

Genetic Diversity and Molecular Evolution of Chinese Waxy Maize Germplasm

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Abstract

Waxy maize (*Zea mays* L. var. *certaina* Kulesh), with many excellent characters in terms of starch composition and economic value, has grown in China for a long history and its production has increased dramatically in recent decades. However, the evolution and origin of waxy maize still remains unclear. We studied the genetic diversity of Chinese waxy maize including typical landraces and inbred lines by SSR analysis and the results showed a wide genetic diversity in the Chinese waxy maize germplasm. We analyzed the origin and evolution of waxy maize by sequencing 108 samples, and downloading 52 sequences from GenBank for the waxy locus in a number of accessions from genus *Zea*. A sharp reduction of nucleotide diversity and significant neutrality tests (Tajima's *D* and Fu and Li's *F**) were observed at the waxy locus in Chinese waxy maize but not in nonglutinous maize. Phylogenetic analysis indicated that Chinese waxy maize originated from the cultivated flint maize and most of the modern waxy maize inbred lines showed a distinct independent origin and evolution process compared with the germplasm from Southwest China. The results indicated that an agronomic trait can be quickly improved to meet production demand by selection.

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Introduction

Waxy maize (*Zea mays* L. var. *certaina* Kulesh) is a special cultivated type of maize, and was first discovered in China in 1908 and then in other Asian countries [1–4]. Waxy maize, with nearly 100% amylopectin in endosperm, is mainly used as food in Asia, and is also an important raw material for food industries, textiles, paper-making and feedstuff worldwide because of its excellent characters in terms of starch composition and economic value [5]. Despite waxy maize was first discovered in China, the origin and evolution of waxy maize is still an enigma [6–11].

There are abundant waxy maize landraces in China, most of which distribute in Southwestern China, particularly in Yunnan, Guizhou and Guangxi [5,7]. Yunnan is an original area of many important plant species with high genetic diversity. Several studies have suggested that Chinese waxy maize originated from Yunnan and Guangxi according to morphology, karyotype, isozymes and DNA markers [3,10,12–14]. There is a wide genetic diversity of waxy maize in agronomic traits such as plant height, maturity, economic characters, resistance to insects and diseases and yield components in those regions [5]. With simple sequence repeats (SSR) markers, comparative analysis of genetic diversity in landraces of waxy maize from Yunnan and Guizhou concluded that both Yunnan and Guizhou would be the center of diversity and origin for waxy maize [10].

According to plant morphology, geographical distribution and biological characters, combined with historical data and folklore, waxy maize was considered to be derived from one single gene mutant from flint corn [5]. However, in 1970, a landrace (termed Four-row Wax due to only four rows of seed set in the cob) was collected from Menghai County, Yunnan Province, which has been planted by the local Dai minority since 1890 and is a primitive cultivar with many characters similar to that of wild species [3,15]. The C-band pattern and karyotype of waxy maize were similar to popcorn, and the latter was considered as the oldest type of maize [16]. In the isozyme patterns of malic dehydrogenase, Chinese waxy maize has six bands as the same as that of Coix, suggesting that the origin of waxy maize might be related to Coix [6]. All these studies have made the origin and evolution of waxy maize more mysterious.

The glutinous phenotype in maize has been shown to be resulted from a dramatic reduction in synthesis of amylose because of mutations or insertions in the waxy gene, which locates on the short arm of chromosome 9 and encodes a granule-bound starch synthase in maize [11,17–18]. The DNA sequence of the wild type waxy locus was determined in 1980s in maize and is composed of 14 exons (Fig. 1) [18]. Recently, waxy sequences have been determined in a number of accessions from *Zea* lineage [4,19–22].

In maize, several genes such as *ae1*, *tb1* and *Y1* have been shown to be under strong selection. In rice, the origin of glutinous rice is

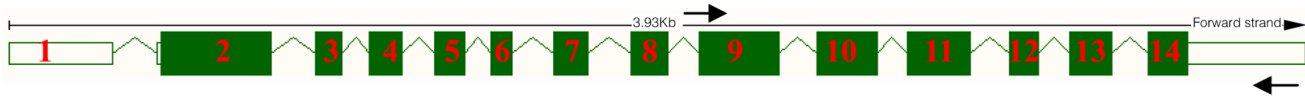


Figure 1. Gene structure of waxy gene in maize (http://www.maizesequence.org/Zea_mays/GRMZM2G024993_T01). Boxes represent exons. Lines between exons represent introns. The region between the two arrows is the fragment used in this study. doi:10.1371/journal.pone.0066606.g001

associated with reduced genetic variation [23], and a selective sweep of about 250 kb in the waxy genomic region was observed [24]. However, there were several contrary reports on selection of waxy gene in maize [11,20–22]. Further studies are needed to elucidate whether a same strong selection in waxy genomic region has happened under domestication in Chinese waxy maize.

With the development of molecular systematics, comparison of DNA sequence variation between closely related species has provided insight into the amount of divergence between sibling species, and the ancestral population size of sibling species [25]. Tian et al determined the systematical position of waxy maize in genus *Zea* and confirmed the relationship among waxy maize, normal maize and progenitor of maize [22].

In recent decades, waxy maize production has increased dramatically especially in the developed areas in Southeast China. It was difficult for breeders to utilize the waxy maize landraces because of their undesirable agronomic traits. Many adapted waxy maize lines have been developed for hybrid production through different selection methods [26]. The unclear relationship between these elite waxy maize lines and old landraces makes the understanding of origin and evolution of waxy maize more complicated.

