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Genome-Wide Identification, Structural Characterization and Phylogenetic Analysis of *NHX* (Na^{+}/H^{+} Exchanger) in Lima Bean (*Phaseolus Lunatus* L.)

Arif Ullah[1], Zamarud Shah[2](Corresponding Author)**, Habiba Ali[3], Osaid Kamran Marwat[4], Kaleem Ullah[5],**

[1] PhD Student, Department of Biotechnology, University of Science and Technology Bannu, Pakistan, Email: *au254837@gmail.com* ORCID: *https://orcid.org/0000-0002-2469-158X*
[2], Assistant Professor, Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan, Email: *zamarud@awkum.edu.pk* ORCID: *https://orcid.org/0009-0008-3561-886X*
[3], BS Student, Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan, Email: *habibaalikhattak@gmail.com* ORCID: *https://orcid.org/0009-0001-1972-8945*
[4], M. Phil Student, School of Interdisciplinary Engineering and Sciences, National University of Science and Technology, Islamabad, Pakistan, Email: *usaidkmarwat@gmail.com* *https://orcid.org/0009-0006-4168-843X*
[5], BS Student, Department of Biotechnology, Abdul Wali Khan University, Mardan, Pakistan, Email: *kaleemullah31220@gmail.com*, ORCID: *https://orcid.org/0009-0001-0818-9667X*

Abstract

The Na+/H+ exchanger (*NHX*), a gene family of membrane transporters, is well recognized for enhancing salt tolerance in plants. The *NHXs* assist in maintaining ion and pH homeostasis in cells by exchanging K+ or Na+ for H+. Genome-wide analysis of *NHX* genes has not been reported in lima bean. The current study aimed at exploring and characterizing the *NHX* gene family across the genome of the lima bean. Eight *PlNHX* genes in the genome of lima bean were detected during the present study, and named as *PlNHX1-PlNHX8* on the basis of predicted sub-cellular localization. *PlNHX8* was recorded as the largest gene while *PlNHX5* was found as the smallest gene based on their CDS, protein length (PL) and protein molecular weight (PMW). Similarly, Isoelectric points and GRAVY were found in the range of 5.33 to 9.18 and 0.111 to 0.647, respectively. Vacoule was predicted as a major residence for *PlNHX* proteins. The varying number of exons reveals that events like deletion/addition have resulted in variation in motif number during the course of evolution, with motif3 conserved in all *PlNHX* genes. Phylogenetic tree reveals that paralogs contributed 25% to the *PlNHX* gene family expansion. Ka/Ks ratios of paralogs reveal that they were all under purifying selection. The promoter region has Box-4, G-box, ABRE and MeJA might be contributing elements for growth, development and defence functions of *PlNHX*. This study provides a basis for the functional validity of *PlNHX* genes.

Keywords: ***Lima bean, NHX, genome-wide analysis, salt tolerance***

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Introduction

Salt stress is considered one of the most important abiotic stress factors that negatively affects plant growth and development and causes serious yield losses in important crop species (Deinlein et al., 2014; Nataraja & Parvathi, 2016). High levels of salinity have limited the cultivation of important crop species, which affect more than 6% of existing farmland and about 20% of irrigated land around the world (Munns & Tester, 2008). The adverse effects of salt can vary, depending on the developmental stages of a plant, such as germination, early growth, and flowering or seeding stages (Chandna et al., 2013). Salt stress could affect essential biological processes, including photosynthesis, water relationships, and nutrient uptake (Parihar et al., 2015). Plants under salt stress conditions accumulate high concentrations of Na+ and Cl ions in the chloroplasts, inhibiting photosynthesis and decreasing chlorophyll content (Chutipaijit et al., 2011; Zhang et al., 2005). Over time, increased Na+ concentrations in plant tissues become toxic, which directly affects intracellular K+ homeostasis (Shabala & Cuin, 2008). A critical Na+/K+ ratio must be reached in the cell for the effectiveness of many cytosolic enzyme activities (Mahajan et al., 2008). It has been demonstrated that many ion transporters play important roles in maintaining the pH and ion homeostasis of plant cells in the presence of salt stress (Hamamoto et al., 2015; Pardo et al., 2006; Ward et al., 2009; Yamaguchi et al., 2013).

