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An *in silico* data mining of the ammonium transporter gene family in *Ananas comosus* L.

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Abstract
Arguably, nitrogen (N) is an important and essential component for plant growth and development. Ammonium is a major inorganic nitrogen source for plants mobilized by ammonium transporter (AMT) among N available sources. In this study, data mining revealed that in the *Ananas comosus* L. genome was identified eight AMT family genes. The eight pineapple AcoAMT proteins were identified and phylogenetically clustered into two groups with AMT proteins from other plants. Two pairs of *AcoAMT* (*AcoAMTa* and *AcoAMTg*) genes located on chromosome 1 and unchromosome appear to be segmental duplications. Based on this information, we conducted a comprehensive analysis using some bioinformatics tools to characterize the identified genes individually. The comprehensive analysis of AMT will provide an essential foundation for further investigation of the regulatory mechanisms of *AcoAMTs* in *A. comosus* L.
Keywords: bioinformatic analysis; pineapple; ammonium; nitrogen transport.

Uma mineração de dados *in silico* da família do gene transportador de amônio em *Ananas comosus* L.

Resumo
Indiscutivelmente, o nitrogênio (N) é um componente importante e essencial para o crescimento e desenvolvimento das plantas. O amônio é a principal fonte de nitrogênio inorgânico para as plantas, sendo mobilizado pelo transportador de amônio (AMT), dentre as fontes de N disponíveis. Neste estudo, a mineração de dados revelou que no genoma de *Ananas comosus* L. foram identificados oito genes da família AMT. As oito proteínas AcoAMT de abacaxi foram identificadas e filogeneticamente agrupadas em dois grupos com proteínas AMT de outras plantas. Dois pares de genes *AcoAMT* (*AcoAMTa* e *AcoAMTg*) localizados no cromossomo 1 e não cromossômicos parecem ser duplicações segmentares. Com base nessas informações, realizamos uma análise abrangente usando algumas ferramentas de bionformática com a finalidade de caracterizar individualmente os genes identificados. A análise abrangente do AMT fornecerá uma base importante para uma investigação mais aprofundada dos mecanismos regulatórios de *AcoAMTs* em *A. comosus* L.
Palavras-chave: análise bioinformática; abacaxi; amônio; transporte de nitrogênio.

Introduction

Agronomically, nitrogen (N) is one of the most necessary macronutrients for plant growth and development (TEGEDER; MASCLAUX-DAUBRESSE, 2018). Nitrogen is a fundamental element of several biological molecules, such as nucleotides, chlorophyll, amino acids, and proteins. Plants can take up the N present in the soil in two ways: either inorganic N (nitrate; FAN *et al*., 2017) or organic N (ammonium, amino acid, and urea; reviewed by TEGEDER; MASCLAUX-DAUBRESSE, 2018). The use of N during plant growth and development can be divided into three main stages: **(I)** absorption; **(II)** assimilation and **(III)** remobilization of N. In addition to N

absorption from the soil, the nitrogen use efficiency (NUE) also depends on the assimilation of nitrate and ammonium, and on how the crop recycles organic N (MASCLAUX-DAUBRESSE *et al*., 2010; XU *et al*., 2012). Mainly, ammonium is the preferential form of N uptake by plants because the ammonium assimilation requires less energy than nitrate (BLOOM, 2015). In plants, the transport of nutrients, water, and metabolites is often mediated by a series of gene families of transporters proteins inserted into cell membranes (NACRY *et al*., 2013).

Specifically, the members of this family in plants belong to the AMT superfamily, which permeates ammonium via $NH4^+$ uniport or NH_3/H^+ co-transport, or belong to the MEP subfamily (Methylammonium Permeases), which includes AmtB from the bacterium *Escherichia coli* and human homologs (AMT/MEP/Rh (*ammonium transporter/methylammonium permease/rhesus*) (LOQUÉ; VON WIRÉN, 2004; LUDEWIG *et al*., 2007). The presence of the transmembrane domains (TM) in this transporter (AMT) can be ranged from 10-12 (TM) and are assembled into homo/or heterotrimers (LUDEWIG *et al*., 2003). Another important description of this transporter is that in plants, they are subdivided into two subfamilies: AMT1 and AMT2 (CASTRO-RODRÍGUEZ *et al*., 2016).

AMTs genes were identified in prokaryotic and eukaryotic organisms (reviewed by McDONALD; WARD, 2016). The first ammonium transporter genes were identified in studies with yeasts (MARINI *et al*., 1997). *AMT* family genes were shown to be highly expanded in plants and have already been characterized in several species, such as *Zea mays* L. (GU *et al*., 2013), *Arabidopsis thaliana* (HUANG *et al*., 2015), *Oryza sativa* L. (FERREIRA *et al*., 2015), *Triticum aestivum* L. (DUAN *et al*., 2016), *Coffea canephora* (DOS SANTOS *et al*., 2017) and *Solanum lycopersicum* L. (FILIZ; AKBUDAK, 2020). Because of the numerous studies in plants that describe the *AMT* gene's identification and characterization, there are no reports on this transporter in pineapple (*Ananas comosus* L.).