In this study, we first used SSR markers to study the genetic diversity of Chinese waxy maize including landraces and inbred lines; we then sampled waxy sequences from 108 maize accessions including 89 waxy and 19 nonglutinous ones and compared with data of waxy sequences downloaded from GenBank to study the systematic position of Chinese waxy maize in genus *Zea*, and investigate the origin and dynamics of population evolution for Chinese waxy maize.

Materials and Methods

SSR Analyses

A set of 165 accessions of Chinese waxy maize including typical landraces and inbred lines were chosen at random and analyzed for the genetic diversity with SSR markers. DNA was extracted employing a modified CTAB procedure [27]. We used the set of 20 SSR markers downloaded from MaizeGDB (<http://www.maizegdb.org>), two from each chromosome (Table 1). PCR conditions and protocols were according to Bassam et al [28]. SSRs were multiplexed for a maximum efficiency. Fragments were separated using acrylamide gels run. The PIC for each marker was determined as described by Smith et al [29]. Allele identity was used for cluster analysis using NTSYS-pc Version 2.1 with UPGMA method to evaluate the genetic diversity [30].

Amylopectin Content Determination

Amylopectin content was determined according to the National Standards of the People's Republic of China, GB 7648–87 (China Standard Press 1987).

DNA Sequences for Waxy Gene

Eighty-nine diverse accessions of Chinese waxy maize, 16 accessions of flint maize and 3 accessions of sweet maize, including

landraces and inbred lines, were selected for amplification and determination of the sequences of the waxy genes (Table 2). The landraces were mainly collected from Yunnan, Guizhou and Guangxi, where Chinese waxy maize was believed to originate. The inbred lines were mainly collected from Shandong, Beijing, Shanghai and Zhejiang where waxy maize production has increased quickly in recent decades. Accessions were sequenced at waxy locus. Primers for two overlapping regions for exon 8–12 (P-F1: 5' GATTTTCATCGACGGGTCTGT -3' and P-R1: 5' TCTGTCCCTCTCGTCAGGAT -3') and exon 11–14 (P-F2: 5' ATCCTGACGAGAGGGACAGA -3' and P-R2: 5' CACC-GAACAGCAGGGATTAT -3') were designed based on conserved regions of the B73 genomic sequence (AF488416). All primers were designed using Primer3 [31]. PCR products were purified with glass milk kit (BioDev Company, China) and were sequenced on both strands using an Applied Biosystems 3730 sequencer with the forward and reverse primers. The alignments were done with DNASTAR (DNASTAR Inc., 2001) and checked manually.

Genetic diversity at waxy gene locus from a wide range of maize (*Zea mays* ssp. *mays*) and its wild relatives (*Z. mays* ssp. *mexicana*, hereafter *mexicana*, and *Z. mays* ssp. *parviglumis*, hereafter *parviglumis*) had been investigated in previous studies [20,32–35], and their sequences were downloaded from GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) as comparison in this study. The sampled accessions were classified into eight populations according to their endosperm texture, geographical origins or taxa (Table 2).

Sequence Variation Analysis for Waxy Gene

Sequence analysis was performed, in part, with the program DNASP version 5.10.01 [36,37]. The average pairwise nucleotide diversity π and Watterson's estimator of θ , an estimate of $4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per nucleotide, were calculated for genetic diversity [38,39]. The Tajima's D and Li & Fu's D^* and F^* were tested for deviation from the neutral equilibrium model of evolution using the tests of Tajima, and Fu and Li [40,41]. Recombination rates were estimated with the methods developed by Hudson et al and Hey et al [42,43].

Phylogenetic Reconstruction

Neighbour-joining (NJ) phylogenies based on the Kimura 2-parameter distance matrix were generated by MEGA version 4.0 to reconstruct the gene tree using waxy gene data [44]. One thousand bootstrap replicates were used to assess confidence in the phylogeny.

Results

Genetic Analyses of Chinese Waxy Maize

We chose 165 accessions of Chinese waxy maize including landraces and inbred lines and analyzed for the genetic diversity with SSR markers. A total of 104 alleles were found at the 20 SSR loci, with a range of 2 to 8 alleles per marker. The average alleles per marker across genotypes were 5.2, which were higher than

Table 1. Chromosome locations of SSR primers and primer sequences.

No.	Primer Name	Bin	Forward chain	Reverse chain
1	bnlg439	1.03	TTGACATCGCCATCTTGGTGACCA	TCTTAATGCGATCGTACGAAGTTGTGGAA
2	phi011	1.09	TGTTGCTCGGTCCACATACC	GCACACACACAGGACGACAGT
3	bnlg381	2.04	TCCCTCTTGAGTGTATACAAAA	GTTTCCATGGGCAGGTGTAT
4	umc1551	2.09	CACCGGAACACCTTCTTACAGTTT	CGAAACCTTCTCGTGATGAGC
5	umc2101	3.00	CCCGGCTAGAGCTATAAAGCAAGT	CTAGCTAGTTTGGTGCCTGGTGTAT
6	bnlg197	3.06	GCGAGAAGAAAGCGAGCAGA	CGCCAAGAAGAAACACATCACA
7	phi072	4.01	ACCGTGATGATTAATTTCTCCAGCCTT	GACAGCGCGCAATGGATTGAACT
8	bnlg2162	4.08	GTCTGCTGCTAGTGGTGGTG	CACCGGCATTCGATATCTTT
9	umc1705	5.03	ATGCGTCTTTCACAAAGCATTACA	AGGTGCAGTTCATAGACTTCTGGG
10	umc1153	5.09	CAGCATCTATAGCTTGTGCTGATT	TGGGTTTTGTTGTTGTTGTTG
11	phi126	6.00	TCCTGCTTATTGCTTTCGTCAT	GAGCTTGCATATTTCTTGAGACA
12	mmc0241	6.05	TATATCCGTGCATTACGTTT	CATCGCTTGTCTGTCGA
13	phi112	7.01	TGCCCTGCAGGTCACATTGAGT	AGGAGTACGCTTGGATGCTCTTC
14	phi328175	7.04	GGGAAGTGCTCCTTGCCAG	CGGTAGGTGAACGCGGTA
15	bnlg2181	8.00	CCAATTCACCAATCATGCAA	TTGGGGTGAAGCAATGTGTA
16	phi080	8.08	CACCCGATGCAACTTGCCTAGA	TCGTACGTTCCACGACATCAC
17	bnlg244	9.02	GATGCTACTACTGGTCTAGTCCAGA	CTCCTCCACTCATCAGCCTTGA
18	umc1277	9.08	TTTGAGAACGGAAGCAAGTACTCC	ACCAACCAACCACTCCCTTTTATG
19	umc1380	10.00	CTGCTGATGTCTGGAAGAACCTT	AGCATCATGCCAGCAGGTTTT
20	umc1196	10.07	CGTGCTACTACTGCTACAAGCGCA	AGTCGTCTGTCTTCCGAAACT