The Na^+/H^+ antiporters **(*NHX*)** are proteins embedded in the vacuolar membrane that exchange sodium (Na^+) or sometimes potassium ions (K^+) for protons (H^+) across cellular membranes (Brett et al., 2005). The genes encoding these transport proteins constitute a family known as the *NHX* family that plays a key role in maintaining ion homeostasis, pH regulation and conferring resistance to salt stress in plants (Van Zelm et al., 2020). Two H^+ pumps, including vacuolar H^+-H-ATPases (V-ATPase) and H^+-pyrophosphatases (H^+-PPase), create a proton gradient across the tonoplast by actively pumping H^+ ions from the cytosol into the vacuole at the expense of energy derived from ATP or pyrophosphate (PPi) (Aharon et al., 2003). This H^+ gradient across membranes is essential to power secondary transporters like *NHX*. Under salt stress, *NHX* exchanges cytosolic Na^+ with vacuolar H^+ across the tonoplast. This helps sequester excess Na^+ into the vacuole, protecting the cytoplasm from Na^+ toxicity and contributing to salt tolerance in plants (Bassil et al., 2011). Potassium (K^+) is a vital macronutrient for plants and is involved in enzyme activation, osmoregulation, and turgor pressure maintenance (Yamaguchi et al., 2003). H^+/K^+ exchangers (particularly in vacuoles) allow K^+ to be sequestered into vacuoles during surplus and released when needed, keeping cytosolic K^+ levels optimal. *NHX1* genes were first identified in Arabidopsis thaliana with three subgroups (Brett et al., 2005). Similarly, previous reports have documented 6 *NHX* genes in *Medicago truncatula* (Sandhu et al., 2018), 9 in *Capsicum annuum* (Luo et al., 2021), 5 in *Beta vulgaris* (Wu et al., 2019) and 10 *NHX* genes in *Glycine max* (Chen et al., 2015).

Among the bean crops, lima bean is the second largest one, both with respect to area under cultivation and consumption, and is only superseded by *Phaseolus vulgaris* (López-Alcocer et al., 2016). Lima bean originated from the USA, but over time its cultivation gained speed and has grown in many regions all across the globe (Baudoin et al., 2006; Martínez-Castillo et al., 2014). A lima bean is a rich source of proteins, fibres, vitamins and minerals (Jin et al., 2014). The fat-free proteins make this crop superior to other legume crops (Adebo et al., 2023). Lima bean has shown enhanced tolerance to abiotic stresses (Nascimento et al., 2017), especially to salt stress. So far, no genome-wide study of the *NHX* family in lima bean has been reported. Thus, the current study was carried out on the assumption of lima bean as a repository of *NHX* genes.

Material and Methods

Identification of *NHX* genes across the lima bean genome

The NHX8 sequence of *Arabidopsis thaliana* was isolated from NCBI (https://www.ncbi.nlm.nih.gov/) and inserted into Motif Finder (https://www.genome.jp/tools/motif/) for detecting Na^+/H^+ exchanger domain. Na^+/H^+ exchanger domain sequence was blasted against Lima bean genome using Phytozome v.13 (https://phytozome-next.jgi.doe.gov. Goodstein et al., 2012) for extracting *PlNHX* transcripts. Short sequences, with no more use in the onward process, were deleted.

Physical characterization of *PlNHXs* proteins

Molecular weight, protein length and CDS of *PlNHXs* were taken from phytozome. Similarly, other features of *PlNHX* proteins, including PI and GRAVY, were obtained from Expasy Protparam (https://web.expasy.org/protparam, Gasteiger et al., 2003).

Conserved domain and interspecific phylogeny of *NHX* Proteins

Two files, including **the** renamed protein and hitdata files, mandatory for finding conserved domains, were obtained from TBtool.v1.09854 (Chen et al., 2018) and conserved domain database (Marchler-Bauer et al., 2015), respectively. The files were subjected to TBtool.v1.09854 for generating domain architecture (Chen et al., 2018). *NHX* sequences were inserted in MEGA 12 (Kumar et al., 2016) for exploring phylogeny among *Phaseolus lunatus, Arabidopsis thaliana, Glycine max*, *Medicago truncatula, Oryza sativa* and *Solanum lycopersicum* based on maximum likelihood (ML, 1,000 bootstrap replicates).

