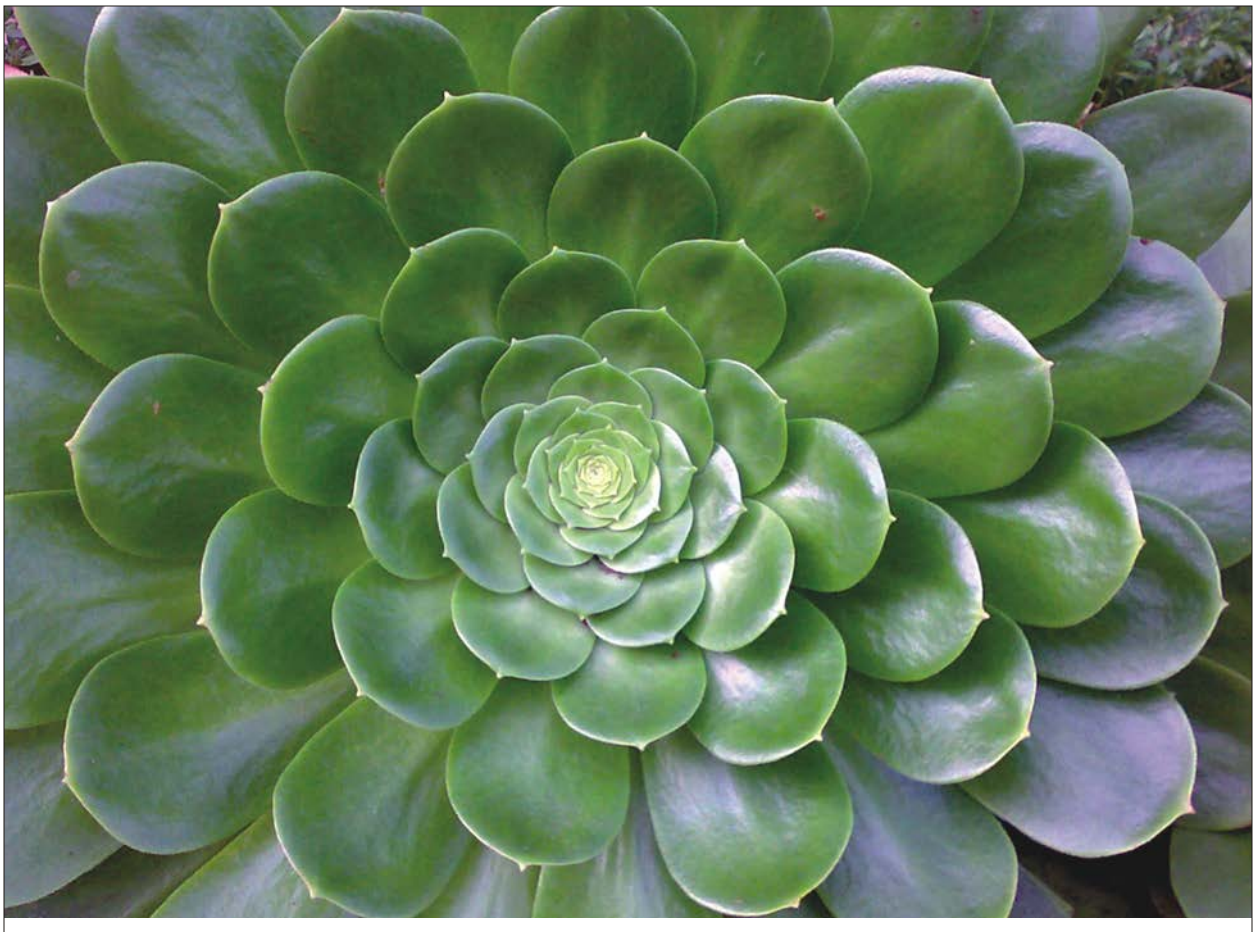

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Molecular Characterization of the *Amy1* Gene in Hexaploid Wheat

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ABSTRACT

Alpha-amylase enzyme is the important member in physiological metabolism of high plant, especially during seed germination stage. The current study identified 19 *amy*1 genes from 19 hexaploid wheat (*Triticum aestivum* L.) accessions. The nucleotide and deduced amino acid sequences of *amy*1 genes were analyzed in detail, respectively. It showed that two main variation types were characterized due to the base substitution and/or indel mutations in genomic nucleotide sequences and the exon and intron domains were presented different variation degree. This study identified 8 clustered haplotypes from hexaploid wheat accessions through characterization of exon domains. The haplotype 8 was the major variation types and the others were low relative frequency. The haplotype 7 was a special kind of haplotypes among *amy*1 genes in wheat accession PI243793 because of the variation of amino acid sequences at 155-161 sites. These results would contribute to the understanding in functional aspects and efficient utilization of *amy*1 genes in hexaploid wheat cultivars.