Pineapple is a tropical fruit, perennial monocot from the Bromeliaceae family, and has significant economic importance in tropical and sub-tropical areas. In world production, the pineapple is considered the third most important tropical fruit after banana and citrus (reviewed by XIE *et al*., 2018). Ming *et al*. (2015) made available pineapple completed genome sequencing. These databases provide valuable information in the search for agronomic interest genes, enabling us to identify the *AMTs* genes in the pineapple genome. Given the above, we performed the analyses of sequence characteristics, gene structures, chromosome distribution, motif compositions, and evolutionary relationships. The present study may understand the transcriptional mechanisms of the *AMTs* genes in response to pineapple's nutritional efficiency.

Material and Methods

We downloaded the protein sequences, genomic sequences and coding sequence (CDS) of pineapple obtained from Phytozome database (GOODSTEIN *et al*., 2012). To confirm the selected candidate gene sequences, belong to the *AMT* gene family, we used the National Center for Biotechnology Information (ALTSCHUL *et al*., 1997) database, using the BLASTP tool. The isoeletric point (pI) and theoretical molecular weight (kDa) of AMTs genes were calculated using the ExPASy server (http://web.expasy.org/compute_pi/). Also was evaluation of the grand average of hydropathicity (GRAVY) of all identified proteins through the GRAVY calculator (GASTEIGER, 2003). The predictions of subcellular localization were verified with the help of Plant-mPloc tool (CHOU; SHEN, 2010). The gene structures (exon/intron) of the *AMTs* genes were determined using the Gene Structure Display Server (HU *et al*., 2015). For these analyses, we used the predicted coding sequence (CDS) and their corresponding genomic DNA sequences. Additionaly, we used the TMHMM server v. 2.0 (KROGH *et al*., 2001) for prediction of transmembrane helices in AMT proteins. To phylogenetic analysis initially, protein sequence alignments and analysis were conducted using ClustalW (LARKIN *et al*., 2007). Posteriorly, the phylogenetic trees were constructed with MEGA7.0 software (KUMAR *et al*., 2016) using the Neighbor-Joining (NJ) method. We also verified the reliability of the obtained trees topology by the bootstrap method (1.000 replicates). Individual, the physical locations of *AcoAMTs* genes were obtained from the database of pineapple genome and posteriorly, the map of the chromosome location of genes was constructed through the Mapchart 2.2

software (VOORRIPS, 2002). We then further investigated the synonymous (*Ks*) and non-synonymous (*Ka*) substitution rates of the paralogs genes by using the *Ka_Ks* calculator 2.0 (ZHANG *et al*., 2006). To analyze the synteny relationship of the *AMT* genes, we used the orthologous genes between *A. comosus* L. and other two species: *A. thaliana* and *O. sativa*. Then, the Circos software was used to represent the syntenic relationships (KRZYWINSKI *et al*., 2009).

Resuts and Discussion

Ammonium acquisition by plants is transporter AMT-mediated that are ubiquitous plasma membrane proteins and essential for the nitrogen demand of plants (VON WIRÉN *et al*., 2000; HAO *et al*., 2020). In this study, we identified and cataloged eight genes coding for putative AMTs. We described the details of all eight pineapple AMT transporter proteins, including gene identifier, chromosome location, protein length, molecular weight, isoelectric point (pI), prediction of the hydrophobicity (GRAVY), subcellular location and their family. Collectively, all the information is listed in Table 1. The number of the genes that we obtained is similar to that in *Setaria italica*, *O. sativa* and *Sorghum bicolor* (Table 2) (see a review of VON WITTGENSTEIN *et al*., 2014; SANTOS *et al*., 2017). In the present study, it is possible to observe the conservation and confirmed of number *AMT* genes in monocot species. Two genes are located on scaffolds (scaffold_558 named *AcoAMTg*) and (scaffold_881 named *AcoAMTh*). We believe that this gene (*AcoAMTg* - Aco021941.1) is an alternate form of the *AcoAMTa* gene, as they have the same characteristics, according to data presented in Table 1. All the genes identified were renamed based on their chromosomal location (Table 1).

The results showed that the lengths of AMT transporter proteins range from 90 (AcoAMTe - Aco003227.1) to 495 (AcoAMTh - Aco030863.1) amino acids. The predicted molecular weights of proteins ranged from 9.95 (AcoAMTe - Aco003227.1) to 52.73 kDa (AcoAMTh - Aco030863.1) and the pI values were between 4.79 (AcoAMTe - Aco003227.1) and 8.53 (AcoAMTa - Aco009722.1 and AcoAMTg - Aco021941.1), respectively. In *S. lycopersicum* L. the molecular weight and pI values ranged from 49.65 to 55.37 kDa and from 5.33 to 7.16 (FILIZ; AKBUDAK, 2020). Generally, the structure of members AMTs family can range from 45–50 kDa and approximately lenght between 400 – 450 amino acids (NINNEMANN *et al*., 1994; BLAKEY *et al*., 2002). The predictions of the hydrophobicity of the deduced amino acid sequences indicated that the GRAVY of all AcoAMT proteins were above zero (Table 1).

Table 1. Characterization *in silico* of *AMT* gene family in pineapple.

