycium shawii (AWSAJ) belongs to family *Solanaceae*, semi-succulent and a thorny perennial shrub. Fukuda *et al*. (2001) reported that *Lycium* species mostly occur in arid and semi-arid climates, and a few are known from coastal zones in somewhat saline habitat types. *Solanaceae* are known for having a diverse range of alkaloids. As far as humans are concerned, these alkaloids can be desirable, toxic, or both. It grows along sandy stone ridges. It has purple, sometimes white, trumpet-like flowers and sharp thorns. The leaves are elliptical and congested in closed clusters (Omar *et al*., 2007). Cherouana *et al*. (2013) isolated two known flavonoid glycosides from arial parts of *Lycium arabicum*, the compounds were identified by spectral analysis.

Zygophyllum album (L.) (El-RETRAT) belongs to subfamily *Zygophylloideae* in family *Zygophyllaceae*. Nine species of *Zygophyllum* are recorded in Egypt. *Zygophyllum album* is a succulent cushion-like under shrub frequently reaching 1 m in height. The leaves and branches are blue-green, mealy pubescent, and present in oases, eastern Egyptian desert, Red sea coastal region and Sinai (Täckholm, 1974). Hassanean *et al*. (1993) characterized besides the two known saponins, quinovic two new glycosides were isolated from the aerial parts of *Zygophyllum album* growing in Egypt. Moustafa *et al*. (2007) detected the chemical constituents of *Zygophyllum album* (L.) (family *Zygophyllaceae*) isolated three flavonoids *via* Kaemferol, Isorhmnetin and Quercetin-3-O-gluoside.

In this study, two modified protocols for RNA isolation are described here as a simple, fast, convenient and does not require DEPC (diethylene pyrocarbonate) treatment. Reverse transcription of the RNA followed by PCR amplification was used to confirm that the RNA produced is able to generate cDNA. One degenerate primer pair with touchdown RT-PCR program is used to amplify a partial middle fragment cDNAs of a novel vacuolar Na$^+$/H$^+$ antiporters (NHX) from *Capparis orientalis, Lycium shawii* and *Zygophyllum album* which were isolated, sequenced and analyzed.

MATERIALS AND METHODS

Plant materials

One hundred milligram of collected frozen tissue samples from three plant species, *Capparis orientalis*, *Lycium shawii* and *Zygophyllum album* were placed in sterile 2 ml microfuge and immediately dipped in liquid nitrogen, and crush into fine powder using Tissue Lyser II machine (Qiagen) for homogenization to avoid browning and degradation during RNA extraction. All reagents and materials including UltraPure™ DNase/RNase-Free distilled water were autoclaved, without treating with DEPC (Diethyl pyrocarbonate).

Total RNA extraction and purification

For establishing the suitable method for isolating total RNA from plants under this study, two different methods were evaluated TRIzol® Reagent and RNeasy Plant Mini Kit. For TRIzol® method, total RNA was extracted using 100 mg tissue/1 mL TRIzol® Reagent according to the manufacturer's instruction (Invitrogen, Cat no. 15596-026). Isolated RNA was cleaned *via* RNeasy Plant Mini Kit. For RNeasy Plant Mini Kit method, 100 mg of plant tissue was used for each reaction. Polyethylene Glycol (PEG- MW 6000) was add to RLC or RLT buffer provided with kits breach of a concentration of 30 mg/1 ml and was incubated at 60°C for 3 hours and keep worm before use. Fine powder of samples was subjected to RNA extraction following the manufacturer's procedure according (RNasy mini plant Kit Cat No: 74904). RNA was suspended in 30-50 µl in RNase free water and stored in -80°C for further analysis. Purified RNA samples were measured using NanoDrop spectrophotometer (NanoDrop, Technologies Inc.). The integrity of total RNA was verified using 1.2% non-denaturing agarose gel electrophoresis.

RNA analysis by one step RT-PCR

Positive RNA (2X 105 copies/ µL), provided by TaKaRa one step RNA PCR Kit was applied as control according to the manufacturer's instruction (TaKaRa Bio INC. Cat. No. RR024A). Two micro liter of total RNA extraction from plant species were used as a template. Primers ubiquitous 18s rRNA universal primer sequences were used as positive control for one step RT-PCR to amplify 1 kb of the 18S rRNA, (18S-F: 5`- CAG TAG TCA TAT GCT TGT CTC AAA-3`/ 18S-R: 5`- GAC

TAG GAC GGT ATC TGA TCG T-3`), (Brunner *et al.*, 2004; Ashoub *et al.*, 2006). Sample amplified products were analyzed using 1.2% agarose gel electrophoreses staining with ethidium bromide.

Amplification of partial cDNA Na^+/H^+ antiporter gene by RT-PCR two steps

Two steps were performed to amplify the cDNA for the NHX gene. For preparing the first strand cDNA synthesis of total RNA 10 pg - 500 ng, oligo (dT) 20 and M-MLV RT (SuperScript III Reverse Transcriptase) were mixed and used according to the manufacturer's instructions (Invitrogen, Cat No. 18080-085). The cDNA synthesis reaction was stored at -20°C to be used for second step PCR.

Oligonucleotide primers designing

One degenerate primer pair was designed from Na^+/H^+ antiporter genes at the conserved nucleotide sequences region which were determined based on the multiple sequence alignment of other Na^+/H^+ antiporter gene families sequences from selected plant species in the universal database. Alignments between related species to plants under this study showed a homology at some regions of the gene (ORF) were species specific.

One degenerate primer pair (NHX-F dp: 5`GGG/CTTTCAA/GGTA/ GAAA/GAAGAAG CAA/G3`) and (NHX-R dp: 5`A/GTC/TACATTGT GCCAG/TGTA/GTAATGA/GGAC3`) was designed to amplify core cDNA fragment of ORF. *Arabidopsis thaliana* (AF510074.1), *Zygophyllum xanthoxylum* (EU103624.1), *Suaeda salsola* (AF370358.1), *Suaeda maritima subsp. Salsa* (*SsNHX1*, AY261806.1), *Atriplex gmelini* (*AgNHX1*, AB038492.1), *Atriplex dimorphostegia* (*AdNHX1*, AY211397.1), *Salsola komarovii* (*SkNHX1*, AB531436.1) and *Salsola soda* (EU073422.1) were used for multiple sequences alignment of nucleotide (BLSTN) to design degenerate primer for amplification of the partial middle fragment of ORF (Fig. 1).

PCR amplification partial cDNA of NHX1 gene

The High-Fidelity DNA polymerase, Phusion® *Taq* (Thermo Scientific, Product codes: F-530L, 500 Unit) with the ability to perform proof reading was used to amplify the cDNA. It generates blunt ends in the amplification products. Reaction was done in a 50 µl total volume. Reaction contained 4 µl cDNA, 10 µl 5X Phusion HF Buffer, 1 µl 10 mM dNTP mix, 2.5 µl primer 1 (10 µM), 2.5 µl primer 2 (10 µM), 0.5 µl Phusion DNA polymerase, 29.5 µl DEPC H_2O and spin for 15 Sec. Touchdown PCR program was used to amplify Na^+/H^+ antiporter partial middle cDNA gene. One cycles 30 sec of preheated at 98°C, 10 cycles 30 sec. for denaturation at 98°C, 30 sec for annealing at 58 - 52°C was decreased (2°C/Cycles) and 30 cycles; 30 sec. of denaturation at 98°C, 30 sec. of annealing 52°C, 1 min of extension at 72°C and followed by final extension at 72°C for 10 min. (Korbie and Mattick, 2008; Hecker and Roux, 1996).