Key words: Hexaploid wheat, alpha-amylase, *amy*1 gene, haplotypes

INTRODUCTION

In hexaploid wheat, *Triticum aestivum* (2n = 6x = 42), alpha-amylase enzyme has received much attention in functional and evolutionary studies and also been regarded as the important enzyme for both agricultural (germination of cereal grains) and industrial (the brewing industry) interests. The genotypes analysis of *amy* gene also attracted more scientists to study including chromosome location, functional analysis, enzyme activity and allelic variation. Gale *et al.* (1983) also developed variety of genetic population to study the *amy* genotype. Alpha-amylase contributes to the grain germination in wheat but it regulates the germination with different tissue of seed (embryo and aleurone) during developing grain (Garcia-Maya *et al.*, 1990; Appleford and Lenton, 1997).

Alpha-amylase has been participated in many physiological processes and metabolic activities (Laurie *et al.*, 2003). The alpha-amylase activity in the grain was used as an indicator for pre-harvest sprouting (Li *et al.*, 2003; Lin *et al.*, 2008). During the germination of wheat grains, alpha-amylase encoded by *amy* gene play a key role in the mobilization of the energy reserves stored in insoluble starch granules (Sugimoto *et al.*, 1998). Furthermore, the transcription of alpha-amylase was regulated by hormone including gibberellins and abscisic acid (Jacobsen and Beach, 1985; Higgins *et al.*, 1976). Alpha-amylase activity is also negative association with Hagberg Falling Number (HFN), an important test of bread-making quality in wheat. Kindred *et al.* (2005) also reported that high alpha-amylase activity could result in the production of sticky doughs which would be difficult to industrial process.

Many alpha-amylase genes have been cloned from various cereals (Mitsui and Itoh, 1997). Great progress has been made in the analysis of alpha-amylase multigene families in cereal, including characterization of protein structure and secretion of the enzyme. Alpha-amylase genes of wheat are expressed at least twice during the life cycle of the plant. They are expressed at a high level during germination in the aleurone cells when the controlling influence at the cellular level is the plant hormone gibberellic acid (Baulcombe *et al*., 1987).

In current study, 19 *amy*1 genes from hexaploid wheat (*Triticum aestivum* L.) accessions were cloned and identified. The present study was conducted to isolate and characterize the *amy*1 genes from hexaploid wheat accessions and to analysis the allelic variation of these genes in the wheat accessions derived from different regions.

MATERIALS AND METHODS

Plant materials and growth conditions: In total, 19 hexaploid wheat accessions derived from various regions including Canada, Eritrea, Mexico, UK, Iraq, China, UK, Ukraine, Australia, Iran, Peru, Ethiopia, US and Tajikistan (Table 1) were used to characterize *amy*1 genes. These experimental wheat materials were grown under a randomized complete block design with two replicates at the Experimental Station of Sichuan Agricultural University at Chengdu-Wenjiang, Sichuan, during the 2012 crop seasons.

DNA extraction and PCR amplification: Genomic DNA was extracted from the seeding leaves of each accession about 2 weeks old using a modified CTAB (Hexadecyltrimethylammonium Bromide) extraction method described by (Murray and Thompson,1980). The specific primers (Forward: 5'-CAACCGGAGAAGAAGAGTGAC-3', Reverse: 5'-ATGGTGGATCAGTGGAG ACTT-3') were designed by bioinformatics software DNAMAN 5.0 (LynnonBioSoft, USA) and Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA) on the basis of the wheat tests sequences

Table 1: Plant materials used in this study

Accessions	Sources
CItr6618	Canada
CItr8195	Canada
CItr14811	Eritrea
CItr17599	Mexico
PI64285	UK
PI70692	Iraq
PI70713	Iraq
PI82755	China
PI94390	UK
PI94460	Ukraine
PI94504	Ukraine
PI106124	Australia
PI113933	Argentina
PI124820	Australia
PI243793	Iran
PI477864	Peru
PI648908	Ethiopia
PI651298	US
PI654219	Tajikistan

derived from NCBI (http://www.ncbi.nlm.nih.gov/) and selected for amplification the target *Taamy1* gene. These TaESTs sequences were aligned by homology gene, *Hvamy1* (Genbank No.: FN179389).

Statistical analysis: The multiple alignment of sequences was carried out by DNAMAN 5.0 (LynnonBiosoft corporation, US), respectively. The phylogenetic tree based on the phylogenetic analysis was performed by Mega 4.0 (Tamura *et al*., 2007).