Gene	Gene identifier	Chromosome location	Protein (aa)	MW (kDa)	pI	GRAVY	Subcellular prediction	Category (family)
AcoAMTa	Aco009722.1	LG01:108464..110894	460	49.73	8.53	0.616	Cell membrane	AMT2
AcoAMTb	Aco011210.1	LG01:13412289..13419316	473	51.51	8.10	0.411	Cell membrane	AMT2
AcoAMTc	Aco014095.1	LG13:57578..58987	469	49.81	6.14	0.570	Cell membrane	AMT1
AcoAMTd	Aco012484.1	LG13:1490708..1492001	342	36.11	7.11	0.594	Cell membrane	AMT2
AcoAMTe	Aco003227.1	LG17:1213248..1213539	90	9.95	4.79	0.361	Chloroplast/ Mitochondrion/Nucleus	AMT1
AcoAMTf	Aco007888.1	LG21:9803845..9808433	482	51.51	5.89	0.543	Cell membrane	AMT2
*AcoAMTg**	Aco021941.1	scaffold_558:72331..74767	460	49.73	8.53	0.616	Cell membrane	AMT2
AcoAMTh	Aco030863.1	scaffold_881:36273..37760	495	52.73	6.69	0.440	Cell membrane	AMT2

* Possible duplication of the *AcoAMTa* gene (see Table 3).

Almost all AMT transporters were predicted to be located in the cell membrane. Only one AMT (AcoAMTe - Aco003227.1) has been indicated with subcellular localization in the chloroplast, mitochondrion, and nucleus (Table 1). Detailed information about copy number of *AMT1* and *AMT2* transporters of different plant species are presented in Table 2.

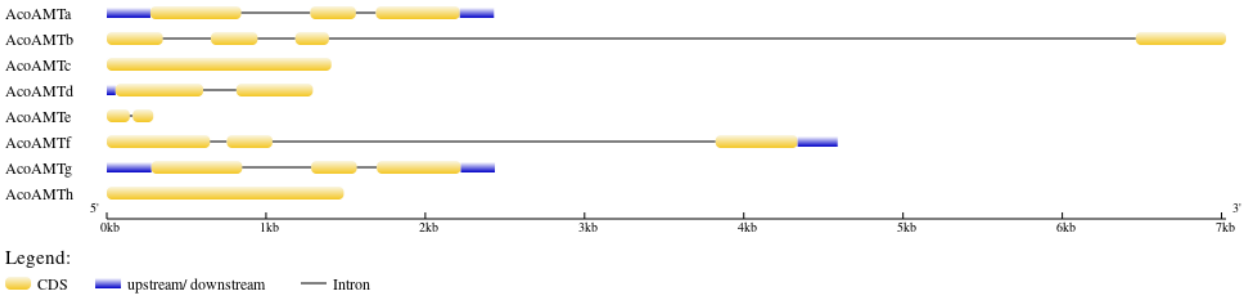
Table 2. List of members from the *AMT1* and *AMT2 genes* identified in different plants species, based in von Wittgenstein *et al*. (2014) and dos Santos *et al*. (2017).

	Number of members	
Species - Monocot/Dicot	**AMT1**	**AMT2**
Arabidopsis thaliana	5	1
Glycine max	5	5
Vitis vinifera	1	1
Populus trichocarpa	6	5
Coffea canephora	4	4
Setaria italica	2	6
Oryza sativa	2	6
Sorghum bicolor	2	6
Manihot esculenta	5	4
Cucumis sativus	4	2
Ricinus communis	4	3
Ananas comosus L.	2	6

In addition, we note that only two *AMT1* transporter members have been identified, which leads us to believe that the duplication mechanisms for this type of transporter in the evolution of the *A. comosus* L. genome were not relevant (Table 1 and Table 2).