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that (3.7) reported by Wu et al using 16 waxy maize landraces and 61 SSR markers [9]. The PIC values for the 20 SSR loci ranged from 0.41 to 0.84, with an average of 0.70. This is consistent with the results of Xue et al., who reported an average PIC value of 0.64 for 184 maize inbred lines including 111 common and 73 waxy inbreds [45]. The average PIC value in combination with the high number of alleles indicates presence of a wide genetic diversity in the Chinese waxy maize germplasm used in this study.

Cluster analysis of 165 waxy maize lines was conducted based on genetic similarities from SSR data with UPGMA method. UPGMA analysis grouped the 165 lines into groups A, B and C (Fig. 2), which was generally consistent with their known pedigree information and breeder's experience. The landraces collected from Southwest China such as Yunnan, Guizhou and Guangxi, and some of inbred lines collected from Northern China formed one group (group A), of which the Southwest Chinese waxy maize land races formed a subgroup. Most of the inbred lines collected from Yangtse River delta including Shanghai and Zhejiang formed one group (group B). Most of the inbred lines collected from North and East China including Shandong, Henan and Beijing formed the group C. The results indicated that most of the modern waxy inbred lines seemed to have little relationship with the Southwest Chinese waxy maize landraces, which were regarded as the origin of Chinese waxy maize [3,12–14]. It would be very helpful to choose the most genetically distant lines for waxy maize genetic study and hybridization breeding based on grouping of waxy maize germplasm.

DNA Sequence Variation and Test for Deviation from Neutrality

We examined DNA sequence variation in about 1,750 bp region of waxy gene in 89 Chinese waxy, 16 flint and 3 sweet maize accessions, of which 72 nucleotides were variable (Table 3).

High amylopectin content (>95%) was observed in the 89 Chinese waxy accessions (Table S1). Waxy sequence data for 52 accessions from maize (*Zea mays* ssp. *mays*) and its wild relatives (*mexicana* and *parviglumis*) were downloaded from GenBank and used as comparison in this study [20,32–35]. Different accessions within each taxon or population showed an apparent difference in genetic variation at waxy locus (Table 3). Average pairwise nucleotide diversity, π , in the nonglutinous maize was more than 3-fold higher than that in the waxy accessions. The waxy maize from East China, mainly being modern waxy maize inbred lines, contained the minimum level of variation among all sampled taxa or populations. The estimate of π for waxy maize from East China was 66.0%, 12.7%, 19.9%, 13.1%, 6.1% and 7.0% of that for waxy maize accessions from Southwest China, flint maize from China, sweet maize from China, flint maize from America, *parviglumis* and *mexicana*, respectively. Similarly, θ and k were lower in the waxy maize compared to others samples. The reduction in genetic diversity at waxy locus in waxy maize suggested that Chinese waxy maize experienced a genetic bottleneck during its improvement, especially in modern waxy maize breeding.

The Tajima's D and Li & Fu's D^* and F^* were tested for deviation from the neutral equilibrium model of evolution based on Tajima, and Fu and Li [40,41]. Estimates of D , D^* and F^* were different within each taxon or population (Table 3). All three tests identified a significantly negative selection on the waxy gene in both waxy subpopulations, but not in any nonglutinous subpopulation or taxon, which suggests that strong selection in improvement has acted on the locus in the Chinese waxy population. The negative selection in East Chinese waxy maize accessions, which mainly consist of modern waxy maize inbred lines, was stronger than that in Southwest Chinese waxy maize accessions, which are landraces. The significant neutral deviation

Table 2. Summary of accessions sampled for *Waxy* gene analysis.