A volume of 10 µl of each sample was analyzed using 1.2% agarose gel electrophoreses and stained with ethidium bromide (Eth-Br). The PCR fragments of each sample were excised and purified from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and the QIAquick® Gel Extraction Kit (Qiagen, cat. no. 28706) was used to elute the DNA from the gel. For PCR product, the sample was centrifuged according to the manufacturer's procedure in the QIAquick PCR Purification Kit (Qiagen, Cat. No. 28106).

Bioinformatic analysis

Nucleotide sequences of NHX genes were searched in NCBI database, The National Center for Biotechnology Information GenBank Database, (http://www.ncbi.nlm.nih.gov), while the European Molecular Biology Laboratory (EMBL), (www.2ebi.ac.uk) were used for sequence analyses. The ClC workbench 6.9 software was used to analyze coding sequences of ORFs of NHX gene sequences (http://www.clcbio.com). Protein sequences was predicted from the obtaining Open Reading Frame (ORF) NHX nucleotide sequence using the web:www.Expasy.org/translate. DNA sequences of amplified NHX genes were determined by GATC, Biotech AG-Germany. The nucleotide and amino acid sequences were then compared with those in GenBank database. Homology comparison and phylogenetic relationship were analyzed using the CLUSTALW multiple sequence alignment algorithm (Thompson *et al.*, 1994), (http://www2.ebiac.uk/clustalw). Evolutional distances were calculated using the neighbor-joining method (Saitou and Nei, 1987). Sequence analysis at BLAST search and ExPASY-translate tool data analysis were conducted on the NCBI platform.

RESULTS AND DISCUSSION

Plant tolerance to salt stress is a multigenic trait and requires the coordinated action of several gene, but it is evident from several reports that over-expression of a single gene can also improve salt tolerance in plants (Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Zhang and Blumwald, 2001; Li *et al.*, 2003; Wang *et al.*, 2003; Ma *et al.*, 2004; Zorb *et al.*, 2005; Li *et al.*, 2008; Wu *et al.*, 2009; Tang *et al.*, 2010; Guan *et al.*, 2011; Wu *et al.*, 2011; Liu *et al.*, 2012, and Rauf *et al.*, 2014).

Partial middle cDNA of vacuolar Na^+/H^+ antiporter genes were used to design degenerate primer pair in conservative region of nucleotide sequence gene of different plant species. The ultimate goal of this study provided basic foundation information about a novel partial vacuolar Na^+/H^+ antiporter genes sequence from three Egyptian native (non-domesticated) plant species belong to three different families; *Capparis orientalis*, *Lycium shawii* and *Zygophyllum album* were collected from North western coast of Marsa Matruh Governorate, Egypt.

Isolating RNA using both the commercial reagent TRIzol® Reagent (Invitrogen) followed by purification step with a Qiagen spin-column and RNeasy plant mini Kit (Qiagen) purified with PEG for RNA extraction showed high efficiency for isolating high-quality and quantity RNA suitable for using in sensitive downstream applications. In RNeasy Plant mini Kits (Qiagen), RLC or RLT extraction buffer contain β-mercaptoethanol to prevent sample oxidation and to inhibit RNase release from tissue prior to chloroform extraction. Polyethylene glycol (PEG, MW: 6000) probably played an important role in separating polyphenols and polysaccharides from RNA. Results showed good quality of the isolated RNA provided by NanoDrop spectrophotometric measurements, as it gave A_{260}/A_{280} absorbance ratio between 1.9-2.0; indicating that RNA was relatively free of DNA and protein contamination. Isolated total RNA was run on 1.2% agarose gel electrophoresis. Clear photo of isolated RNA was obtained and both 28S rRNA with molecular size (3.7 Kb) and 18S rRNA with molecular size (1.9 Kb) were separated clearly from mRNA, discrete ribosomal RNA with no apparent RNA degradation, indicating that RNA is also relatively free of RNase (Fig. 2). Similar results were also reported by Kiefer *et al.* (2000), Tattersall *et al.* (2005) and Portillo *et al.* (2006).

The RNA quality was tested by one step RT-PCR for TaKaRa kit using universal oligonucleotide primers, which were designed based on the conserved 18S rRNA. Reaction products and 1 Kbp DNA leader plus, were separated on 1.2% agarose TAE of gel electrophoresis and visualized under UV light following Et-Br staining (Fig. 3).

Results showed that the expected 1 kb DNA fragment of the 18S rRNA was amplified as a positive control from plant species. This result is in agreement with those observed by Brunner *et al.* (2004) and Ashoub *et al.* (2006). This protocol allowed RNA isolation with high purity from plant species under this study. The method may be suitable for other plant species from different families rich in polyphenols and polysaccharides.

Amplification of NHX1 partial cDNA by RT-PCR two steps

One degenerate primer pair (NHX F-dp and NHX R-dp) was used to amplify a partial middle fragment of cDNA Na^+/H^+ antiporter gene in plant species (*Capparis orientalis*, *Lycium shawii* and *Zygophyllum album*) which gave one fragment with size about 600 base pair (Fig. 4). Using touchdown PCR program (TD-PCR) of cDNA was able to increase specificity and sensitivity to amplify partial a according to Hecker and Roux (1996) and Korbie and Mattik (2008).

Phylogenetic relationship based on sequence analyses

All purified PCR products were sequenced. The partial fragment sequence showed sequence identity with Na^+/H^+ antiporter sequence gene from data. Results of sequences data analysis of purified PCR fragment of Na^+/H^+ antiporter gene were submitted by dried submission of sequence data to NCBI GenBank as BankIt online with GenBank accession number KJ452345.1, KJ452346.1 and KJ452347.1 for *CoNHX1*, *LsNHX*, and *ZaNHX* cDNA, and amino acid sequences with GenBank accession no. AHY19036.1, AHY19037.1 and AHY19038.1, respectively (Figs 5, 6 and 7).

The sequence analysis of the cDNA for vacuolar Na^+/H^+ antiporter gene *CoNHX1* revealed that it has 548 bp for core fragment of ORF, while the core fragment of the *LsNHX* ORF was 557 bp, and core fragment of the *ZaNHX* ORF was also 557 bp.

Result of BLASTN pairwise alignment analysis of nucleotide sequence revealed that cDNA core fragment of a vacuolar Na^+/H^+ antiporter gene from *Capparis orientalis* (*CoNHX*, 548 bp) showed 89% similarity with Na^+/H^+ antiporter, *Nitraria trichocarpa*, 89% with *Atriplex gemlini*, 85% with *Brassica napus,* 84% with *Arabidopsis thaliana* and 83% with *Brassica oleraceal*.