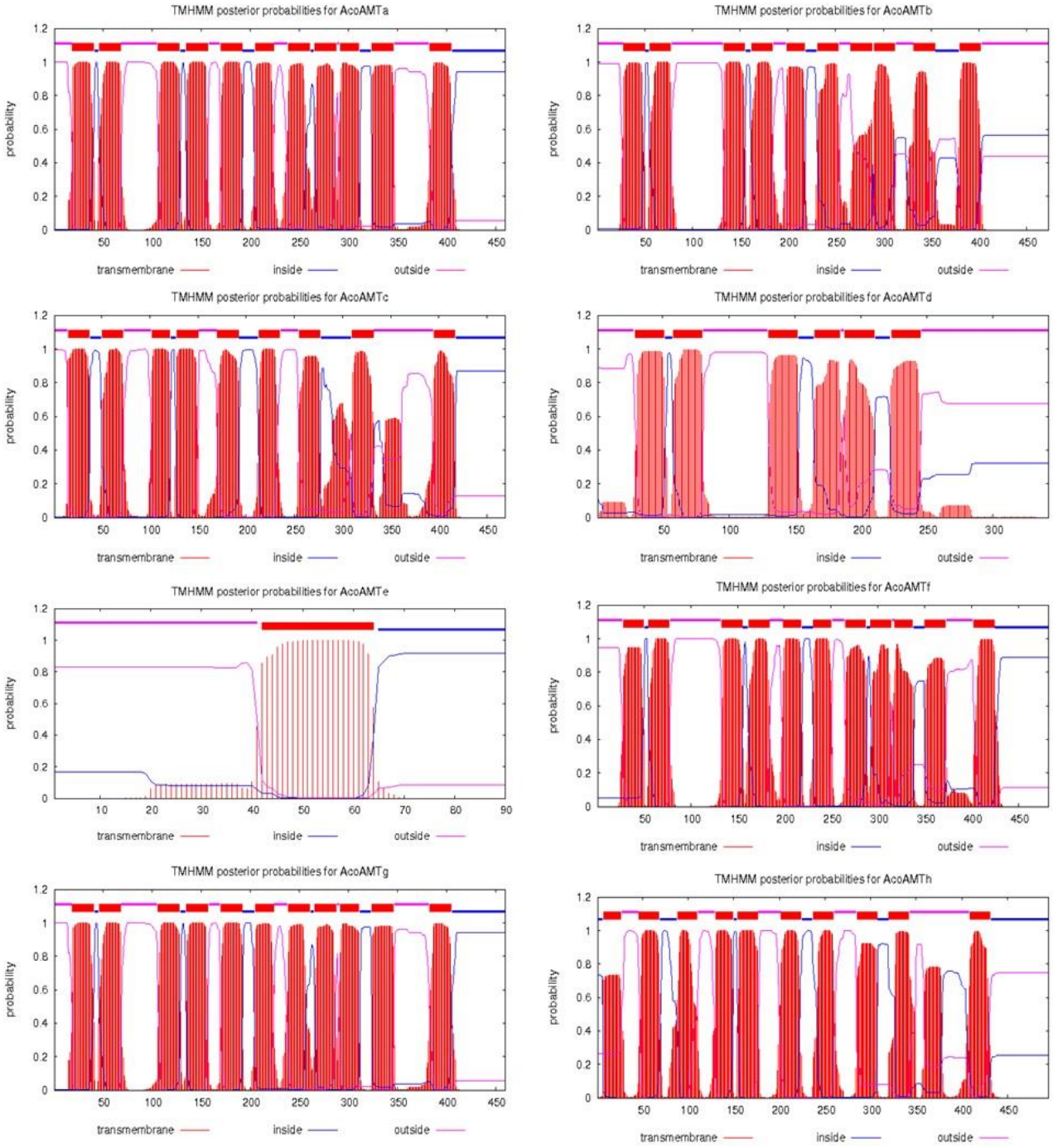
Evolutionarily, it is known that the structural diversity of the gene is one of the main evidence, besides that it also provides valuable information for the evolution of the innumerable multigenic families (CAO; SHI, 2012). In this sense, we analyzed the putative gene structure based on the CDS and genomic DNA sequences of each gene (Figure 1). The number of introns in *AcoAMT* genes varied from one to three. These results indicated that *AcoAMT* in pineapple shows lower gene structure diversity.

Figure 1. Representation of the *AcoAMT* genes structure, the yellow box, blue and black lines represent exons, upstream/downstream regions of the gene and introns, respectively.



The number of transmembrane helices (TMHs) in pineapple showed variations between 1 and 11 TMs (Figure 2).

Figure 2. Prediction of transmembrane domains of AcoAMT sequences were performed using TMHMM server v. 2.0.