Population/taxon	Sample name	Type	Origin	Sequence or GenBank no.	Seed source or Reference
Waxy maize from Southwest China	Banqiaohuangnuo	LR	Guizhou, China	CWM050	CAAS
	Chaoyangbainuo	LR	Guizhou, China	CWM052	CAAS
	Diannuobaogu	LR	Yunnan, China	CWM056	CAAS
	Bainuoyumi	LR	Yunnan, China	CWM057	CAAS
	Huangnuobaogu	LR	Yunnan, China	CWM069	CAAS
	Bendinuobaogu	LR	Yunnan, China	CWM074	CAAS
	Nuobaogu	LR	Shanxi, China	CWM080	CAAS
	Liuchengnuo	LR	Guangxi, China	DQ369863	Tian,M.L. et. al 2006
	Dalinuoyumi	LR	Guangxi, China	DQ369864	Tian,M.L. et. al 2006
	Laonuo	LR	Guangxi, China	DQ369865	Tian,M.L. et. al 2006
	Wunonglaonuo	LR	Guangxi, China	DQ369866	Tian,M.L. et. al 2006
	Zaibainuo	LR	Yunnan, China	DQ369886	Tian,M.L. et. al 2006
	Piantounuo	LR	Yunnan, China	DQ369887	Tian,M.L. et. al 2006
	Huanuoyumi	LR	Yunnan, China	DQ369888	Tian,M.L. et. al 2006
	Landihuanuo	LR	Yunnan, China	DQ369891	Tian,M.L. et. al 2006
	Changchongbainuo	LR	Guizhou, China	DQ369895	Tian,M.L. et. al 2006
	Huangyounuo	LR	Guizhou, China	DQ369897	Tian,M.L. et. al 2006
	Jinhuangnuo	LR	Guizhou, China	DQ369899	Tian,M.L. et. al 2006
	Xiaobainuo	LR	Guizhou, China	DQ369901	Tian,M.L. et. al 2006
	Waxy maize from East China	Baigengyumi	LR	Shanghai, China	SWM011
Huangnuoyumi		LR	Shanghai, China	SWM012	SAGC
Gengbaidayumi		LR	Shanghai, China	SWM017	SAGC
SWL089		IL	Shandong, China	SWL089	SAAS
SWL094		IL	Shandong, China	SWL094	SAAS
SWL097		IL	Shandong, China	SWL097	SAAS
SWL102		IL	Shandong, China	SWL102	SAAS
SWL105		IL	Shandong, China	SWL105	SAAS
SWL107		IL	Shandong, China	SWL107	SAAS
SWL108		IL	Shandong, China	SWL108	SAAS
SWL124		IL	Shanghai,China	SWL124	SAAS
SWL125		IL	Shanghai,China	SWL125	SAAS
SWL127		IL	Shanghai,China	SWL127	SAAS
SWL129		IL	Shanghai,China	SWL129	SAAS
SWL130		IL	Shandong,China	SWL130	SAAS
SWL131		IL	Shandong,China	SWL131	SAAS
SWL132		IL	Guangdong, China	SWL132	SAAS
SWL135		IL	Shandong, China	SWL135	SAAS
SWL138		IL	Shandong, China	SWL138	SAAS
SWL155		IL	Shanghai, China	SWL155	SAAS
SWL161	IL	Shanghai, China	SWL161	SAAS	
SWL162	IL	Shandong, China	SWL162	SAAS	
SWL165	IL	Beijing, China	SWL165	SAAS	
SWL166	IL	Jiangsu, China	SWL166	SAAS	
SWL169	IL	Guizhou, China	SWL169	SAAS	
SWL170	IL	Guizhou, China	SWL170	SAAS	
SWL171	IL	Guizhou, China	SWL171	SAAS	
Waxy maize from East China	SWL172	IL	Shanghai, China	SWL172	SAAS
	SWL174	IL	Shanghai, China	SWL174	SAAS
	SWL175	IL	Shanghai, China	SWL175	SAAS

Table 2. Cont.

Population/taxon	Sample name	Type	Origin	Sequence or GenBank no.	Seed source or Reference
	SWL176	IL	Shandong, China	SWL176	SAAS
	SWL177	IL	Shandong, China	SWL177	SAAS
	SWL178	IL	Shandong, China	SWL178	SAAS
	SWL179	IL	Shandong, China	SWL179	SAAS
	SWL180	IL	Shandong, China	SWL180	SAAS
	SWL182	IL	Hubei, China	SWL182	SAAS
	SWL183	IL	Shandong, China	SWL183	SAAS
	SWL190	IL	Shanghai, China	SWL190	SAAS
	SWL191	IL	Shanghai, China	SWL191	SAAS
	SWL193	IL	Shanghai, China	SWL193	SAAS
	SWL194	IL	Shanghai, China	SWL194	SAAS
	SWL196	IL	Shanghai, China	SWL196	SAAS
	SWL197	IL	Shandong, China	SWL197	SAAS
	SWL198	IL	Shanghai, China	SWL198	SAAS
	SWL200	IL	Shanghai, China	SWL200	SAAS
	SWL201	IL	Shanghai, China	SWL201	SAAS
	SWL202	IL	Shanghai, China	SWL202	SAAS
	SWL231	IL	Shanghai, China	SWL231	SAAS
	SWL258	IL	Shanghai, China	SWL258	SAAS
	SWL269	IL	Shanghai, China	SWL269	SAAS
	SWL271	IL	Shanghai, China	SWL271	SAAS
	SWL273	IL	Shanghai, China	SWL273	SAAS
	SWL276	IL	Beijing, China	SWL276	SAAS
	SWL277	IL	Beijing, China	SWL277	SAAS
	SWL278	IL	Jiangsu, China	SWL278	SAAS
	SWL283	IL	Shanghai, China	SWL283	SAAS
	SWL284	IL	Shanghai, China	SWL284	SAAS
	SWL285	IL	Shanghai, China	SWL285	SAAS
	SWL286	IL	Shanghai, China	SWL286	SAAS
	SWL287	IL	Shanghai, China	SWL287	SAAS
	SWL290	IL	Shanghai, China	SWL290	SAAS
	SWL294	IL	Shanghai, China	SWL294	SAAS
	SWL310	IL	Shanghai, China	SWL310	SAAS
	SWL312	IL	Shanghai, China	SWL312	SAAS
	SWL324	IL	Shanghai, China	SWL324	SAAS
	SWL326	IL	Shanghai, China	SWL326	SAAS
	SWL328	IL	Shanghai, China	SWL328	SAAS
	SWL329	IL	Shanghai, China	SWL329	SAAS
	SWL333	IL	Shanghai, China	SWL333	SAAS
	SWL335	IL	Shanghai, China	SWL335	SAAS
	SWL337	IL	Shanghai, China	SWL337	SAAS
	SWL339	IL	Shanghai, China	SWL339	SAAS
	SWL343	IL	Shanghai, China	SWL343	SAAS
	SWL345	IL	Shanghai, China	SWL345	SAAS
	SWL348	IL	Shanghai, China	SWL348	SAAS
Waxy maize from East China	SWL359	IL	Jiangsu, China	SWL359	SAAS
	SWL362	IL	Shanghai, China	SWL362	SAAS
	SWL370	IL	Shanghai, China	SWL370	SAAS
	SWL371	IL	Shanghai, China	SWL371	SAAS

Table 2. Cont.