Conserved motif and structure of *PlNHX* genes

Conserved motifs were visualized in *PlNHX* proteins using Multiple Em for Motif Elicitation (http://memesuite.org). CDS and genomic sequences of *PlNHX* genes were extracted from Pytozome, and subjected to Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/. Hu et al., 2015) for visualizing the gene structure.

Mapping and duplication of *PlNHX* genes

The mandatory information required for locating *PlNHX* genes, including chromosome number, position and length of *PlNHX* genes, was extracted from phytozome. The information was inserted in PhenoGram Plot for mapping *PlNHX* on the chromosomes (http://visualization.ritchielab.psu.edu/phenogram s/plot). The values of non-synonymous (Ka) and synonymous (Ks) substitutions, *PlNHX* paralog pairs, were obtained from TBTool (Chen et al., 2018). The Ka/Ks ratio was analyzed to determine the pattern of duplicated *PlNHX* genes.

Promoter Region Analysis

A sequence of 1500bp upstream to the 5 UTR was taken from the Phytozome of each gene. PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used for exploring *cis*-regulatory elements in *PlNHX* genes (Lescot et al., 2002).

Results

Detection of *NHX* genes across the lima bean genome and physico-chemical characterization of respective proteins

Eight *PlNHX* genes across the genome of lima bean were detected during the present study, and named as *PlNHX1*-*PlNHX8* based on their location on the chromosome in ascending order. *PlNHX8* was recorded as the largest gene with 3435 bp CDS, 1144 amino acids protein length (PL) and 126.875 kDa protein molecular weight (PMW), while *PlNHX5* was found as the smallest one with only 1395 bp CDS, 494 PL and 52.447 kDa PMW, respectively (Table 1). Isoelectric points and GRAVY were found in the range of 5.33 to 9.18 and 0.111 to 0.647, respectively (Table 1).

Table 1: Physico-chemical properties of *PlNHX* proteins (Annexed – A)

Interspecific phylogeny of *NHX* Proteins

Forty-seven *NHX* proteins across different species, including *Phaseolus lunatus*, *Arabidopsis thaliana*, *Glycine max*, *Medicago truncatula*, *Oryza sativa* and *Solanum lycopersicum*, were clustered into three groups based on their predicted subcellular localization, which are *NHX1*, *NHX2* and *NHX3*. *NHX1* group genes were localized on the vacuole membranes (Vac group); *NHX2* group genes were located on the endosomal region (Endo group); and *NHX3* group genes were localized on the plasma membrane (PM group). The *NHX1* group comprise 30 genes, *NHX2* have 10 genes, and *NHX3* have 7 genes (Figure 1). The *NHX1* group have the genes of all plants, and *Phaseolus lunatus* has the 5 genes (*PlNHX1*-*PlNHX5*) in the *NHX1* group. The *NHX2* group have the genes of all plants and has 2 genes (*PlNHX6*-*PlNHX7*) of *Phaseolus lunatus*. The *NHX3* group have the genes of all plants except *M.truncatula*, and one gene (*NHX8*) of *Phaseolus lunatus* is present.

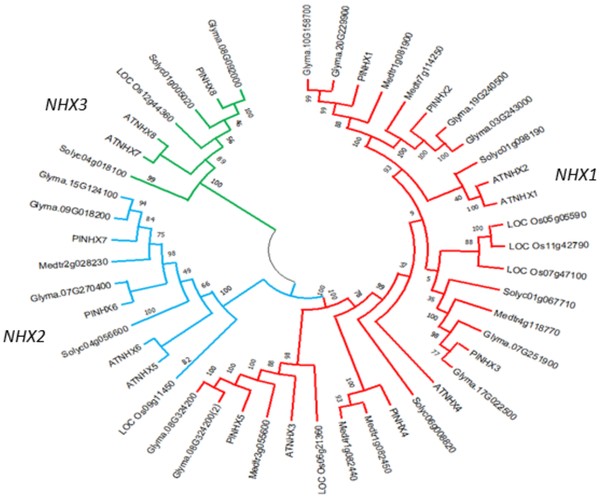

Figure 1. Comparative analysis of *NHX* proteins across different plant species. A phylogenetic tree was constructed through MEGA 12 using the Maximum Likelihood (ML) method. Different subgroups are highlighted with different colours.