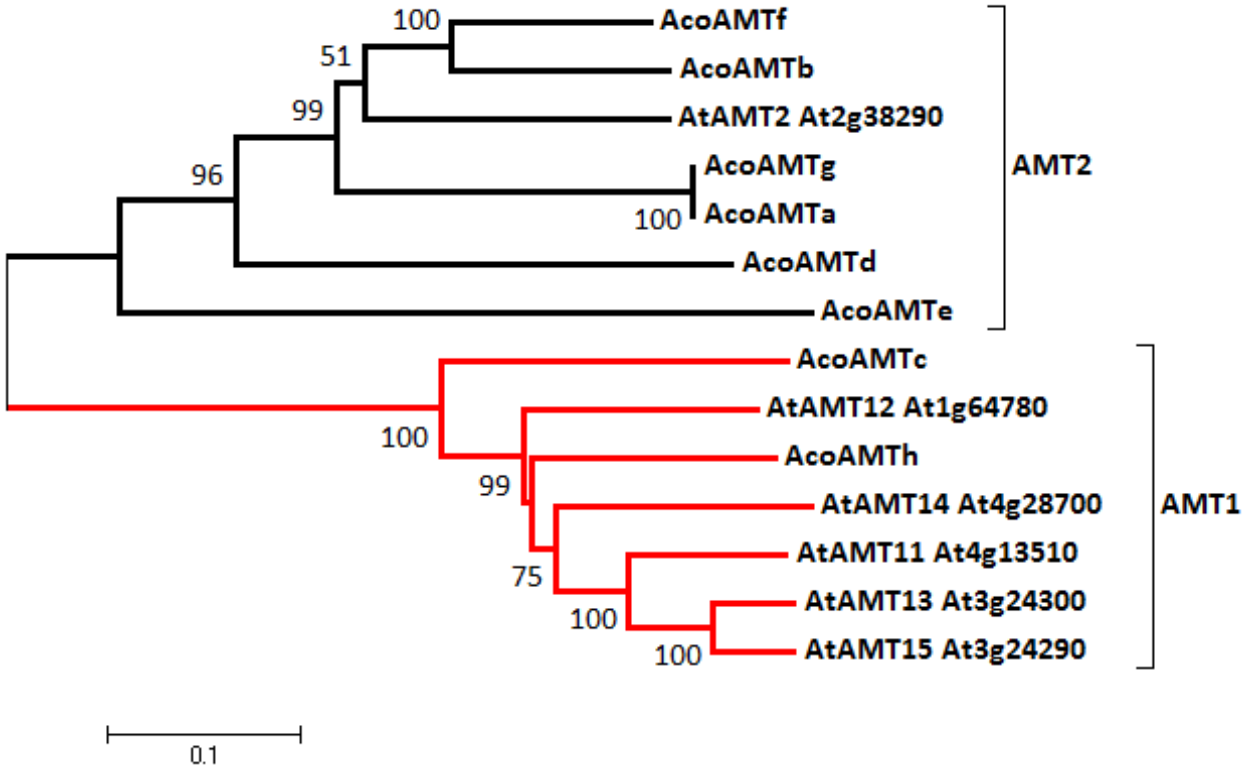


It is important to clarify to readers that the little transmembrane domain presented by AcoAMTe is due to the size of the protein sequence (90 aa). Although only one AcoAMT sequence showed little TM, ours analyzes corroborate with previous studies, about on the indicative of the amount of the transmembrane domain (McDONALD *et al.*, 2012; FILIZ; AKBUDAK, 2019). This result may be indicative of the complexity of ammonium homeostasis in pineapple. The structural formation of an AMT is formed by 11–12 transmembrane regions, standing out with feature sequences "D (F Y W S) A G (G S C) X2 (L I V) (E H) X2 (G A S) (G A) X2 (G A S) (L F)" located at its transmembrane region 5 and "D D X (L I V M F C) (E D G A) (L I V AC) X3 H (G A L I V) X2 (G S) X (L I V A W) G" at transmembrane region 10 (VON WIRÉN; MERRICK, 2004; reviewed by HAO *et al.*, 2020).

Firstly, to understand the evolutionary relationships of AMTs between pineapple and *A. thaliana*, we aligned multiple AMTs protein

sequences and constructed an unrooted phylogenetic tree for the identified multiple AMTs genes using ClustalW and MEGA 7.0 (Figure 3).

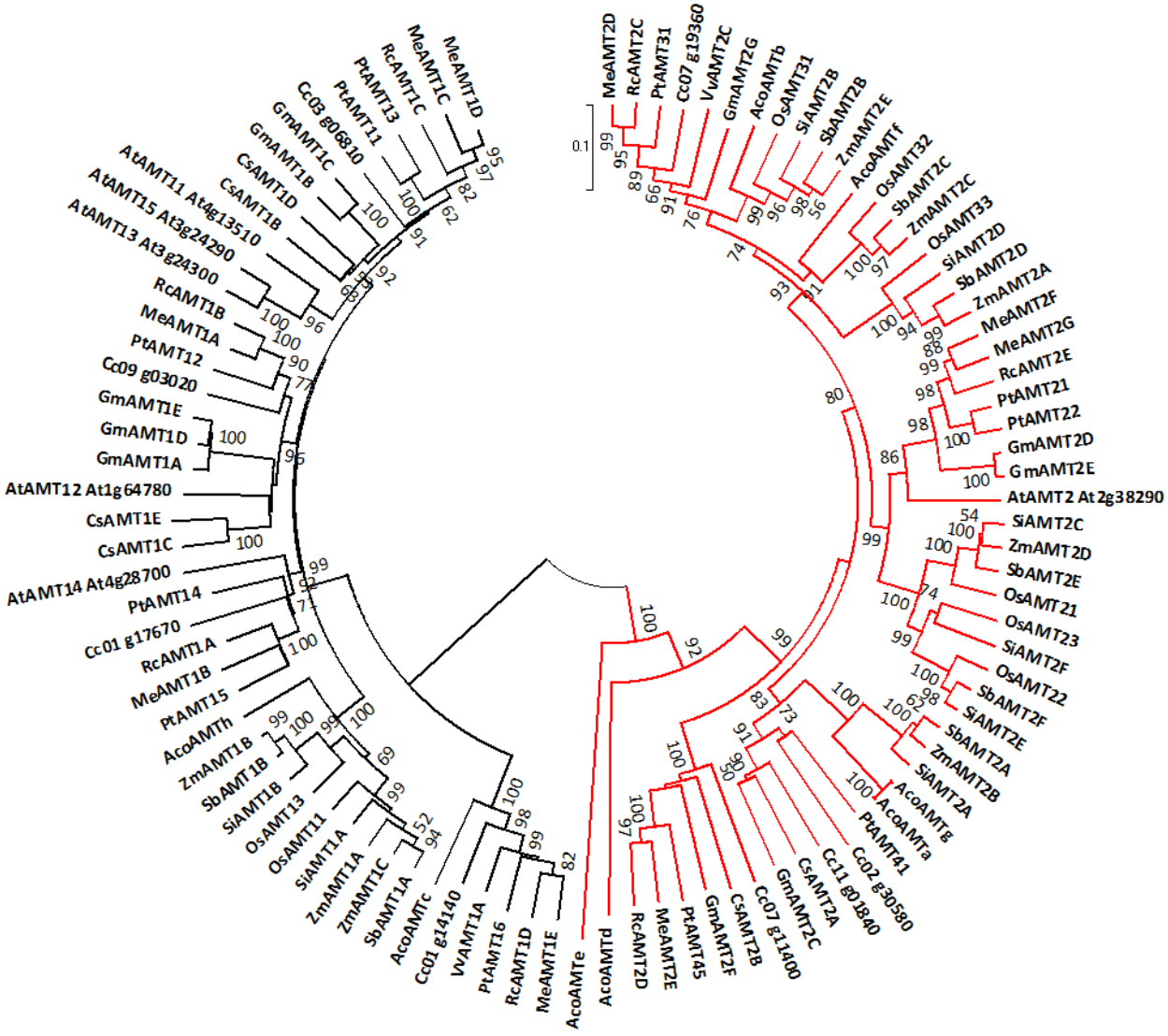
Figure 3. Phylogenetic tree of AMT proteins sequence from *A. comosus* L. and *A. thaliana*. Phylogenetic tree was constructed with the Neighbour-Joining (NJ) method using MEGA7.0 program with 1.000 bootstrap replicates. Branches with less than 50% bootstrap support were collapsed. The different colors highlight different groups of AMT1 and AMT2 family.