BLASTN pairwise alignment analysis of nucleotide sequence analysis revealed that core cDNA fragment of a vacuolar Na^+/H^+ antiporter gene from *Lycium shawii* (*LsNHX*, 557 bp) showed 95% similarity with Na^+/H^+ antiporter, *Solanum lycopersicum,* 80% with *Pyrus ussriensis,* 80% *with Salicornia europaea*, 79% with *Atriplex gemlini*, 79% with *Ipomoea tricolor* and 79% with *Citrus paradise.*

BLASTN pairwise alignment analysis of nucleotide sequence analysis revealed that core cDNA fragment of a vacuolar Na^+/H^+ antiporter gene from of *Zygophyllum album* (*ZaNHX*, 557 bp) 90% similarity with Na^+/H^+ antiporter, *Zygophyllum xanthoxylum,* 81% with *Ricinus communis,* 79% with *Populus trichocarpa*, 79% with *Citrus paradisi* and 79% with *Atriplex gemlini.*

BLASTN of result and phylogenetic relationship analysis indicated that all obtained fragments were clustered into the vacuolar Na^+/H^+ antiporter group. The deduced amino acid sequences showed high identities with other plant vacuolar-type Na^+/H^+ antiporters (Figs 8 and 9). Taken together, these results suggest that *CoNHX*, *LsNHX* and *ZaNHX* are new members of the vacuolar Na^+/H^+ antiporter families. Multiple alignments of vacuolar Na^+/H^+ antiporters (the deduced amino acid sequence) showed that CoNHX, LsNHX and ZaNHX share high identity with other plant vacuolar Na^+/H^+ antiporters as shown in Table (1) and in Figs (8 and 9). Some putative membrane spanning domains (M4, M5, M6 and M7) were recognized by SOSUI software program (http://harrier. nagahama-i-bio.ac.jp/sosui/ cgi- bin/adv_sosui.cgi; Hirokawa *et al*., 1998).

SUMMARY

Egyptian native (non-domesticated) plant species from different

families such as *Capparis orientalis*, *Lycium shawii* and *Zygophyllum album* were collected from North Western coast of Marsa Matrouh Governorate, Egypt. Plant vacuolar Na^+/H^+ antiporter candidate gene from tolerant plant species; several are localized on the tonoplast, plays an important role in several plant species (Halophytes and xerophytes) under abiotic stress. Then, once the genes will be identified from tolerant plant species, the overall goal of this study is to identify partial the vacuolar antiporter NHX1 candidate gene. According to NHX1 family homologous sequence conservative region; one degenerate oligonucleotide primer pair was used to amplify core (partial middle) fragment of cDNAs vacuolar Na^+/H^+ antiporter with about size of 600 bp, approximately. Touchdown PCR program (TD-PCR) of cDNAs were success to increase specificity, sensitivity and yield to amplify core cDNA of vacuolar Na^+/H^+ antiporter gene.

Sequence analysis provided us with a novel partial fragment length of cDNAs about 548 bp, 557 bp and 557 bp of a novel CoNHX1, LsNHX and ZaNHX was deposited in GenBank database with NCBI, GenBank accession no. KJ452345.1, KJ452346.1 and KJ452347.1 and amino acid sequences about 182 a.a, 185 a.a and 185 a.a with GenBank accession no. AHY19036.1, AHY19037.1 and AHY19038.1, respectively. BLASTN of sequences result and phylogenetic relationship analysis indicated that all were clustered into the vacuolar Na^+/H^+ antiporter group. The deduced amino acid sequences showed high identities with other plant vacuolar-type Na^+/H^+ antiporters. Taken together, these results suggest that *Co*NHX, *Ls*NHX and *Za*NHX are new members of the vacuolar Na^+/H^+ antiporter family.

The ultimate goal of this study provided a basic foundation information about a Novel partial vacuolar Na^+/H^+ antiporter gene to develop 5` and 3` RACE technique (Rapid amplification cDNA Ends).

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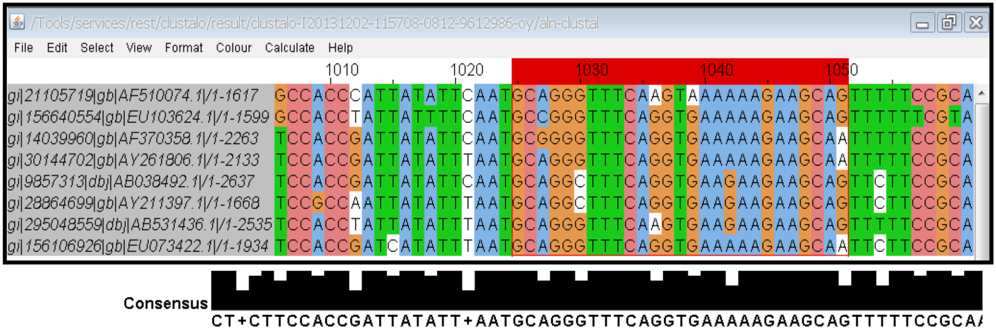
Table (1): Sequences homology of plant vacuolar Na$^+$/H$^+$ antiporter gene (nucleotide sequences and amino acid sequences) for *Capparis orientalis* CoNHX1 (AHY19036.1), *Lycium shawii* LsNHX (AHY19037.1) and *Zygophyllum album* ZaNHX (AHY19038.1) to various Na$^+$/H$^+$ antiporters genes.

Plant species	Accession no. nucleotide seq.	Accession no. amino acid	Identity* bp (a.a)%
***Capparis orientalis* (CoNHX1)**	**KJ452345.1**	**AHY19036.1**	
Cochlearia danica	JQ435893.1	AFH37921.1	**82**(86)
Brassica oleraceal	JQ435891.1	AFH37919.1	**83**(86)
Tetragonia tetragnioides	AF527625.1	AAQ08988.1	**78**(89)
Brassica napus	AY189676.1	AAO38856.1	**85**(87)
Nitraria trichocarpa	KF751928.1	AID55215.1	**89**(87)
Zygophyllum xanthoxylum	EU103624.1	ABU92562.1	**77**(85)
Atriplex gmelini	AB038492.1	BAB11940.1	**89**(86)
Oxybasis glauca	AY371319.1	AAQ72785.1	**78**(86)
Arabidopsis thaliana	AY685183.1	AAT95387.1	**84**(89)
***Lycium shawii* (LaNHX)**	**KJ452346.1**	**AHY19037.1**	
Solanum lycopersicum	NM_001246956.1	NP_001233885.1	**95**(95)
Pyrus ussriensis	KC136359.1	AGE13943.1	**80**(88)
Salicornia europaea	AY131235.1	AAN08157.1	**80**(85)
Salsola komarovii	AB531436.1	BAJ06110.1	**78**(86)
Zygophyllum Xanthoxylum	EU103624.1	ABU92562.1	**78**(86)
Ipomoea tricolor	AB29774.1	BAF75378.1	**79**(83)
Atriplex gmelini	AB038492.1	BAB11940.1	**79**(85)
Citrus x paradise	AY028416.2	AAK27314.2	**79**(86)
***Zygophyllum album* (ZaNHX)**	**KJ452347.1**	**AHY19038.1**	
Zygophyllum Xanthoxylum	EU103624.1	ABU92562.1	**90**(92)
Nitraria tangutourum	KF751928.1	AID552215.1	**77**(83)
Glycine max	AY972078.1	AAY43006.1	**78**(82)
Populus trichocarpa	XM_002319556.2	XP_002307194.2	**79**(82)
Ricinus communis	XM_002512236.1	XP_002512282.1	**81**(81)
Citrus x paradisi	AY028416.2	AAK27314.2	79(82)
Pyrus ussriensis	KC136359.1	AGE13943.1	78(82)
Atriplex gmelini	AB038492.1	BAB11940.1	**79**(81)
Cochlearia danica	JQ435893.1	AFH37921.1	**76**(81)

*Homology values of nucleotide are bold. Homology values of amino acid are in brackets.