RESULTS

Variation analysis: Currently, 19 nucleotide sequences of *amy*1 gene sequences were obtained from the hexaploid wheat accessions basis on the homology-cloning according to *Hvamy1* gene (Genbank No.: FN179389). The similarity of nucleotide sequences of exon domains between *amy*1 genes in wheat accessions and *Hvamy1* gene was 97.18% and the similarity of deduced amino acid residue sequences among them was 91.30%. *Amy*1 gene derived from hexaploid wheat contained 4 exons and 3 introns (Fig. 1). In current study, the genomic sequences of *amy1* genes obtained from hexaploid wheat accessions contained 1691 to 1715 nucleotides (exon domains, 1314 to 1339 nucleotides and intron domains, 376 to 402 nucleotides). The nucleotide sequences of nineteen *amy*1 genes shared a high sequence identity (94.07%) and the coding and noncoding domains sequences shared 96.71 and 91.69%, respectively. Furthermore, these nucleotides in entire coding domains might encode 437 to 440 amino acid residues. There were 51 variation positions in entire exon domains and it could be divided into two large genotypes (Table 2). Among those variation positions, it presented 42 Single Nucleotide Polymorphisms (SNPs) and 9 polynucleotide variation sites (Table 2), including 4 indel sites. These SNPs of coding sequences resulted in diverse variation of deduced amino acids sequences. Most of them might lead to the structural variation of alpha-amylase I enzyme protein and contribute to different function due to the different binding sites.

Haplotype diversity: Currently, it indicated that seven haplotypes were evaluated in accordance with SNP and indel variation analysis. The haplotype 8 was presented most frequently among the genotypes and it was involved in 9 genes simples (PI70713, PI94390, PI94460, PI94504, PI113933, PI124820, PI648908, PI651298 and PI654219). The second frequent haplotype was haplotype 1, which were *amy*1 genes from four genotypes including CItr6618, CItr8195, CItr14811 and CItr17599. The other 6 cluster of haplotypes was only had one genotype respectively. It demonstrated that *amy*1 genes from hexaploid wheat accessions presented high polymorphism. Referring to *amy*1 gene in hexaploid wheat, the diverse variation of coding domains could be lead to variation of amino acid sequences, even result in the change of base protein structure. More new alleles of *amy*1 genes could be frequently observed in hexaploid wheat.

Deduced amino acid analysis: The deduced amino acid sequences of alpha-amylase presented certain polymorphism. Herein, the coding domains of 18 *amy*1 gens could encode the normal amino acid reduces and develop the normal alpha-amylase proteins and only one from PI477864 could not conduct the transcript process because of the abnormal present of termination codon in coding domain of *amy*1 gene. The changes in the deduced amino acid residues at 35 positions could be presented high polymorphism (Table 3), among which four deduced amino acid sequences showed

Table 2: Distribution patterns of SNPs and indels of haplotypes in coding domain of nineteen *amy*1 genes from hexaploid wheat accessions

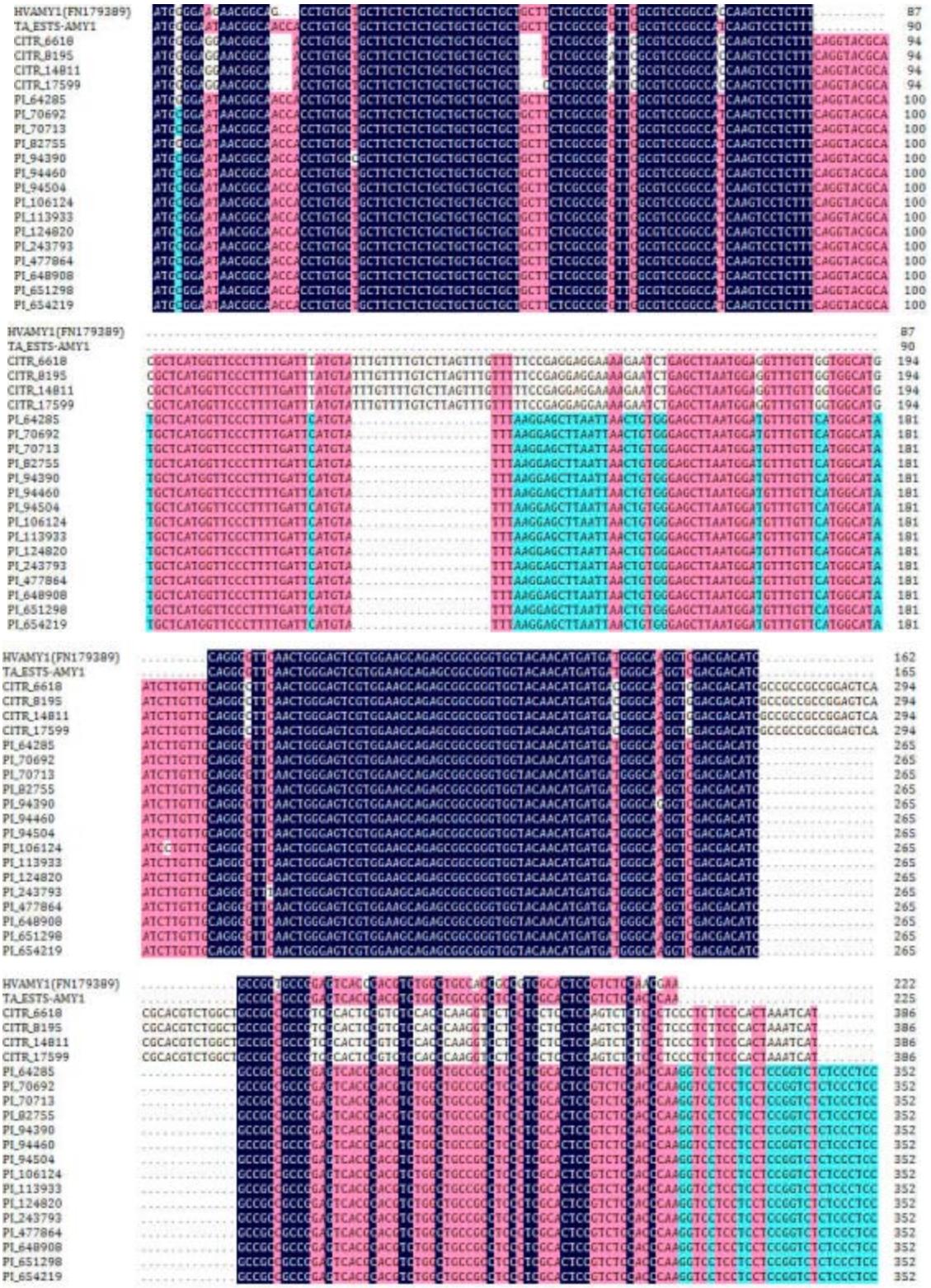
	Variation sites (bp)																
Haplotype	4	8-9	16-18	36-38	62	65	77	145	155	200	203	245	260	284	300-301	461-463	479
1	G	GG	-	-	A	C	C	G	G	G	G	C	G	C	CG	CAC	C
2	G	GG	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A
3	C	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A
4	G	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A
5	C	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	A--	A
6	C	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A
7	C	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A
8	C	AT	ACC	CTG	G	G	G	T	C	T	C	G	A	G	GC	CAC	A