These results indicated that the AcoAMTs proteins were clustered into two subgroups, AMT1 and AMT2, according to their affinity type. Group AMT1 is composed by transporters of high-affinity NH_4^+ (represented in red, Figure 3) (NINNEMANN *et al*., 1994; VON WIRÉN; MERRICK, 2004; YUAN *et al*., 2007). According to the literature, the group AMT2 members (represented in black, Figure 3) are consists of representatives can participate in the to the transfer of net NH_3, however without the presence of current across the membrane (GUETHER *et al*., 2009) and also are not permeable to the NH_4^+ analog methylammonium (SIMON-ROSIN *et al*., 2003; review by SUN *et al*., 2018).

To further confirm that there were two major AMT transporters groups and to study and understand the evolutionary relationships of the AMTs of other plants, we selected proteins sequences of 12 species (including 10 from *Glycine max*, 2 from *Vitis vinifera*, 11 from *Populus trichocarpa*, 8 from *C. canephora*, 8 from *Setaria italica*, 8 from *O. sativa*, 8 from *S. bicolor*, 9 from *Manihot esculenta*, 6 from *Cucumis sativus*, 7 from *Ricinus communis*, 8 from *A. comosus* L.), and we constructed a phylogenetic tree (Figure 4).

Figure 4. Phylogenetic tree analysis for all AMT proteins from 12 plants (*A. thaliana*, *G. max*, *V. vinifera*, *P. trichocarpa*, *C. canephora*, *S. italica*, *O. sativa*, *S. bicolor*, *M. esculenta*, *C. sativus*, *R. communis*, *A. comosus* L.), totally 99 protein sequences. Phylogenetic tree was constructed with the Neighbour-Joining (NJ) method using MEGA7.0 program with 1.000 bootstrap replicates. Branches with less than 50% bootstrap support were collapsed. The different colors highlight different groups of AMT1 and AMT2 family. The sequences of *A. comosus* L. was obtained in Phytozome database (http://www.phytozome.net/pineapple.php) and other sequences were obtained based in von Wittgenstein *et al*. (2014) publication.



Genome chromosomal location showed that *AcoAMT* genes were randomly distributed on chromosomes (Figure 5). The largest number of *AcoAMT* genes occurred on chromosomes 1 and 13 (two *AcoAMT* genes), followed by one gene located on chromosomes 17 and 21. In addition, two genes are located on scaffold558 (one gene) and scaffold881 (one gene), in which they are represented by the unchromosome in Figure 5. Expansion analysis of the *AcoAMT* genes in the *A. comosus* L. genome was examined. Based on their chromosomal distribution and the high rate of sequence similarity, we determined that two duplication pairs arose from segmental events, one between *AcoAMTa* and *AcoAMTg* genes and another between *AcoAMTb* and *AcoAMTf* genes in *A. comosus* L. (Table 3); the lines red in Figure 5 shows the connections among these paralogs. Our results indicated that these *AcoAMT* genes were possibly generated by gene duplication and the segmental duplication events played and a major driving force for *AcoAMT* expansion. The duplication of genes increases the functional

divergence, which is an essential factor in adaptability under changing environmental conditions (CONANT; WOLFE, 2008).

We have calculated the divergence and evolutionary relationship by *Ka*, *Ks*, and *Ka*/*Ks* ratios between two paralogs *AcoAMT* genes. *Ka*/*Ks* ratio was 0 for *AcoAMTa* and *AcoAMTg* and 0.22 for *AcoAMTb* and *AcoAMTf* (Table 3). In general, *Ka*/*Ks* ratio less than 1, equal to 1, and greater than 1 means negative or stabilizing selection, neutral selection, and positive selection, respectively (ALI *et al*., 2017). The *Ka*/*Ks* ratio trend in the coding sequences of two duplicate pairs indicated that the *AcoAMT* genes have undergone a positive Darwinian or purifying selection (*Ka*/*Ks* < 1). Positive selection of a gene during evolution means that it increases its potential and has more transcription levels under stress conditions (BOWERS *et al*., 2003).

Figure 5. Chromosomal distribution of *AMT* genes in *A. comosus* L. Chromosome number is labeled above the chromosomes. Red lines reflect segmental duplications. (Data extracted from Table 3).