A. Degenerate primer design forward primer (NHX-F dp) location



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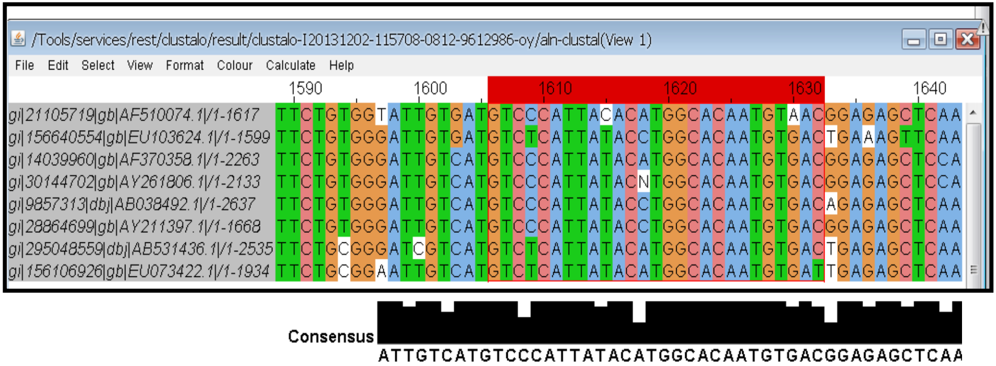
gi|156640554|gb|EU103624.1|/1-1599   GCCACCTATTATTTTCAATGCCGGGTTTCAGGTGAAAAAGAAGCAGTTTTTTCGTA
gi|14039960|gb|AF370358.1|/1-2263    TCCACCGATTATATTCAATGCGGGGTTTCAGGTGAAAAAGAAGCAATTTTTCCGCA
gi|30144702|gb|AY261806.1|/1-2133    TCCACCGATTATATTTAATGCAGGGTTTCAGGTGAAAAAGAAGCAATTTTTCCGCA
gi|9857313|dbj|AB038492.1|/1-2637    TCCACCGATTATATTCAATGCAGGCTTTCAGGTGAAGAAGAAGCAGTTCTTCCGCA
gi|28864699|gb|AY211397.1|/1-1668    TCCGCCAATTATATTTAATGCAGGCTTTCAGGTGAAGAAGAAGCAGTTCTTCCGCA
gi|295048559|dbj|AB531436.1|/1-2535  TCCACCTATTATATTTAATGCAGGGTTTCAAGTGAAGAAGAAGCAGTTTTTCCGCA
gi|156106926|gb|EU073422.1|/1-1934   TCCACCGATCATATTTAATGCAGGGTTTCAGGTGAAAAAGAAGCAATTCTTCCGCA

Consensus  CT+CTTCCACCGATTATATT+AATGCAGGGTTTCAGGTGAAAAAGAAGCAGTTTTTCCGCA
```

B. Degenerate primer design reveres primer (NHX-R dp).



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gi|156640554|gb|EU103624.1|/1-1599   TTCTGTGGGATTGTGATGTCTCATTATACCTGGCACAATGTGACTGAAAGTTCAA
gi|14039960|gb|AF370358.1|/1-2263    TTCTGTGGGATTGTCATGTCCCATTATACATGGCACAATGTGACGGAGAGCTCCA
gi|30144702|gb|AY261806.1|/1-2133    TTCTGTGGGATTGTCATGTCCCATTATACNTGGCACAATGTGACGGAGAGCTCCA
gi|9857313|dbj|AB038492.1|/1-2637    TTCTGTGGGATTGTCATGTCCCATTATACCTGGCACAATGTGACAGAGAGCTCAA
gi|28864699|gb|AY211397.1|/1-1668    TTCTGTGGGATTGTCATGTCCCATTATACCTGGCACAATGTGACGGAGAGCTCAA
gi|295048559|dbj|AB531436.1|/1-2535  TTCTGCGGGATCGTCATGTCTCATTATACATGGCACAATGTGACTGAGAGCTCAA
gi|156106926|gb|EU073422.1|/1-1934   TTCTGCGGAATTGTCATGTCTCATTATACATGGCACAATGTGATTGAGAGCTCAA

Consensus  ATTGTCATGTCCCATTATACATGGCACAATGTGACGGAGAGCTCAA
```

Fig. (1): Multiple alignment nucleotide sequences of some Na+/H+ antiporter genes from some plant species to design degenerate primer (Dp). *Arabidopsis thaliana* (AF510074.1), *Zygophyllum xanthoxylum* (EU103624.1), *Suaeda salsola* (AF370358.1), *Suaeda maritima subsp. salsa* (AY261806.1), *Atriplex gmelini* (AB038492.1), *Atriplex dimorphostegia* (AY 211397.1), *Salsola komarovii* (AB531436.1), and *Salsola soda* (EU073422.1). A. Degenerate primer design forward primer (NHX-F dp). B. Reveres primer (NHX-R dp) location.

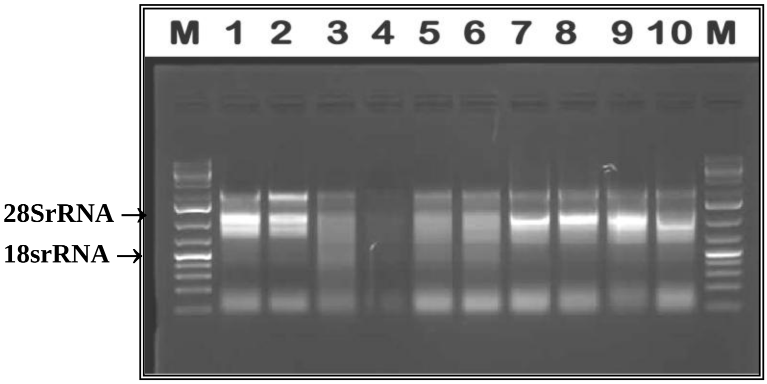


Fig. (2): RNA extracts from Egyptian native plant species.
For TRIzol Reagent method: Lane (1) Total RNA from *Arabidopsis thaliana* (Ecotype Colombia, Co-0), Lane (3 and 4) Total RNA from *Capparis orientalis*, Lane (7) Total RNA from *Lycium shawii*, Lane (9) Total RNA from *Zygophyllum album*. **For RNeasy plant mini kit (Qiagen) with PEG:** Lane (2) Total RNA from *Arabidopsis thaliana* (Ecotype Colombia, Co-0), Lane (5 and 6) Total RNA from *Capparis orientalis*, Lane (8) Total RNA from *Lycium shawii*, Lane (10) Total RNA from *Zygophyllum album* and (M) DNA size markers GeneRuler 1Kb plus DNA Ladder.