Population/taxon	Sample name	Type	Origin	Sequence or GenBank no.	Seed source or Reference
	SWL378	IL	Shanghai, China	SWL378	SAAS
	SWL385	IL	Shanghai, China	SWL385	SAAS
	SWL386	IL	Shanghai, China	SWL386	SAAS
Waxy maize from America	915B	IL	Argentina	EU747862	Zhao,Y. et al 2008
	923A	IL	Argentina	EU747863	Zhao,Y. et al 2008
Flint maize from China	Laorenya	LR	Shanghai, China	SMV002	SAGC
	Ziyuya	LR	Shanghai, China	SMV003	SAGC
	Jinmeihuang	LR	Shanghai, China	SMV004	SAGC
	Matuan	LR	Shanghai, China	SMV005	SAGC
	Zaobai	LR	Shanghai, China	SMV006	SAGC
	Xiaojinhuang	LR	Shanghai, China	SMV015	SAGC
	Huangnuo	LR	Guizhou, China	CMV054	CAAS
	Bainuoyumai	LR	Yunnan, China	CMV065	CAAS
	Heinuobaogu	LR	Yunnan, China	CMV075	CAAS
	SML090	IL	Shandong, China	SML090	SAAS
	SML136	IL	Shandong, China	SML136	SAAS
	SML137	IL	Shandong, China	SML137	SAAS
	SML173	IL	Jiangsu, China	SML173	SAAS
	SML280	IL	Shanghai, China	SML280	SAAS
	SML306	IL	Shanghai, China	SML306	SAAS
	SML383	IL	Shangdong, China	SML383	SAAS
Sweet maize from China	SHL391	IL	Zhejiang, China	SHL391	SAAS
	SHL403	IL	Zhejiang, China	SHL403	SAAS
	SHL408	IL	Zhejiang, China	SHL408	SAAS
Flint maze from America	EP1	IL	America	AF544080	Whitt,S.R., et al. 2002
	Mo17	IL	America	AF544090	Whitt,S.R., et al. 2002
	M162W	IL	America	AF544089	Whitt,S.R., et al. 2002
	Oh43	IL	America	AF544094	Whitt,S.R., et al. 2002
	Ki21	IL	America	AF544087	Whitt,S.R., et al. 2002
	P39	IL	America	AF544095	Whitt,S.R., et al. 2002
	IL101	IL	America	AF544085	Whitt,S.R., et al. 2002
	A6	IL	America	AF544069	Whitt,S.R., et al. 2002
	B37	IL	America	AF544072	Whitt,S.R., et al. 2002
	Pa91	IL	America	AF544096	Whitt,S.R., et al. 2002
	Tx601	IL	America	AF544098	Whitt,S.R., et al. 2002
	NC348	IL	America	AF544093	Whitt,S.R., et al. 2002
	CML254	IL	America	AF544076	Whitt,S.R., et al. 2002
	T232	IL	America	AF544097	Whitt,S.R., et al. 2002
	Ky21	IL	America	AF544088	Whitt,S.R., et al. 2002
	B97	IL	America	AF544074	Whitt,S.R., et al. 2002
	CI187	IL	America	AF544075	Whitt,S.R., et al. 2002
	CML333	IL	America	AF544078	Whitt,S.R., et al. 2002
	W153R	IL	America	AF544099	Whitt,S.R., et al. 2002
	I205	IL	America	AF544083	Whitt,S.R., et al. 2002
	B14A	IL	America	AF544071	Whitt,S.R., et al. 2002
	N28Ht	IL	America	AF544091	Whitt,S.R., et al. 2002
	NC260	IL	America	AF544092	Whitt,S.R., et al. 2002
	IDS28	IL	America	AF544084	Whitt,S.R., et al. 2002
	a2132	IL	America	AF544082	Whitt,S.R., et al. 2002

Table 2. Cont.

Population/taxon	Sample name	Type	Origin	Sequence or GenBank no.	Seed source or Reference
<i>Zea mays</i> ssp.	PI566683	LR	America	mAF292525	Gaut,B.S. et al 2000
<i>Mexicana</i>	PI566691	LR	America	mAF292530	Gaut,B.S. et al 2000
	PI566685	LR	America	mAF292524	Gaut,B.S. et al 2000
	PI566673	LR	America	mAF292528	Gaut,B.S. et al 2000
	PI566682	LR	America	mAF292526	Gaut,B.S. et al 2000
	-	LR	America	mAF079260	Mason-Gamer, 1998
<i>Zea mays</i> ssp.	PI331786	LR	America	pAF292518	Gaut,B.S. et al 2000
<i>Parviglumis</i>	M106	LR	America	pAF292523	Gaut,B.S. et al 2000
	PI331785	LR	America	pAF292517	Gaut,B.S. et al 2000
	PI384061	LR	America	pAF292520	Gaut,B.S. et al 2000
	PI331783	LR	America	pAF292516	Gaut,B.S. et al 2000
	PI384062	LR	America	pAF292521	Gaut,B.S. et al 2000
	PI331787	LR	America	pAF292519	Gaut,B.S. et al 2000

LR: Landrace; IL: Inbred line; CAAS: Chinese Academy of Agricultural Sciences; SAGC: Shanghai Agrobiological Gene Center; SAAS: Shanghai Academy of Agricultural Sciences.