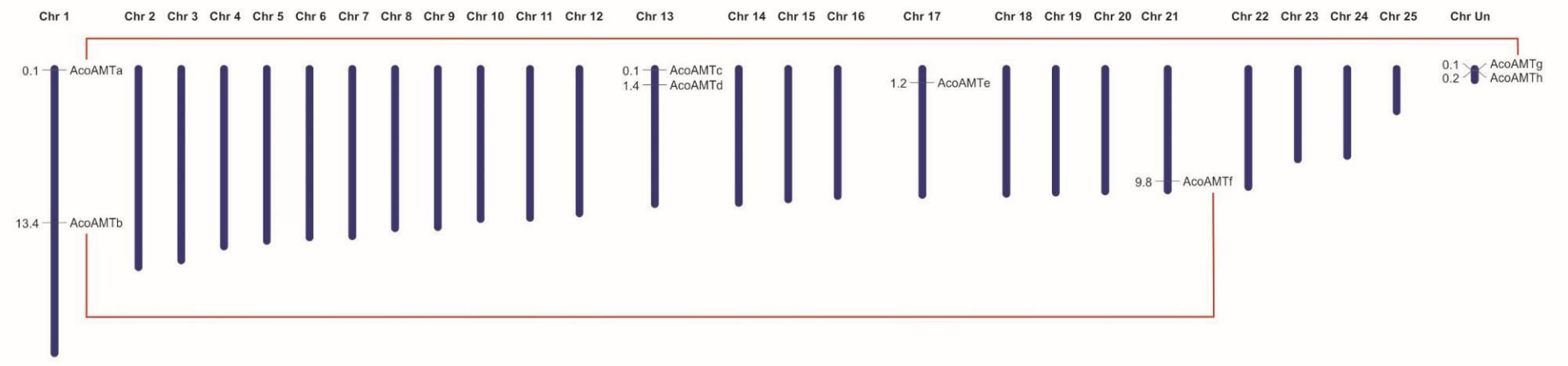


Table 3. Duplication date of paralogous genes pairs in among *A. comosus* L. *AcoAMT* genes. *Ka* represents the non-synonymous substitution number per non-synonymous site, *Ks* is the number of the synonymous substitution site; *Ka*/*Ks* represents the ratio of non-synonymous (*Ka*) to synonymous (*Ks*) substitutions.

Paralogous Pairs	Chromosomal location	Duplication event	*Ka*	*Ks*	*Ka*/*Ks*	Selection
AcoAMTa/ *AcoAMTg*	Chr1/ scaffold_881	segmental	0.00	0.003	0	Purifying
AcoAMTb/ *AcoAMTf*	Chr1/Chr21	segmental	0.16	0.74	0.22	Purifying

To examine the origin and evolutionary history among *A. comosus* L. with other species (*A. thaliana* and *O. sativa*), a comparative analysis was performed to identify the orthologous pairs of *Aco*AMT genes (Figure 6; Table 4). A total of 5 *AcoAMT* genes showed a syntenic relationship with *A. thaliana*, followed by 8 in *O. sativa* (Table 4). *AcoAMTh* was found to be associated with five genes pairs in *A. thaliana* (*AtAMT11, AtAMT12, AtAMT15, AtAMT13* and *AtAMT14*) and two genes pairs in *O. sativa* (*OsAMT11 and OsAMT13*), indicating that *AcoAMTh* gene might be derived from the same ancestor and arose before the divergence of eudicots and monocots. Indeed, we suggest that this gene may have played an essential adaptative role during evolution. Besides, we also found that all other *AcoAMT* genes were present in *A. comosus* L. and were not found in *Arabidopsis* (*AcoAMTa, AcoAMTb, AcoAMTc, AcoAMTd, AcoAMTe, AcoAMTf* and *AcoAMTg* - Figure 6; Table 4), which may indicate that these genes were formed after the divergence of eudicot and monocot plants. Interestingly, we found an extensive genetic synteny between *A. comosus* L. and *O. sativa,* two monocot species. This was expected because the genome-sequenced CAM (pineapple) share conserved syntenic relationships with several important cereal species (like rice and sorghum) and has been regarded as an important crop for studying CAM photosynthesis and abiotic stress tolerance (MING *et al*., 2015).

Figure 6. Synteny between *A. comosus* L. and *A. thaliana* (A) and *O. sativa* (B). (Data extracted from Table 4).