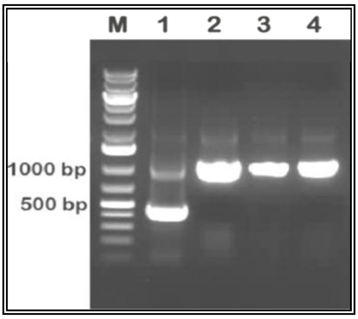


Fig. (3): RT-PCR amplification for Ubiquitous 18S rRNA 1Kbp from plant species. M. DNA size markers GeneRuler 1Kb plus DNA Ladder. Lane (1) PCR product (462 bp) from RNA positive control provide by TaKaRa Kit, Lane (2) PCR product from *Capparis orientalis*, Lane (3) PCR product from *Lycium shawii*, and Lane (4) PCR product from *Zygophyllum album.*

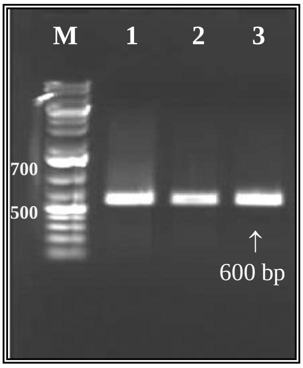
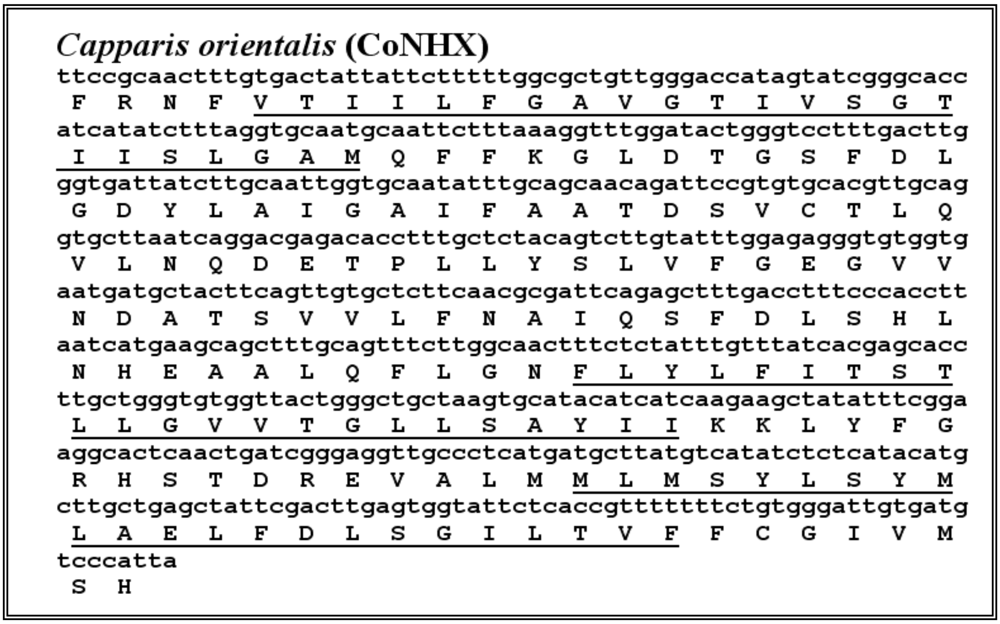


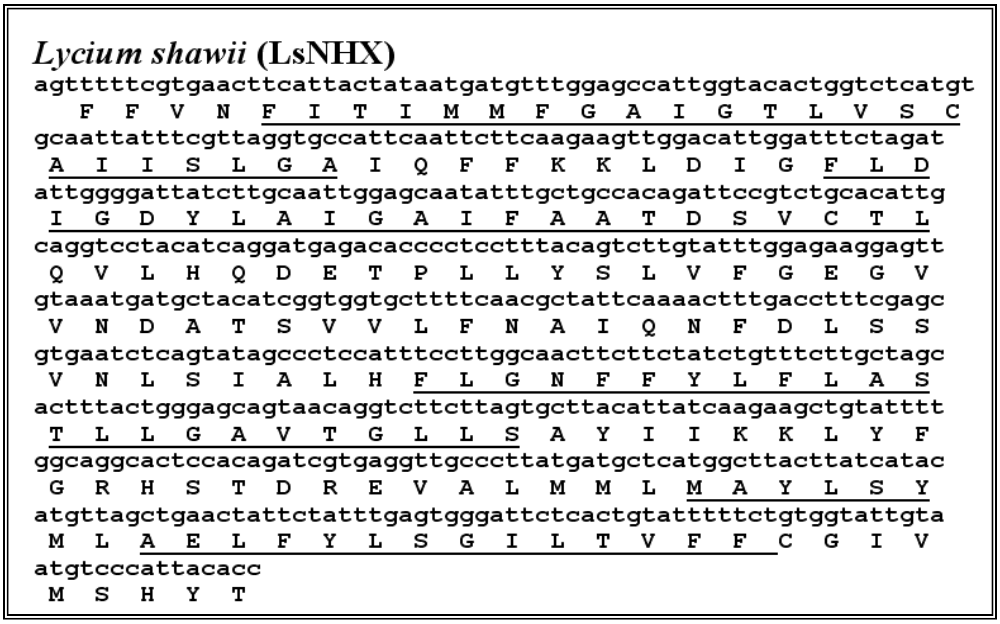
Fig. (4): RT-PCR product of cDNA of middle partial fragment ORF (600 bp) of Na$^+$/H$^+$ antiporter gene using degenerate primer pair for plant species. M. DNA size marker GeneRuler 1Kb plus DNA Ladder, Lane (1) PCR product from *Capparis orientalis*, Lane (2) PCR product from *Lycium shawii*, and Lane (3) PCR product from *Zygophyllum album.*



***Capparis orientalis* (CoNHX)**

```
ttccgcaactttgtgactattattctttttggcgctgttgggaccatagtatcgggcacc
 F  R  N  F  V  T  I  I  L  F  G  A  V  G  T  I  V  S  G  T
atcatatctttaggtgcaatgcaattctttaaaggtttggatactgggtcctttgacttg
 I  I  S  L  G  A  M  Q  F  F  K  G  L  D  T  G  S  F  D  L
ggtgattatcttgcaattggtgcaatatttgcagcaacagattccgtgtgcacgttgcag
 G  D  Y  L  A  I  G  A  I  F  A  A  T  D  S  V  C  T  L  Q
gtgcttaatcaggacgagacacctttgctctacagtcttgtatttggagagggtgtggtg
 V  L  N  Q  D  E  T  P  L  L  Y  S  L  V  F  G  E  G  V  V
aatgatgctacttcagttgtgctcttcaacgcgattcagagctttgacctttcccacctt
 N  D  A  T  S  V  V  L  F  N  A  I  Q  S  F  D  L  S  H  L
aatcatgaagcagctttgcagtttcttggcaactttctctatttgtttatcacgagcacc
 N  H  E  A  A  L  Q  F  L  G  N  F  L  Y  L  F  I  T  S  T
ttgctgggtgtggttactgggctgctaagtgcatacatcatcaagaagctatatttcgga
 L  L  G  V  V  T  G  L  L  S  A  Y  I  I  K  K  L  Y  F  G
aggcactcaactgatcgggaggttgccctcatgatgcttatgtcatatctctcatacatg
 R  H  S  T  D  R  E  V  A  L  M  M  L  M  S  Y  L  S  Y  M
cttgctgagctattcgacttgagtggtattctcaccgtttttttctgtgggattgtgatg
 L  A  E  L  F  D  L  S  G  I  L  T  V  F  F  C  G  I  V  M
tcccatta
 S  H
```

Fig. (5): Partial fragment (548 bp) of Na$^+$/H$^+$ antiporter gene of *CoNHX1* and deduced amino acid sequence of CoNHX1 from *Capparis orientalis*, GenBank accession number is KJ452345.1/ AHY19036.1. Nucleotide sequence and deduced sequence of amino acid residues of Na$^+$/H$^+$ antiporter gene is indicated by a single letter code.