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at this locus in the waxy population compared to the nonglutinous population is consistent with the sharp reduction in polymorphism.

We estimated the minimum number of recombination events (R_m) within the samples of waxy maize sequences (Table 3). The recombination events were 0, 0, 6, 5, 8 and 2 for the waxy maize from Southwest China, waxy maize from East China, Chinese flint maize, American flint, *parviglumis* and *mexicana* accessions. High-frequency recombination appears to somewhat increase the sequence diversity at waxy locus.

Phylogenetics for Chinese Waxy Maize Based on Sequence Polymorphisms

Based on the waxy sequences, we constructed a phylogenetic tree including Chinese waxy and other nonglutinous maize accessions by neighbor joint method (Fig. 3), which is helpful to elucidate their origins. The tree indicated that Chinese waxy maize were grouped two distinct independent branches, one mainly containing Southwest Chinese waxy maize and the other mainly East Chinese waxy maize. It suggests that the Chinese

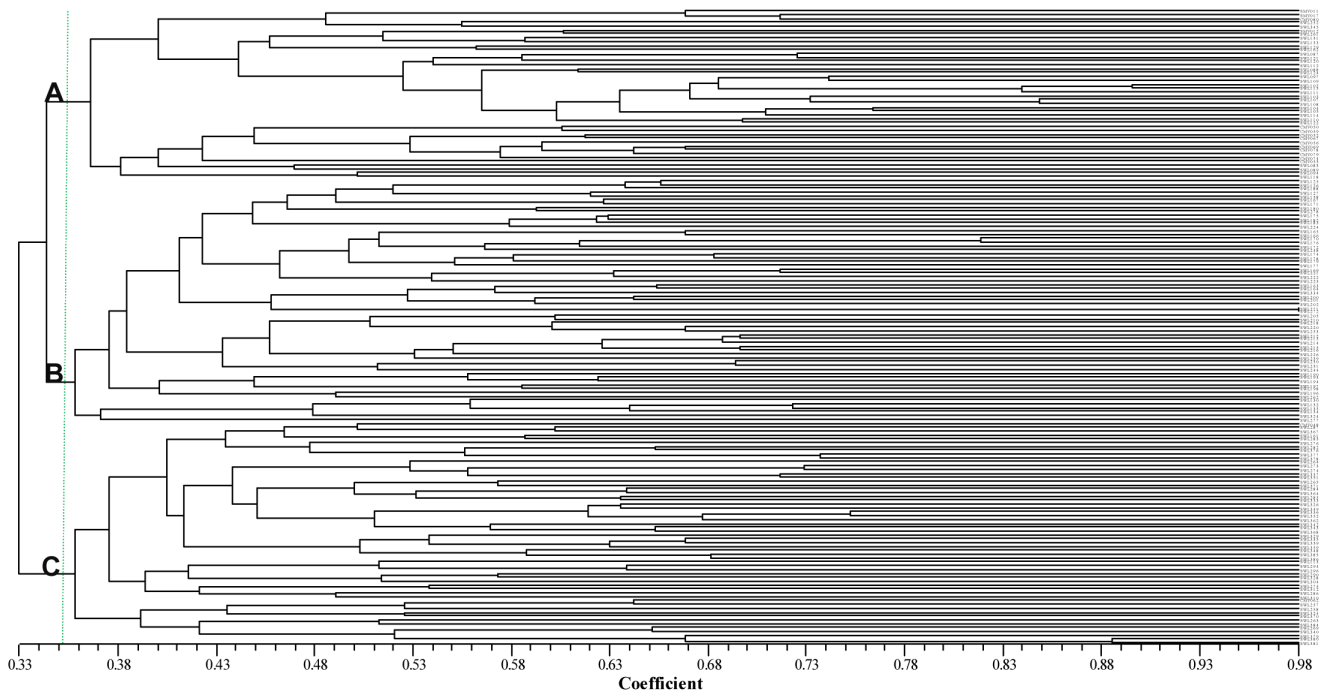


Figure 2. Dendrogram for 165 maize accessions based on a cluster analysis of genetic similarities from SSR data.

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Table 3. Summary of nucleotide diversity within maize taxon or population.

Taxon or Population	N^b	Site ^b	S^b	H^b	π^b	θ^b	K^b	D	D^*	F^*	Rm
Waxy maize from Southwest China ^a	19	1775	12	6	0.00103	0.00260	1.357	-2.20087**	-2.97230*	-3.18559*	0
Waxy maize from East China	82	1791	26	8	0.00068	0.00204	1.170	-2.38092**	-4.10102*	-4.12647*	0
Flint maize from China	16	1793	27	11	0.00535	0.00472	9.225	0.54969	0.86751	0.86751	6
Sweet maize from China	3	1763	9	4	0.00341	0.00341	6.000	-	-	-	-
Flint maize from America ^a	25	1788	23	15	0.00521	0.00634	5.481	-0.68060	-0.92668	-0.99414	5
<i>parviglumis</i> ^a	7	1388	37	7	0.01106	0.01139	14.667	-0.16551	-0.13036	-0.15287	8
<i>mexicana</i> ^a	6	1373	29	6	0.00974	0.01067	12.400	-0.54951	-0.49755	-0.55619	2

^aSome sequence data were from Mason et al (1998), Gaut et al (2000); Whitt et al. (2002), Tian et al. (2006), and Zhao et al. (2008).