	Variation sites (bp)														
Haplotype	500	509	517-520	533	543-562	564	573	631	633	644	672	731	750	759
1	C	-	CGCC	C	-	C	C	T	T	A	G	C	C	C
2	T	-	CGCC	T	-	A	G	C	C	G	C	G	T	C
3	T	-	CGCC	T	-	C	G	C	C	G	C	G	T	G
4	T	-	CGCC	T	-	A	G	C	C	G	C	G	T	C
5	T	GCG	ACAT	T	-	C	G	C	C	G	C	G	T	C
6	T	-	CGCC	T	-	C	G	C	C	G	C	G	T	G
7	T	-	CGCC	T	GCGTCCAGCGGGAGCTCAA	C	G	C	C	G	C	G	T	C
8	T	-	CGCC	T	-	C	G	C	C	G	C	G	T	C

	Variation sites (bp)																			
Haplotype	816	822	849	855-856	900	912	972	1029	1099	1149	1152	1204	1213	1260	1275	1278	1311	1314	1323	1328
1	G	A	G	AT	G	T	A	A	C	A	C	A	C	C	A	T	A	C	A	G
2	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
3	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
4	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
5	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
6	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
7	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T
8	C	C	A	TC	A	C	G	G	G	G	T	G	G	A	G	A	C	G	G	T

the high homology with the same variation sites differing in the others. Interestingly, PI243793 indicated that the only variation pattern from 153 to 176 amino acids reduces (Fig. 2). Possibility, it would contribute to the special mechanism function of alpha-amylase though the whole plant lifespan.

Phylogenetic analysis: The phylogenetic relationships among the *amy1* genes in these hexaploid wheat accessions derived from different countries around the world was evaluated and indicated by phylogenetic distances in neighbor-joining trees (Fig. 3) developed by MEGA 4.0. The clustering results indicated that the *amy*1 gene was obviously divided into two groups. Four genes from CItr6618, CItr8195, CItr14811 and CItr17599 were clustered into one group and the other genes were included in the second group (Fig. 3). Furthermore, *amy*1 gene from wheat accession PI243793 with special variation of amino acid sequences had genetic relationship from the others in the second clustered group.