***Lycium shawii* (LsNHX)**

```
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gcaattatttcgttaggtgccattcaattcttcaagaagttggacattggatttctagat
 A  I  I  S  L  G  A  I  Q  F  F  K  K  L  D  I  G  F  L  D
attggggattatcttgcaattggagcaatatttgctgccacagattccgtctgcacattg
 I  G  D  Y  L  A  I  G  A  I  F  A  A  T  D  S  V  C  T  L
caggtcctacatcaggatgagacacccctcctttacagtcttgtatttggagaaggagtt
 Q  V  L  H  Q  D  E  T  P  L  L  Y  S  L  V  F  G  E  G  V
gtaaatgatgctacatcggtggtgcttttcaacgctattcaaaactttgacctttcgagc
 V  N  D  A  T  S  V  V  L  F  N  A  I  Q  N  F  D  L  S  S
gtgaatctcagtatagccctccatttccttggcaacttcttctatctgtttcttgctagc
 V  N  L  S  I  A  L  H  F  L  G  N  F  F  Y  L  F  L  A  S
actttactgggagcagtaacaggtcttcttagtgcttacattatcaagaagctgtatttt
 T  L  L  G  A  V  T  G  L  L  S  A  Y  I  I  K  K  L  Y  F
ggcaggcactccacagatcgtgaggttgcccttatgatgctcatggcttacttatcatac
 G  R  H  S  T  D  R  E  V  A  L  M  M  L  M  A  Y  L  S  Y
atgttagctgaactattctatttgagtggattctcactgtattttctgtggtattgta
 M  L  A  E  L  F  Y  L  S  G  I  L  T  V  F  F  C  G  I  V
atgtcccattacacc
 M  S  H  Y  T
```
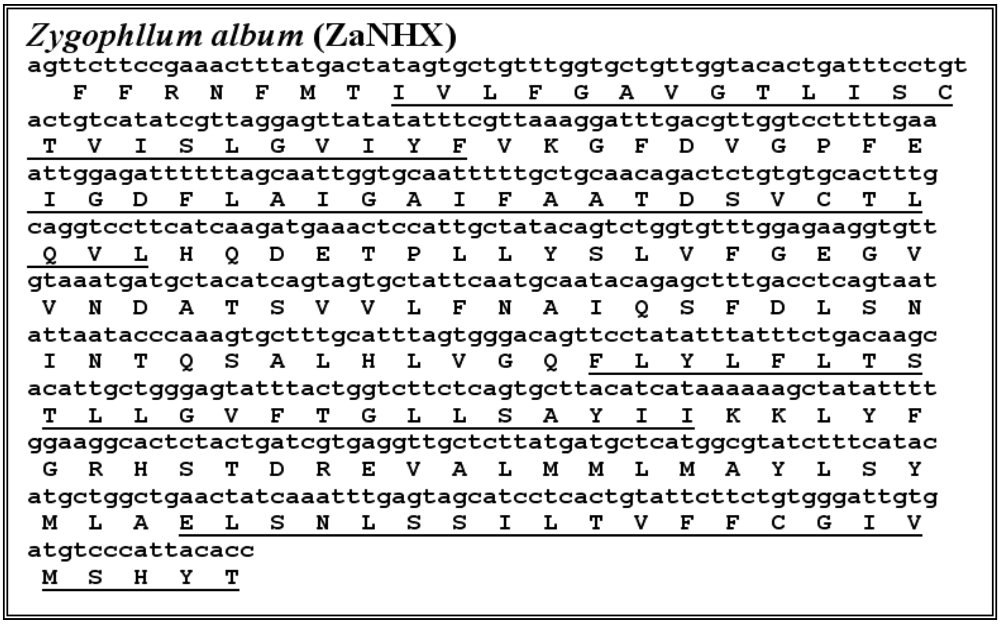

Fig. (6): Partial fragment (557 bp) of Na$^+$/H$^+$ antiporter gene of *LsNHX* and deduced amino acid sequence of LsNHX from *Lycium shawii*, GenBank accession number is KJ452346.1/AHY19037.1. Nucleotide sequence and deduced sequence of amino acid residues of Na$^+$/H$^+$ antiporter gene are indicated by a single letter code.



***Zygophllum album* (ZaNHX)**

```
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actgtcatatcgttaggagttatatatttcgttaaaggatttgacgttggtccttttgaa
 T  V  I  S  L  G  V  I  Y  F  V  K  G  F  D  V  G  P  F  E
attggagatttttagcaattggtgcaattttgctgcaacagactctgtgtgcactttg
 I  G  D  F  L  A  I  G  A  I  F  A  A  T  D  S  V  C  T  L
caggtccttcatcaagatgaaactccattgctatacagtctggtgtttggagaaggtgtt
 Q  V  L  H  Q  D  E  T  P  L  L  Y  S  L  V  F  G  E  G  V
gtaaatgatgctacatcagtagtgctattcaatgcaatacagagctttgacctcagtaat
 V  N  D  A  T  S  V  V  L  F  N  A  I  Q  S  F  D  L  S  N
attaatacccaaagtgctttgcatttagtgggacagttcctatatttatttctgacaagc
 I  N  T  Q  S  A  L  H  L  V  G  Q  F  L  Y  L  F  L  T  S
acattgctgggagtatttactggtcttctcagtgcttacatcataaaaaagctatatttt
 T  L  L  G  V  F  T  G  L  L  S  A  Y  I  I  K  K  L  Y  F
ggaaggcactctactgatcgtgaggttgctcttatgatgctcatggcgtatctttcatac
 G  R  H  S  T  D  R  E  V  A  L  M  M  L  M  A  Y  L  S  Y
atgctggctgaactatcaaatttgagtagcatcctcactgtattcttctgtgggattgtg
 M  L  A  E  L  S  N  L  S  S  I  L  T  V  F  F  C  G  I  V
atgtcccattacacc
 M  S  H  Y  T
```
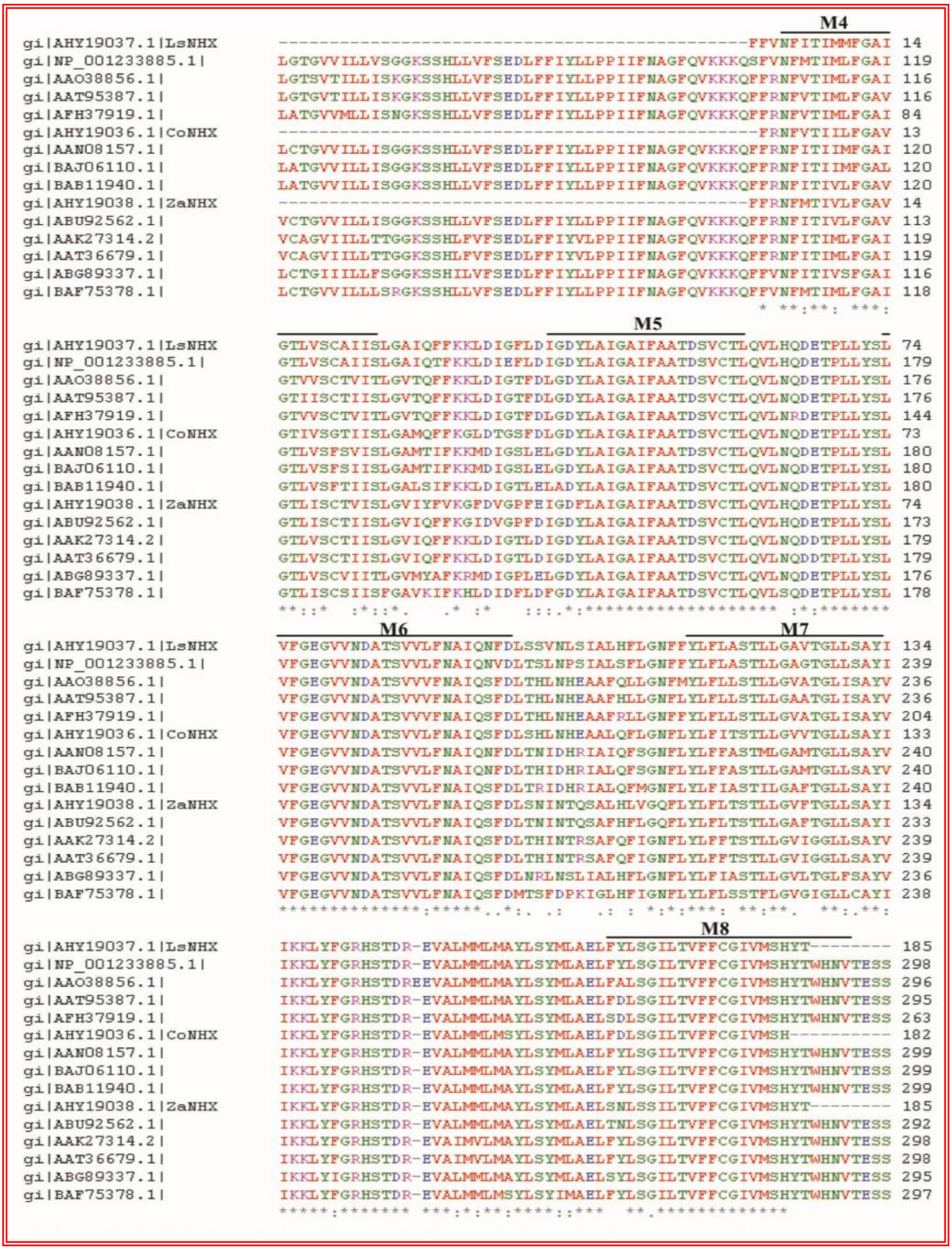