^b n , number of samples; S : number of polymorphic (segregating) sites; H , haploid; Hd : haplotype (gene) diversity; π , The average pairwise nucleotide diversity (Tajima 1983); θ , an estimate of $4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per nucleotide (Watterson 1975); K , average number of nucleotide differences; D : Tajima's D (1989); D^* :Fu and Li's D (1993); F^* : Fu and Li's F (1993); Rm : Minimum number of recombination events.

** $P < 0.01$; * $P < 0.05$.

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waxy maize should have two independent origins and evolution processes.

One subset of waxy maize sequences forms one distinct branch except one Chinese flint maize sequence SML137. This group contains most of the waxy maize inbred lines from East China and three landraces Baigengyumi, Huangnuoyumi and Gengbaidayumi. The remaining waxy maize accessions, which contained all other Southwest Chinese waxy landraces and some East Chinese waxy accessions, were mixed with several Chinese flint and America flint accessions. Two American waxy maize accessions (915B and 923A) formed a distinct group mixed with some American flint maize, which was different from Chinese waxy maize. The intermixing is reasonable and essential if waxy maize might be domesticated from flint maize.

Three wild maize accessions (*parviglumis* AF292516, and *mexicana* AF292525 and AF079260) formed a distinct branch which is basal to the flint and waxy maize groups. On the other hand, two different branches were formed with flint maize, which were basal to the two different Chinese waxy maize groups. The results suggest that flint maize maybe be the ancestor of waxy maize, and wild maize relatives *parviglumis* or *mexicana* maybe be the ancestor of flint maize. This suggestion was consistent with the low nucleotide diversity in waxy maize compared to flint maize, *parviglumis* and *mexicana*.

Discussion

Due to the special dietary habit for glutinous food in China, selection in maize has been made for waxy phenotype with high amylopectin since the maize was introduced into China from the new world about 400 years ago [3]. Although there are abundant waxy maize landraces in south China such as Yunnan, Guizhou and Guangxi, where were regarded as the origin of Chinese waxy maize [3,12–14], they have seldom been used directly or indirectly in modern waxy maize breeding in recent decades [25]. Our study was a try to elucidate the relationship between modern waxy inbred lines and waxy maize landraces.

The waxy mutants give rise to the waxy maize phenotype because the mutants hinder amylase synthesis leading to accumulate nearly 100% amylopectin in starch [25]. In our collection, high amylopectin content (>95%) was observed in the 89 Chinese waxy accessions sequenced by ourselves (Table S1). We tried to include the main typical landraces and inbred lines currently used for commercial production in China to reveal the genetic diversity of Chinese waxy maize in our study.

Genetic Diversity of Chinese Waxy Maize

Our SSR analysis have shown a wide genetic diversity in Chinese waxy maize accessions including landraces and inbred lines. It is also very interesting to find that the landraces collected from Southwest China formed a subgroup, indicating that most of the modern waxy inbred lines seemed to have little relationship with the Southwest Chinese landraces. A further study on DNA sequence variation at waxy locus for Chinese waxy maize and its wild relatives provided insight to the relationship between modern waxy inbred lines and waxy maize landraces, and information on the origin and dynamics of population evolution for Chinese waxy maize in genus *Zea*.

It is commonly thought that crops are bereft of genetic variation compared to their wild relatives [46]. In our study, the Chinese waxy maize only contained 16.0%, 16.3%, 7.7% and 8.8% of sequence diversity in flint maize accessions from China, flint maize from America, *parviglumis*, and *mexicana*, respectively (Table 3). The decrease of genetic diversity at waxy locus was caused by only one possible reason that waxy gene experienced a genetic bottleneck and strong selection during its improvement, especially in modern waxy maize breeding, for waxy maize from Eastern China contained 66.0% of the sequence diversity in waxy maize from Southwest.

Our test for deviation from neutrality revealed a significantly negative selection on the waxy gene in waxy maize, but not in normal maize and their wild relatives. On the other hand, the negative selection in Eastern Chinese waxy maize was stronger than that in Southwest Chinese waxy maize. The neutral test result is consistent with the sharp reduction in polymorphism at this locus in waxy maize compared to the normal maize and their wild relatives. The results suggest that strong selection in improvement has acted on the locus in the Chinese waxy maize.

Origin and Evolution of Chinese Waxy Maize

Resolving the issues related to the origin and evolution for a domesticated crop is a fascinating and challenging endeavor that requires the integration of botanical, archeological and genetic evidence [47–49]. Our data have provided us an explicit relationship among waxy maize, flint maize and the wild relatives for two reasons. First, in the phylogenetic tree, the sequences of Chinese waxy maize formed two groups which are mixed with several Chinese flint maize sequences, and two distinct branches formed with flint maize were basal to the two Chinese waxy maize groups. It showed that waxy maize undergoes the most recent