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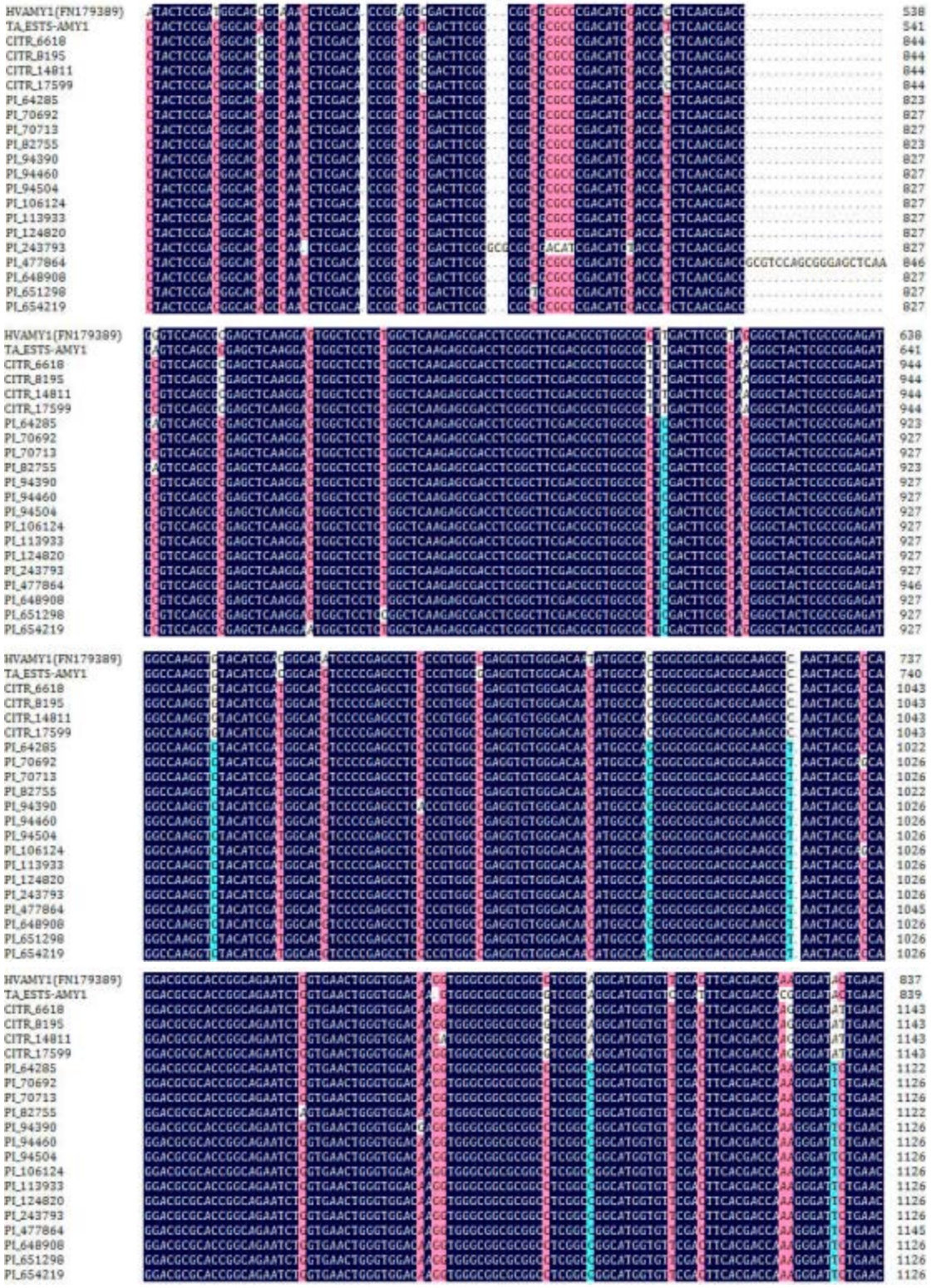

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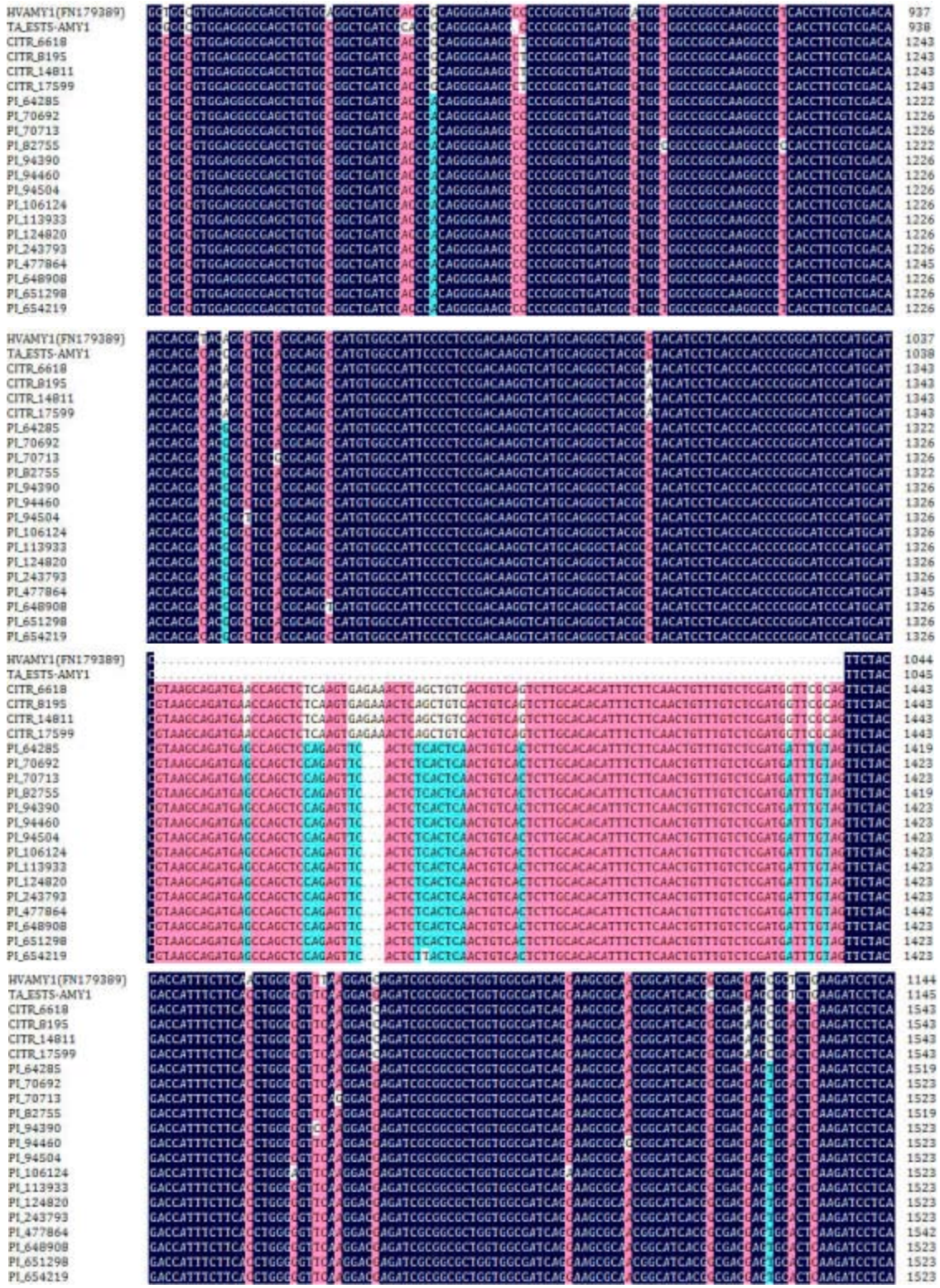