Fig. (7): Partial fragment (557 bp) of Na^+/H^+ antiporter gene of *ZaNHX* and deduced amino acid sequence of ZaNHX from *Zygophyllum album*, GenBank accession number is KJ452347.1/ AHY19038.1. Nucleotide sequence and deduced sequence of amino acid residues of Na^+/H^+ antiporter gene are indicated by a single letter code.



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gi|AAO38856.1|           LGTSVTILLISKGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAI 116
gi|AAT95387.1|           LGTGVTILLISKGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAV 116
gi|AFH37919.1|           LATGVVMLLISNGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFVTIMLFGAI 84
gi|AHY19036.1|CoNHX      -----------------------------------------------FRNFVTIILFGAV 13
gi|AAN08157.1|           LCTGVVILLISGGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIIMFGAI 120
gi|BAJ06110.1|           LATGVVILLISGGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIIMFGAL 120
gi|BAB11940.1|           LATGVVILLISGGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIVLFGAV 120
gi|AHY19038.1|ZaNHX      ----------------------------------------------FFRNFMTIVLFGAV 14
gi|ABU92562.1|           VCTGVVILLISGGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIVLFGAV 113
gi|AAK27314.2|           VCAGVIILLTTGGKSSHLFVFSEDLFFIYVLPPIIFNAGFQVKKKQFFRNFITIMLFGAI 119
gi|AAT36679.1|           VCAGVIILLTTGGKSSHLFVFSEDLFFIYVLPPIIFNAGFQVKKKQFFRNFITIMLFGAI 119
gi|ABG89337.1|           LCTGIIILLFSGGKSSHILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFVNFITIVSFGAI 116
gi|BAF75378.1|           LCTGVVILLLSRGKSSHLLVFSEDLFFIYLLPPIIFNAGFQVKKKQFFVNFMTIMLFGAI 118
                                                                * **:**: ***:

                                                    M5
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gi|NP_001233885.1|       GTLVSCAIISLGAIQTFKKLDIEFLDIGDYLAIGAIFAATDSVCTLQVLHQDETPLLYSL 179
gi|AAO38856.1|           GTVVSCTVITLGVTQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 176
gi|AAT95387.1|           GTIISCTIISLGVTQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 176
gi|AFH37919.1|           GTVVSCTVITLGVTQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNRDETPLLYSL 144
gi|AHY19036.1|CoNHX      GTIVSGTIISLGAMQFFKGLDTGSFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 73
gi|AAN08157.1|           GTLVSFSVISLGAMTIFKKMDIGSLELGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 180
gi|BAJ06110.1|           GTLVSFSIISLGAMTIFKKMDIGSLELGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 180
gi|BAB11940.1|           GTLVSFTIISLGALSIFKKLDIGTLELADYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 180
gi|AHY19038.1|ZaNHX      GTLISCTVISLGVIYFVKGFDVGPFEIGDFLAIGAIFAATDSVCTLQVLHQDETPLLYSL 74
gi|ABU92562.1|           GTLISCTIISLGVIQFFKGIDVGPFDIGDYLAIGAIFAATDSVCTLQVLHQDETPLLYSL 173
gi|AAK27314.2|           GTLVSCTIISLGVIQFFKKLDIGTLDIGDYLAIGAIFAATDSVCTLQVLNQDDTPLLYSL 179
gi|AAT36679.1|           GTLVSCTIISLGVIQFFKKLDIGTLDIGDYLAIGAIFAATDSVCTLQVLNQDDTPLLYSL 179
gi|ABG89337.1|           GTLVSCVIITLGVMYAFKRMDIGPLELGDYLAIGAIFAATDSVCTLQVLNQDETPLLYSL 176
gi|BAF75378.1|           GTLISCSIISFGAVKIFKHLDIDFLDFGDYLAIGAIFAATDSVCTLQVLSQDETPLLYSL 178
                         **::*  :*::*.   .* :*   :::.*:******************* :*:*******
                                   M6                                 M7
gi|AHY19037.1|LsNHX      VFGEGVVNDATSVVLFNAIQNFDLSSVNLSIALHFLGNFFYLFLASTLLGAVTGLLSAYI 134
gi|NP_001233885.1|       VFGEGVVNDATSVVLFNAIQNVDLTSLNPSIALSFLGNFFYLFLASTLLGAGTGLLSAYI 239
gi|AAO38856.1|           VFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFQLLGNFMYLFLLSTLLGVATGLISAYV 236
gi|AAT95387.1|           VFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFHLLGNFLYLFLLSTLLGAATGLISAYV 236
gi|AFH37919.1|           VFGEGVVNDATSVVVFNAIQSFDLTHLNHEAAFRLLGNFFYLFLLSTLLGVATGLISAYV 204
gi|AHY19036.1|CoNHX      VFGEGVVNDATSVVLFNAIQSFDLSHLNHEAALQFLGNFLYLFITSTLLGVVTGLLSAYI 133
gi|AAN08157.1|           VFGEGVVNDATSVVLFNAIQNFDLTNIDHRIAIQFSGNFLYLFFASTMLGAMTGLLSAYV 240
gi|BAJ06110.1|           VFGEGVVNDATSVVLFNAIQNFDLTHIDHRIALQFSGNFLYLFFASTLLGAMTGLLSAYV 240
gi|BAB11940.1|           VFGEGVVNDATSVVLFNAIQSFDLTRIDHRIALQFMGNFLYLFIASTILGAFTGLLSAYI 240
gi|AHY19038.1|ZaNHX      VFGEGVVNDATSVVLFNAIQSFDLSNINTQSALHLVGQFLYLFLTSTLLGVFTGLLSAYI 134
gi|ABU92562.1|           VFGEGVVNDATSVVLFNAIQSFDLTNINTQSAFHFLGQFLYLFLTSTLLGAFTGLLSAYI 233
gi|AAK27314.2|           VFGEGVVNDATSVVLFNAIQSFDLTHINTRSAFQFIGNFLYLFFTSTLLGVIGGLLSAYV 239
gi|AAT36679.1|           VFGEGVVNDATSVVLFNAIQSFDLTHINTRSAFQFIGNFLYLFFTSTLLGVIGGLLSAYV 239
gi|ABG89337.1|           VFGEGVVNDATSVVLFNAIQSFDLNRLNSLIALHFLGNFLYLFIASTLLGVLTGLFSAYV 236
gi|BAF75378.1|           VFGEGVVNDATSVVLFNAIQSFDMTSFDPKIGLHFIGNFLYLFLSSTFLGVGIGLLCAYI 238
                         **************:*****..*:.  .:  .: : *:*:***: **:**.  **:.**:

                                                                  M8
gi|AHY19037.1|LsNHX      IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYT-------- 185
gi|NP_001233885.1|       IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 298
gi|AAO38856.1|           IKKLYFGRHSTDREEVALMMLMAYLSYMLAELFALSGILTVFFCGIVMSHYTWHNVTESS 296
gi|AAT95387.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESS 295
gi|AFH37919.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELSDLSGILTVFFCGIVMSHYTWHNVTESS 263
gi|AHY19036.1|CoNHX      IKKLYFGRHSTDR-EVALMMLMSYLSYMLAELFDLSGILTVFFCGIVMSH---------- 182
gi|AAN08157.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 299
gi|BAJ06110.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 299
gi|BAB11940.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 299
gi|AHY19038.1|ZaNHX      IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELSNLSSILTVFFCGIVMSHYT-------- 185
gi|ABU92562.1|           IKKLYFGRHSTDR-EVALMMLMAYLSYMLAELTNLSGILTVFFCGIVMSHYTWHNVTESS 292
gi|AAK27314.2|           IKKLYFGRHSTDR-EVAIMVLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 298
gi|AAT36679.1|           IKKLYFGRHSTDR-EVAIMVLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESS 298
gi|ABG89337.1|           IKKLYIGRHSTDR-EVALMMLMAYLSYMLAELSYLSGILTVFFCGIVMSHYTWHNVTESS 295
gi|BAF75378.1|           IKKLYFGRHSTDR-EVALMMLMSYLSYIMAELFYLSGILTVFFCGIVMSHYTWHNVTESS 297
                         *****:******* ***:*:**:****::***  **.*************
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Fig. (8): Multiple sequence alignment of Na$^+$/H$^+$ antiporters protein sequence. Alignment of amino acid sequence of partial Na$^+$/H$^+$ antiporters of each LsNHX, CoNHX, and ZaNHX with other putative Na$^+$/H$^+$ antiporters proteins. Some Putative membrane spanning domains (M4, M5, M6, and M7) are indicated by (http://harrier.nagahama-i-bio.ac.jp/sosui/ cgi- bin/adv_sosui.cgi; Hirokawa *et al*., 1998).

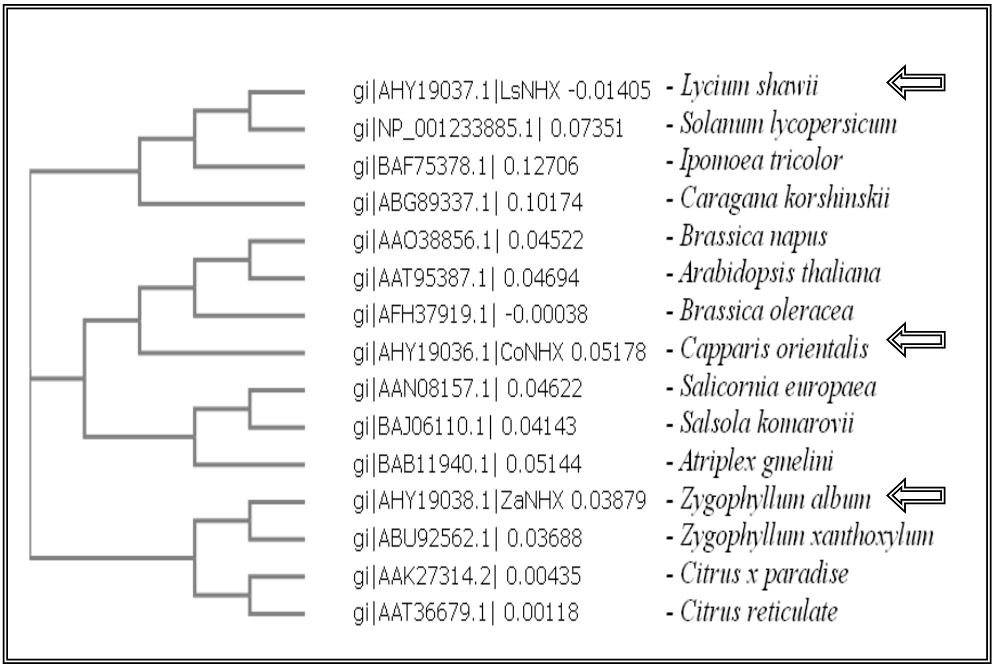

Fig. (9): Multiple sequence alignment of Na$^+$/H$^+$ antiporters protein sequence. Software packages, ClustalW2 and phylogeny were used to do multiple sequences alignment and generate phylogenetic trees. The accession numbers of Na$^+$/H$^+$ antiporters are: *Lycium shawii* (AHY19037.1), *Solanum lycopersicum* (NP_001233885.1), *Ipomoea tricolor* (BAF75378.1), *Caragana korshinskii* (ABG89337.1), *Brassica napus* (AAO38856.1), *Arabidopsis thaliana* (AAT95387.1), *Brassica oleracea* (AFH37919.1), *Capparis orientalis* (AHY19036.1), *Salicornia europaea* (AAN08157.1), *Salsola komarovii* (BAJ06110.1), *Atriplex gmelini* (BAB11940.1), *Zygophyllum album* (AHY19036.1), *Zygophyllum xanthoxylum* (ABU92562.1), *Citrus x paradise* (AAK27314.2) and *Citrus reticulate* (AAT36679.1).