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 non-glutinous 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.

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 Guizhou 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 amylase 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 αel, βel and Y1 have been shown to be under strong selection. In rice, the origin of glutinous rice is
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 strong selection in the 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 systematic 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 MaizeDB (http://www.maizesequence.org/Zea_mays/GRMZM2G024993_T01), two from each chromosome (Table 1). PCR conditions and protocols were according to Bassam et al [20]. SSRs were multiplexed for a maximum efficiency. Fragments were separated using acrylamide gels run. The PIC for each marker was estimated with the methods developed by Hudson et al and Hey, Tajima, and Fu and Li [40,41]. Recombination rates were estimated with the methods developed by Hudson et al [42] and Hey et al [43].

**Amylopectin Content Determination**

Amylopectin content was determined according to the National Standards of the People’s Republic of China, GB 7649–87 (China Standard Press 1907).

**DNA Sequences for Waxy Gene**

Eighty-nine diverse accessions of Chinese waxy maize, 16 accessions of flint maize and 5 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’-GATTTCATCGACGGGTCTGT-3’ and P-R1: 5’-TCTGTGCCCTCTCGGTACAG-3’) and exon 11–14 (P-F2: 5’-ATCTGTACGAGGGACAGAGAC-3’ and P-R2: 5’-CACC-GAACAGGAGGATTTAT-3’) were designed based on conserved regions of the B73 genomic sequence (AF483416). 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.

The inbred lines were mainly collected from Shandong, Beijing, Guangxi, where Chinese waxy maize was believed to originate. 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’-GATTTCATCGACGGGTCTGT-3’ and P-R1: 5’-TCTGTGCCCTCTCGGTACAG-3’) and exon 11–14 (P-F2: 5’-ATCTGTACGAGGGACAGAGAC-3’ and P-R2: 5’-CACC-GAACAGGAGGATTTAT-3’) were designed based on conserved regions of the B73 genomic sequence (AF483416). 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.

**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 test 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 3.2, which were higher than
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 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, formed a subgroup. 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).

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

| No. | Primer Name | Bin | Forward chain | Reverse chain |
|-----|-------------|-----|---------------|---------------|
| 1   | bnlg439     | 1.03| TTGACATGCCCATTTGCTGACCA | TCCTATAGCCATGTACAGAATGTTGAGAA |
| 2   | phi011      | 1.09| TGTGTGCTGCTTACAACTTCCA | GCACACACACACAGACAGACAG |
| 3   | bnlg381     | 2.04| TCCCTCTTGAGTTTATCAAAAA | GTTTCCATGGGGCAGGGTGAT |
| 4   | umc1551     | 2.09| CACGGACACACACACATCAAGTGT | GGAAACCTCGGCTGAGAT |
| 5   | umc2101     | 3.00| CCCGGCTGACAGTATAAGCAAGT | CTAGTAGTTTGGCCGTTGGAG |
| 6   | bnlg197     | 3.06| CGGAGAAGAAGACAGCACAG | CGGCAAGAAACACACATCAAC |
| 7   | phi072      | 4.01| ACCGTGCTATGATTTTCTCCAGCTT | GAGCCGCGGCAAATAGTTGAGACT |
| 8   | bnlg2162    | 4.08| GTCTGCTGCTGATGTTGGGAGTG | CACGGGCTACATGATCTT |
| 9   | umc1705     | 5.03| ATGCGTCTTTCCAAACAGCTTCAA | AGGTCGTTCATAGACTCTCTGG |
| 10  | umc1153     | 5.09| CACGGCTACATAGCTTCATGAT | TGGGTGGGTGTTGGTTGGTTG |
| 11  | phi126      | 6.00| TCTGCTTATGTTTCTGGTCT | GAGGCATTATATCCTGTTGGAAC |
| 12  | mmc0241     | 6.05| TATATCCGCTGCATTTACGCCG | CATCGGTGTGTGCAG |
| 13  | phi112      | 7.01| TGGCTGGAGGTCCTCAATGTAGT | AGGAGTACCTTGATGTCCTT |
| 14  | phi328175   | 7.04| GGGAAATGTCCTTGCGCAG | CCACTGATAATCGTGCAA |
| 15  | bnlg2181    | 8.00| CCAATTCAACACATGCAA | TTGGGTGGAACAGATATGTA |
| 16  | phi080      | 8.08| CACGGCGGTACATGCCTGTAGA | TCCTCGGATCGACAGCCACATC |
| 17  | bnlg244     | 9.02| GATGCTACTCTGCTATGGCTAGCAGA | CTCTCCACACTCATAGCTT |
| 18  | umc1277     | 9.08| TTGGAGAAGCGGAAAGAAGTACTCC | ACCAAACACACACTCTCTGTTT |
| 19  | umc1380     | 10.00| CTGCTGCTGCTGCTGCTGAAACCCT | AGTATGCTGAGACAGGGTTT |
| 20  | umc1196     | 10.07| CTGCTGCTGCTGCTGCTGAAACCCT | AGTATGCTGAGACAGGGTTT |

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  |
|                   | Dalnuoyumi      | 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  |
|                   | Plantoungo      | 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  |
|                   | Huangyuonuo     | LR  | Guizhou, China  | DQ369897                | Tian, M.L. et. al 2006  |
|                   | Jinhuangnuo     | LR  | Guizhou, China  | DQ369899                | Tian, M.L. et. al 2006  |
|                   | Xiadainuo       | LR  | Guizhou, China  | DQ369901                | Tian, M.L. et. al 2006  |
| Waxy maize from East China | Baigengyumi   | LR  | Shanghai, China | SWM011                  | SAGC                     |
|                   | 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                     |
| 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                    |
| 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   | Laorenayo   | LR    | Shanghai, China | SMV002                 | SAGC                     |
|                          | Ziyuaya     | 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                     |
|                          | Heimuobaogu | 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    | Shandong, 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 maize 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 |
|                          | Ohv43       | IL    | America      | AF544094                | Whitt, S.R., et al. 2002 |
|                          | K21         | 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 |
|                          | C1187       | 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 |
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

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**Table 2.** Cont.

| Population/taxon     | Sample name | Type | Origin   | Sequence or GenBank no. | Seed source or Reference |
|----------------------|-------------|------|----------|-------------------------|--------------------------|
| *Zea mays* ssp.      | PI566683    | LR   | America  | mAF292525               | Gaut, B.S. et al 2000    |
| *Mexicana*           | 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        |
| *Zea mays* ssp.      | PI331786    | LR   | America  | pAF292518               | Gaut, B.S. et al 2000    |
| *Parviglumis*        | 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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Figure 2. Dendrogram for 165 maize accessions based on a cluster analysis of genetic similarities from SSR data.

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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 Gengbai-dayumi. 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 (91SB 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 AF929518, and mexicana AF929525 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, for waxy maize from Eastern China only contained 16.0%, 16.3%, 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.

**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.

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**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
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

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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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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