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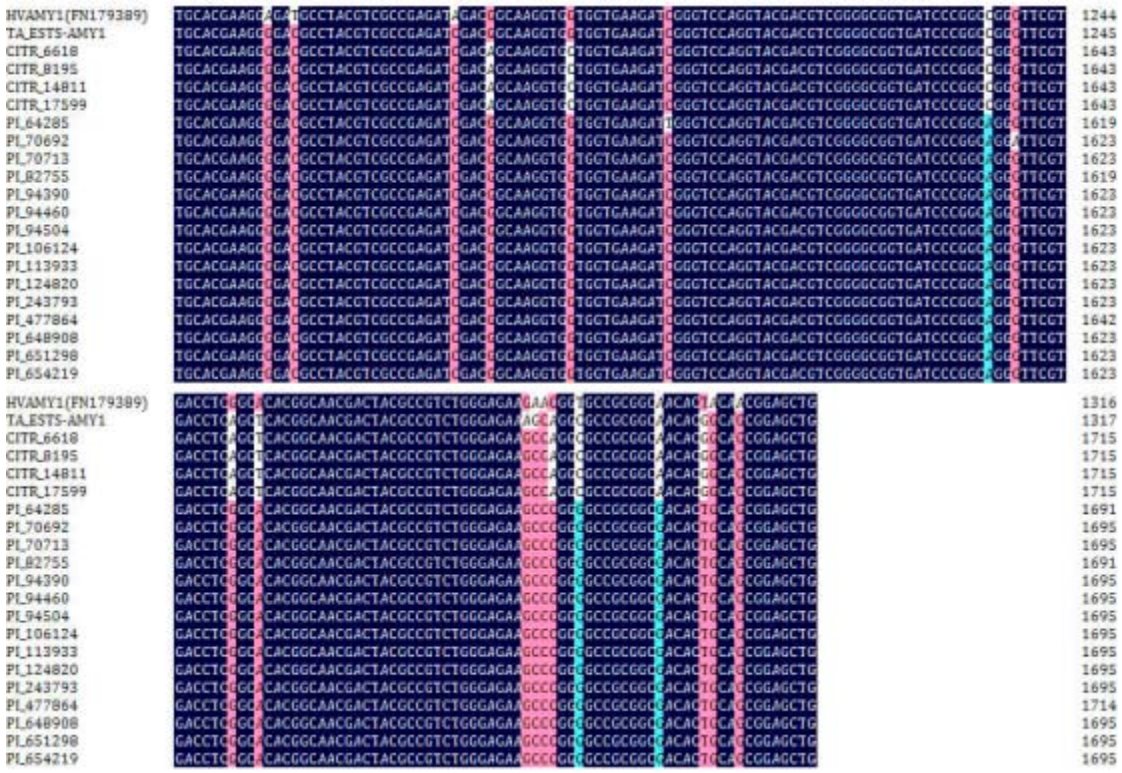

Fig. 1: The chart of alignment genomic sequence of *amy*1 genes from hexaploid wheat accessions