PlNHX intra-specific phylogeny, gene structure, motifs configuration and domain

Non-specific tree is divided into 3 groups (*NHX1*, *NHX2* and *NHX3*). *NHX1*, *NHX2* and *NHX3* groups have 5, 2 and 1 genes, respectively. The tree reveals 2 paralogs (*PlNHX1*-*PlNHX2* and *PlNHX6*-*PlNHX7*) (Figure 2A).

NHX1 group members (*PlNHXs*) have several exons from 12 to 14, *NHX2* group members have several exons from 16 to 22, and *NHX3* group members have 1 gene (*PlNHX8*) and 23 exons present in it. Introns are one number shorter than exons, so the range of introns is from 11 to 22 (Figure 2B). Each member of the *NHX1* group had 12 motifs except *PlNHX5*, which had 9 motifs (the first 12 motifs in Figure 4b); all the motifs in the four members of this group are the same in order and type. The *NHX2* group had 6-8 motifs; they have 6 conserved motifs with each other member, and their order is similar. The *NHX3* group has only one gene (*NHX8*), and it has 2 motifs (motif3 and motif15)

(Figure 2C). The *PlNHX* proteins have a range of motifs from 21-50. Each motif 10, 11, 13 and 14 had a maximum of 50 amino acids, while the minimum amino acids (21) was reported in this study for motif 3 (Figure 2C). Both motif finder and TBtool results revealed that all the *PlNHX* genes have a Na^+/H^+ exchanger domain.

Figure 2: (Annexed-B) Phylogenetic tree (Figure 2A), Gene structure (Figure 2B) and Motifs (Figure 2C) of *PINHX* in Lima bean.

Chromosomal mapping and duplication of *PlNHX* genes

Eight *PlNHX* genes were distributed across 4 chromosomes. Three *PlNHXs* (maximum) were placed on chromosome number 6, while only 1 *PlNHX* (minimum) was found on chromosome number 2 (Figure 3). Segmental duplication was witnessed in the two paralog pairs. The Ka/Ks ratio was less than 1 for 2 paralogs, so they were under purifying selection (Table 2).

Figure 3. (Annexed-C) Chromosomal locations and duplication patterns of *PlNHX* genes across the lima bean genome. The chromosome number is mentioned in each bar. Each line connects genes of a paralog pair.

Table 2: The Ka/Ks ratio of paralog pairs

Seq_1	Seq_2	Ka	Ks	Ka_Ks
PlNHX 1	*PlNHX* 2	0.112785	0.639077	0.176481
PlNHX 6	*PlNHX* 7	0.218238	0.816933	0.267143

Promoter Analysis

From PlantCARE, a total of 170 cis-acting regulatory elements were explored 1500bp upstream region of 8 *PlNHX* genes. Cis-regulatory elements were dominated by light-responsive elements (60%), followed by hormone-responsive elements (23%), environmental stress (11.1%), site binding (3.5%), and developmental elements (2.3%) (Figure 4). The detection of a large number of cis-regulatory elements indicates the involvement of *PlDof* genes in functions with major emphasis on growth and response to environmental stresses.

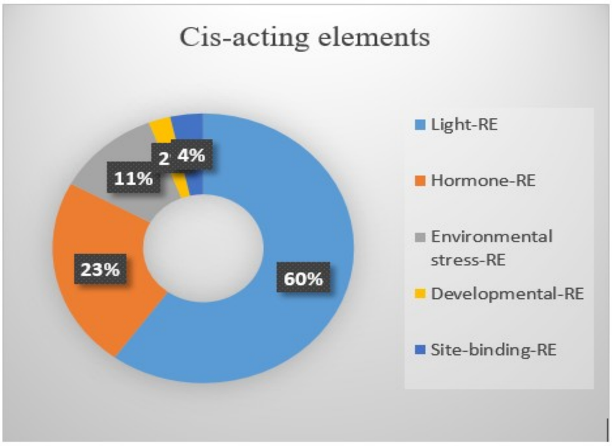

Figure 4: Different cis-regulatory elements (%) in the promoter region of *PINHX* genes

Discussion

The Na+/H+ antiporters play a significant role in ion homeostasis, vesicle trafficking, plant growth, salt tolerance, and cellular pH regulation in chickpea (Pehlivan et al., 2016; Qiu and Q.S., 2012). These antiporters are encoded by the *NHX* gene family in plants. Eight numbers of *PlNHX* were in line with 10 genes in G. max (Chen et al., 2015), 9 in *Camellia sinensis* (Paul et al., 2021), 8 in *A. thaliana* (Brett et al., 2005), 7 in *S. bicolor* (Kumari et al., 2018), and 6 in *C. reinhardtii* (Bassil et al., 2012). It indicates that lima bean has relatively lower genetic diversity for this gene family.