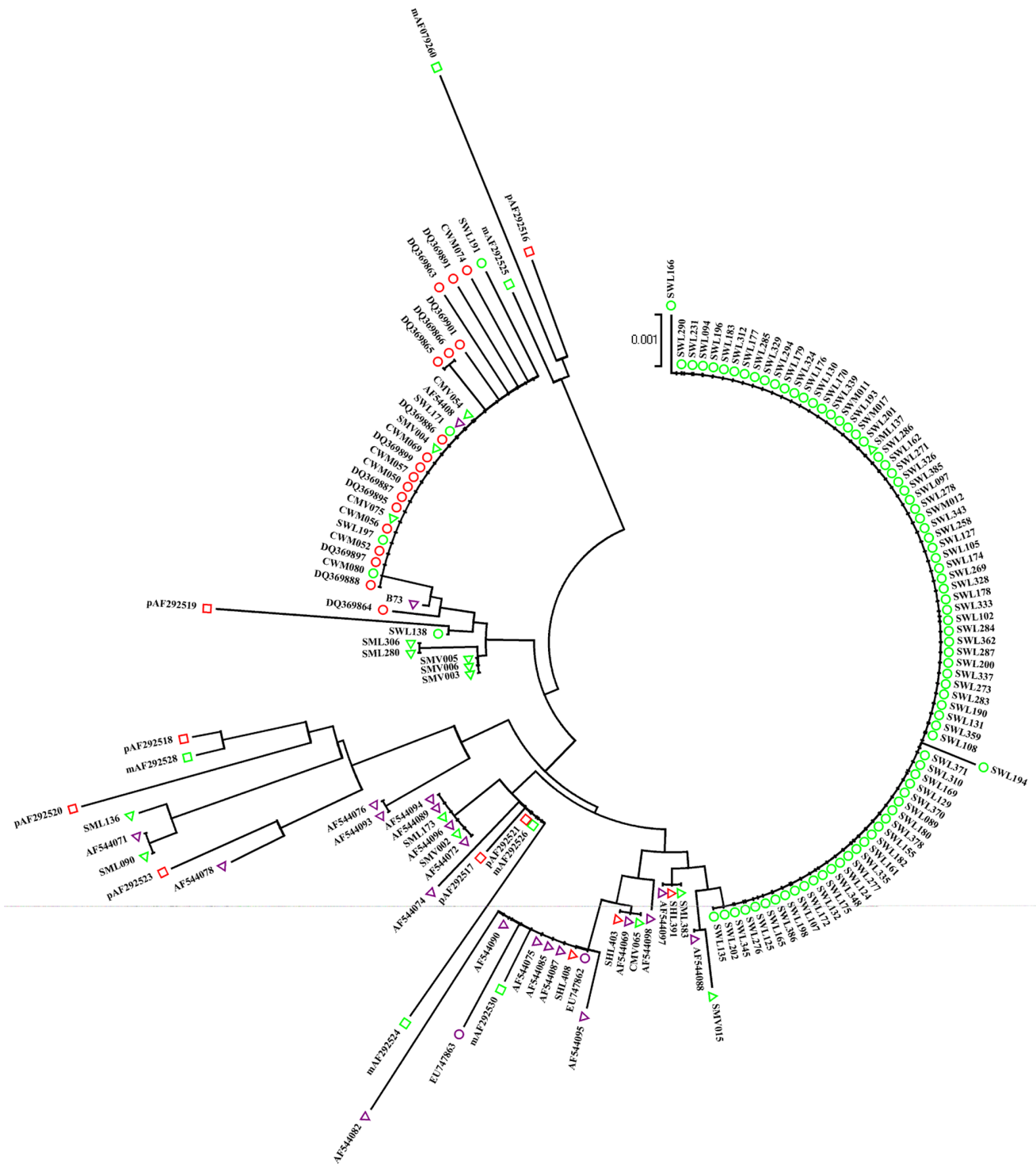


Figure 3. Phylogenetic tree of the Chinese waxy maize accessions based on waxy gene. The waxy maize from Southwest China, East China and America are shown with red, green and purple circles, respectively. The sweet maize, flint maize from China and flint maize from America are shown with red, green and purple triangles, respectively. Wild maize *Zea mays* spp. *parviglumis* and *Zea mays* spp. *mexicana* are shown with red and green squares, respectively.
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divergence event from flint maize. Second, three wild maize accessions (*parviglumis* AF292516, and *mexicana* AF292525 and AF079260) formed a distinct branch which is basal to the flint and waxy maize groups. The results suggest that wild maize relatives *parviglumis* or *mexicana* maybe be the ancestor of flint maize.

It seems difficult to explain the observation that the intermixture of two subspecies (*parviglumis* and *mexicana*) formed a basal branch in the phylogenetic tree, given that these two subspecies do not grow sympatrically [49,50–52]. There are two possible explanations: (1) there is a long distance dispersal from *parviglumis* to

mexicana populations; (2) the gene pools of the two subspecies were diverged too late to be fully differentiated. The first explanation seems unlikely but cannot be excluded. The second explanation seems more likely, given that the gene pools of *mexicana*, *parviglumis* and maize differ more in allele frequencies than by allele presence/absence. The result is consistent with the observation of Fukunaga et al [51]. Of course, this viewpoint needs to be supported by more molecular evidences.

Tian et al concluded that Southwest China origin for waxy maize is consistent with local cultural practices [5]. In the past, only in Southwest China does waxy maize attain the importance of a staple crop. Ethnographic studies suggest that waxy maize cultivation is associated with upland agriculture in Southwest China. Indeed, Yunnan, Guizhou and Guangxi in Southwest China are referred to as “the genetic diversity centre” for waxy maize, reflecting the importance of waxy maize to the economy and culture of the area. In recent decades, waxy maize production has increased dramatically especially in the developed areas of Southeast China. Many waxy maize germplasm and new inbred lines have been developed for hybrid production through genetic improvement [26]. Our study suggests that most of the modern waxy maize germplasm showed a distinct, independent origin and evolution compared with the germplasm in Southwest China. The

results indicate that an agronomic trait can be quickly improved to meet production demand by artificial selection. It is reasonable to speculate that production demand plays a critical role in crop genetic improvement and can speed the artificial selection and evolution at a target gene locus.

Supporting Information

Table S1 Waxy gene structure of maize accessions sequenced by Shanghai Academy of Agricultural Sciences.

(DOC)

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Author Contributions

Conceived and designed the experiments: HZ LL AW. Performed the experiments: HW HY JW. Analyzed the data: HZ HW BS. Contributed reagents/materials/analysis tools: RC YX. Wrote the paper: HZ YX.

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