DISCUSSION

Alpha-amylases encoded by *amy* genes play an essential role in the germination and has been regarded as the most important enzymes and the trigger for regulating germination in wheat grain (Huang *et al*., 1993; Karrer and Rodriguez, 1992). Variety of alpha-amylase genes including *amy*1, *amy2* and *amy3* (Baulcombe *et al*., 1987) and Late Maturity Alpha-amylase (LMA) gene (Mrva and Mares, 1996, 2001; Mrva *et al*., 2009) have also been reported in previous published literatures. The three types of *amy*1 genes shared a common evolutionary ancestor (Baulcombe *et al*., 1987). Similarly, the SNPs of *amy*1 genes in barley were analyzed and indicated many variation sites were associated with malting quality index (MQI) (Matthies *et al*., 2009). The allelic variation of *amy*1 genes from 19 hexaploid wheat accessions was identified in this study. Integrated genomic sequence of *Taamy1* gene was not reported in NCBI (http://www.ncbi.nlm.nih.gov/). This study provided the reference genomic sequence of *Taamy1* gene for further research. The *Taamy1* gene contained 1691 to 1715 nucleotides and 4 exons and 3 introns and the noncoding domain shared more variation than coding domain. Nevertheless, the structure of *Taamy1* in this study was different from *Hvamy1*gene which just contained two introns (Knox *et al*., 1987).

In current study, by the alignment and phylogenetic analysis, the *amy*1 gene sequences from 19 hexaploid wheat accessions could be distinguished into two different types (Fig. 3) and had relative great variation in nucleotides and amino acids sequences (Fig. 1, 2). Apparently, many studies just compared the difference of amy multigene family (Lazarus *et al*., 1985; Baulcombe *et al*., 1987). Little was known about the characterization of amy1 gene. Possibility, this