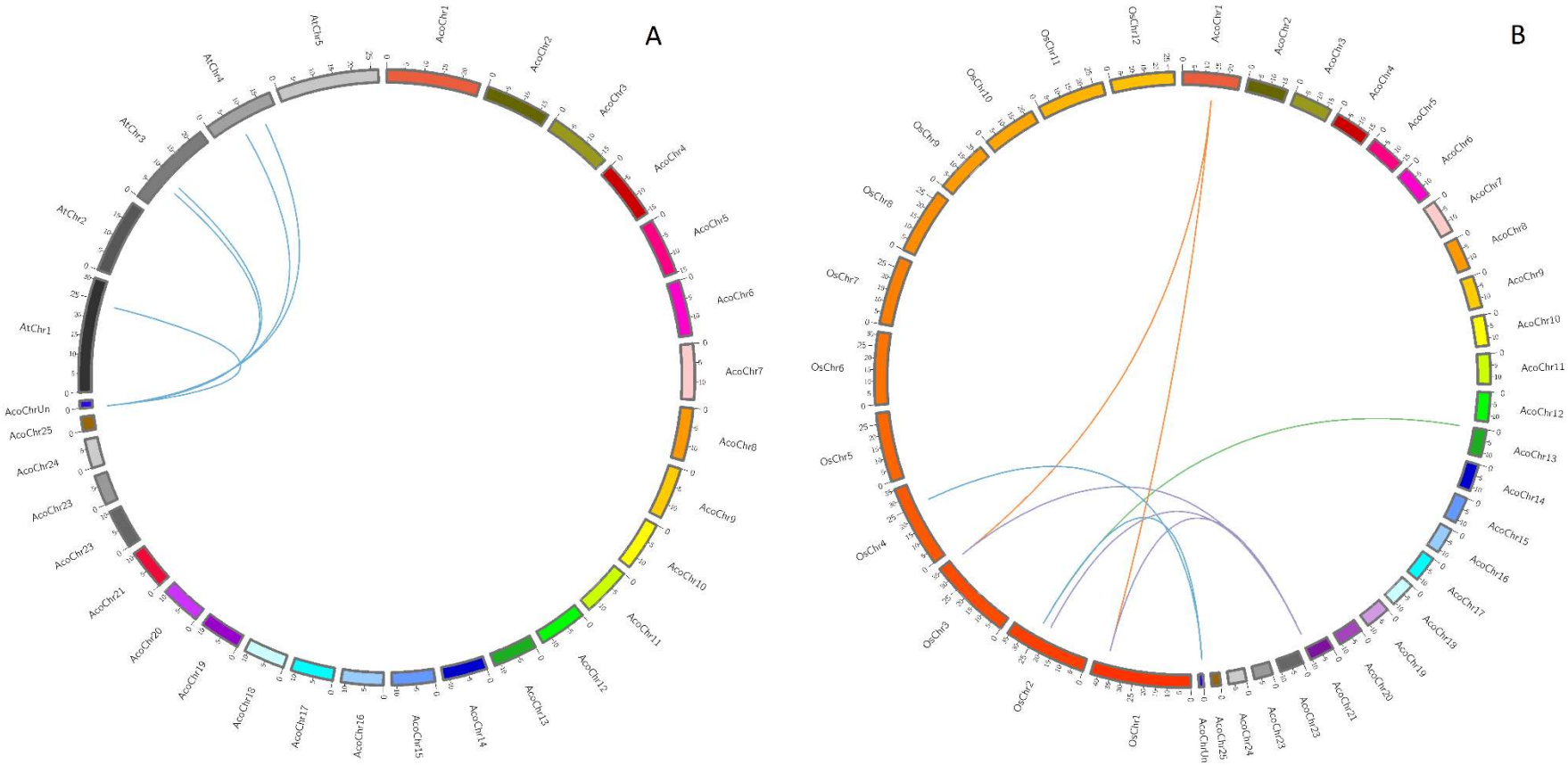


Table 4. *AcoAMT* genes synteny among *A. comosus* L. and *A. thaliana* and *A. comosus* L. and *O. sativa.*

N	***A. comosus***	**Chromosome Position**	***A. thaliana***	**Chromosome Position**
1	*AcoAMTh*	scaffold_881:36273..37760	*AtAMT11*	Chr4:7858183..7859918
2	*AcoAMTh*	scaffold_881:36273..37760	*AtAMT12*	Chr1:24060770..24062630
3	*AcoAMTh*	scaffold_881:36273..37760	*AtAMT15*	Chr3:8801400..8802890
4	*AcoAMTh*	scaffold_881:36273..37760	*AtAMT13*	Chr3:8805631..8807388
5	*AcoAMTh*	scaffold_881:36273..37760	*AtAMT14*	Chr4:14161681..14163195
N	***A. comosus***	**Chromosome Position**	***O. sativa***	**Chromosome Position**
1	*AcoAMTb*	LG01:13412289..13419316	*OsAMT31*	Chr1:37735280..37737641
2	*AcoAMTb*	LG01:13412289..13419316	*OsAMT33*	Chr3:35238255..35241976
3	*AcoAMTc*	LG13:57578..58987	*OsAMT13*	Chr2:24690884..24692884
4	*AcoAMTf*	LG21:9803845..98084333	*OsAMT32*	Chr2:20734076..20737331
5	*AcoAMTf*	LG21:9803845..98084333	*OsAMT31*	Chr1:37735280..37737641
6	*AcoAMTf*	LG21:9803845..98084333	*OsAMT33*	Chr3:35238255..35241976
7	*AcoAMTh*	scaffold_881:36273..37760	*OsAMT11*	Chr4:25500158..25502633
8	*AcoAMTh*	scaffold_881:36273..37760	*OsAMT13*	Chr2:24690884..24692884

Conclusion

The current study identified eight ammonium transporters genes in the pineapple genome and provides an overview of the structure, phylogeny, chromosomal distribution, and synteny relationship of the *AMT* genes. This study provides comprehensive information and can help the researchers better understand the functioning of these genes, besides assisting breeding programs pineapple culture.

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