PlNHX genes were present in all three groups of the phylogenetic tree and in line with previous studies of rice, Pima cotton and pomegranate (Solis et al., 2022; Akram et al., 2020; Dong et al., 2021). The *NHX* proteins of lima bean revealed a closest evolutionary relationship with *G. max*; however, *M. truncatula* is closely related to lima bean and may share a common ancestor (Ling et al., 2020).

The subcellular localization of *NHX* genes has important implications for plant stress tolerance. Targeted manipulation of *NHX* genes based on their subcellular localization can result in the development of stress-tolerant crops, which are important in agriculture. For example, a recent study found that overexpression of the plasma membrane-localized wheat *NHX* gene, Ta*NHX*3, enhanced the salt tolerance in maize (Shah et al., 2021). In Arabidopsis thaliana, the *NHX*1 gene is localized in the tonoplast, where it functions in the vacuolar compartmentalization of Na+ ions and plays an important role in plant growth and abiotic stress tolerance (Apse et al., 1999; Zhang et al., 2012). In lima bean, five genes (*PlNHX1-PlNHX5*) are localized in the tonoplast. The *PlNHX8* protein localization in the plasma membrane suggests that this protein may play a role in controlling the pH and ions at the cell's surface. It might participate in the plasma membrane's exchange of protons and sodium, which can impact various cellular processes, including cell volume regulation, pH homeostasis, and cell signalling (Wang et al., 2015).

The existence of highly conserved Na+/H+ exchanger domains in *PlNHX* factor, as reflected in the present study, highlights the conservation of *NHX* transcription factors in plants during the course of evolution. Though a differential distribution of 15 motifs among *PlNHXs* was witnessed, motif 3 was present across all members of the *PlNHX* family. Motif 1 and motif 2 were conserved in all the *PlNHX* genes except *PlNHX8*. The results about the number of introns in *PlNHX* genes (11-22) were found in agreement with soybean (Chen et al., 2015) and sugar beet (Wu et al., 2019).

Less than 1 value of the Ka/Ks ratio, for *PlNHX* paralogs, pointing towards purifying selection as reported in Car*NHX* genes (Parveen et al., 2023). The positive GRAVY value of *PlNHXs* (0.111 to 0.647) shows their hydrophobic nature. The maximum GRAVY value was in agreement with the maximum value of the Car*NHX* gene (Parveen et al., 2023). The pH range of *PlNHXs* (5.32- 9.12) in the present study strengthened earlier reports of (Parveen et al. 2023) in Car*NHXs*.

Promoter analysis revealed that *PlNHX* gene expression is influenced by a range of cis-acting elements within its promoter regions. These elements are associated with light responsiveness, MeJA-responsiveness, ABA-related pathways, and fundamental plant growth and development functions. These elements are involved in hormone and stress responses and are also responsive to light. Notably, the Box-4 and G-box elements were present in all the *PlNHX1*-8 genes, indicating their potential significance in regulating lima bean development through light-related processes. The widespread presence of ABRE elements in all genes except suggests a strong link between *PlNHXs* and the regulation of the ABA metabolic pathway, potentially influencing various physiological responses in lima bean (Ain-Ali et al., 2021). The presence of MeJA is a known trigger that helps plants activate their defence mechanisms. These mechanisms work effectively to protect the plant against many types of insects and harmful microorganisms (Wang et al., 2021c). This comprehensive understanding enhances our insight into the regulatory mechanisms controlling the *PlNHX* gene family's involvement in lima bean physiological processes.