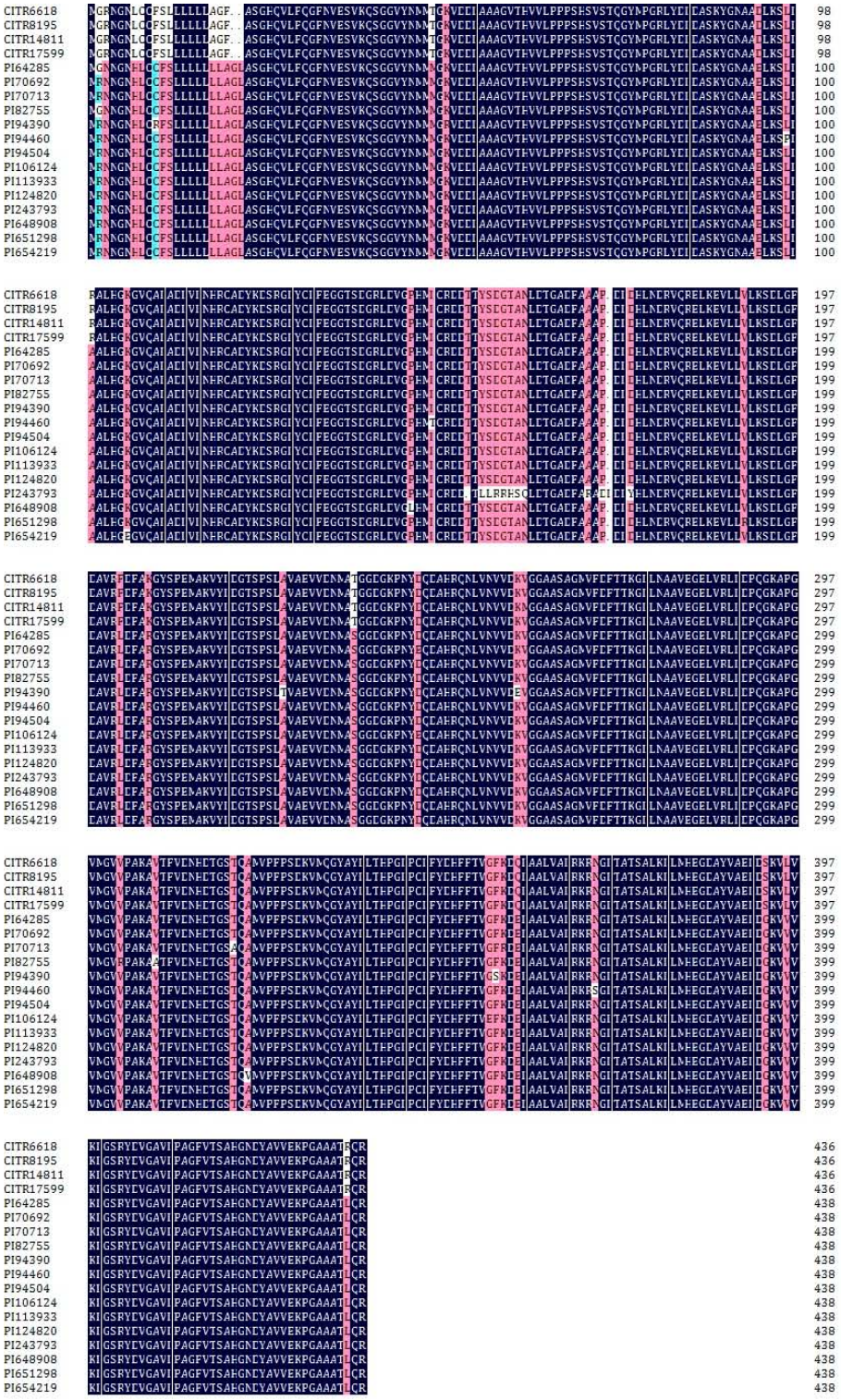

Fig. 2: The chart of alignment of amino acid sequences of *amy1* genes from hexaploid wheat accessions

Table 3: Variation of amino acids in *amy*1 genes from hexaploid wheat accessions

Variation of amino acids	Amino acid position	Frequency
G/R	2	6/12
R/N	3	4/14
LC/HL	7-8	4/14
F/R/C	9	4/1/14
SL/FS	10-11	4/14
AGF/LLAGL	17-21	4/14
T/M	48	4/14
R/K	50	1/17
D/E	94	4/14
P/L	98	1/17
R/A	100	4/14
K/E	105	1/17
L/P	145	1/17
T/I	148	1/17
-/T	153	1/17
LLRRHSQ/YSDGTAN	155-161	1/17
R/A	170	1/17
DI/P	172-173	1/17
Y/D	176	1/17
R/V	192	1/17
F/L	204	4/14
K/R	208	4/14
T/A	227	1/17
T/S	237	4/14
E/D	246	2/16
E/K	260	1/17
M/V	261	1/17
A/V	309	1/17
A/T	320	1/17
V/A	322	1/17
S/F	357	1/17
S/N	371	1/17
S/G	395	4/14
L/V	398	3/14
R/L	436	4/14

study provided a new insight that was two copies of *amy*1 gene from hexaploid wheat. Additionally, the sequence alignment and haplotypes analysis revealed 8 haplotypes in 19 hexaploid wheat accessions from the diverse countries. It showed that *amy*1 gene has more variation and haplotype 8 was found to be the major type. Furthermore, the haplotype 7 would be used to develop the potential marker for different function in plant metabolize.

Variation of *amy*1 gene influences the grain germination in wheat greatly, especially in PHS (Lin *et al*., 2008; Li *et al*., 2004). And the activation of alpha-amylases was reported to associated with the grain quality and adaption of cereal crops by high temperature (Hakata *et al*., 2012). It would be important for identification of germplasm resources from current hexaploid wheat accessions via characterization of *amy*1 genes. Two clustered types might perform different molecular function in plant physiological development. Moreover, the special variation of amino

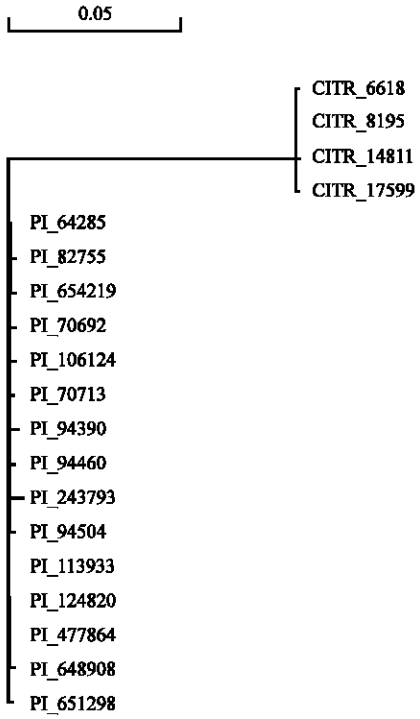

Fig. 3: Phylogenetic tree of *amy*1 genes

acid sequences was at 155 to 161 sites (Table 3, Fig. 2) and the relevant nucleotide sequence of *amy*1 gene would be the potential marker for new function developing. A further understanding for allelic variation of *amy*1 gene in hexaploid wheat would benefit our breeder to improve our wheat cultivars during breeding process, particularly in resistance to PHS. All discovered SNPs could be converted into high-throughput markers for pyrosequencing and might be used for marker assisted selection.

CONCLUSION

In current study, we have isolated the *amy*1 genes in 19 hexaploid wheat accessions and identified natural variation for nucleotides and amino acids sequences of *amy*1 genes. *amy*1 gene in hexaploid wheat accessions presented high variation degree. It demonstrated that two main variation types and eight clustered haplotypes appeared in hexaploid wheat accessions. Furthermore, it was interesting that haplotype 7 as a special kind of haplotypes among *a*my1 genes was just cloned in wheat accession PI243793 due to the unique variation sites of nucleotides and amino acids sequences, which might be severed as a special material for future utilization of *amy*1 genes in hexaploid wheat cultivars.

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