Conclusion

The in silico analysis revealed 8 *PlNHX* genes in lima bean. The protein length, pI, molecular mass, Gene structure and subcellular localization of *PlNHX* protein synchronized with previous reported *NHX*s. The gene duplication events contributed 25% to the expansion of the *PlNHX* gene family. The ka/ks ratio was less than 1 for all the paralogs, revealing purifying selection. The detection of many cis-acting elements, in the upstream region of *PINHX*, uncovered elements associated with growth, development and defence mechanisms.

Recommendation

Overexpression of the most promising *PlNHX* genes in model plants, like Arabidopsis thaliana, is recommended for functional validation of these genes under salt stress.

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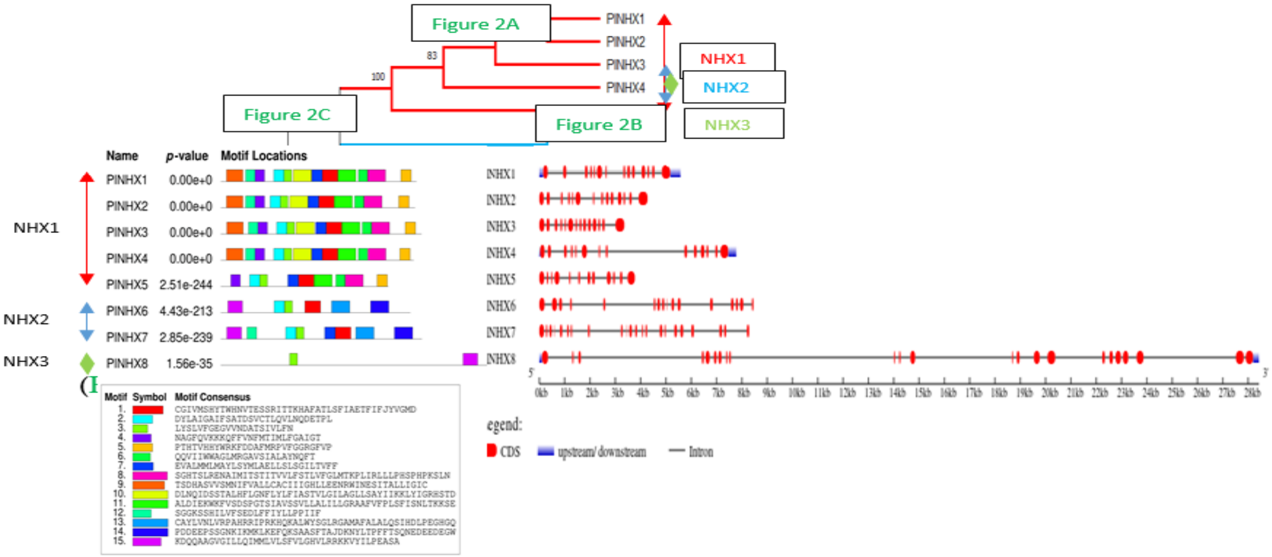
Gene names	start	end	strand	Protein length	MW	CDS	Pi	formula	extinction coefficient	instability index	aliphatic index	GRAVY
PINHX1	36678361	36683940	reverse	542	59627.01	1629	6.92	C2745H4315N681O750S24	56755	36.46	114.91	0.576
PINHX2	25968812	25973085	reverse	538	59619.66	1617	7.71	C2740H4282N686O753S23	65110	40.2	109.41	0.466
PINHX3	20882751	20886105	forward	556	61453.51	1671	9.18	C2850H4459N703O755S25	63745	34.45	112.73	0.584
PINHX4	36509026	36516806	reverse	534	59047.43	1605	7.1	C2737H4286N660O744S22	56880	29.16	117.04	0.647
PINHX5	20847753	20851524	reverse	464	52447.3	1395	8.81	C2421H3746N606O652S21	62590	40.61	104.61	0.365
PINHX6	18937621	18946080	reverse	525	58162.66	1578	6.01	C2669H4125N661O741S26	56185	44.42	104.95	0.409
PINHX7	29380403	29388689	reverse	557	60713.23	1674	5.33	C2789H4289N685O785S23	49195	41.77	105.76	0.465
PINHX8	37165106	37193534	reverse	1144	126875.5	3435	6.67	C5741H9039N1521O1639S40	137920	41.2	102.26	0.111

Annexed – B



Annexed- C