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Molecular evidence for post-domestication selection in the *Waxy* gene of Chinese waxy maize

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Abstract Waxy maize was first reported in China in 1909 and is mainly used in food production in Asia. The evidence for strong domestication selection in the *Waxy* locus of rice and a selective sweep around its genomic region make us to wonder whether there has been similar selection in *Waxy* in glutinous maize. To address this issue, DNA sequences of *Waxy*, three flanking genes and an unlinked gene (*Adh1*) of 30 accessions sampled from Chinese waxy maize accessions, including representative landraces and inbred lines, were determined in this study. Sharp reduction of nucleotide diversity and significant neutrality tests (Tajima's *D* and Fu and Li's *F**) were observed in the *Waxy* locus in Chinese waxy maize but not in nonglutinous maize; comparison with the unlinked gene confirmed that this pattern was different to *Waxy*. Sequence analysis across a 143 kb genomic segment centered on the *Waxy* locus revealed patterns consistent with a selective sweep in the upstream region of *Waxy*. The selective sweep detected based on current limited genomic sequences exceeded over 50 kb, indicating strong selection in this or a bigger region. However, No sweep effect was detected in the repetitive downstream region of *Waxy*. Phylogenetic analysis indicated that Chinese waxy maize was domesticated from the cultivated flint maize (*Zea mays* ssp. *mays*) that was introduced from the new world. At least two independent deletions in exon 7 (30 bp) and 10 (15 bp) were identified in the Chinese accessions respectively. These findings demonstrate a similar pattern of domestication selection in the *Waxy* genomic region in both glutinous maize and rice, suggesting that this pattern in the rise of glutinous phenotype is likely in other cereal crops.

Keywords Chinese waxy maize · *Waxy* locus · Domestication selection · Selective sweep · Origin

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Introduction

Waxy maize was first reported in China in 1909. A botanist named G.N. Collins first planted and reported the new type of maize collected from China by a Presbyterian missionary (Collins 1909) and reported that "The corn is much more glutinous than

the other varieties, so far as I know" according to a note by the missionary in the archives of the US Department of Agriculture (USDA). Later, waxy maize was also discovered in other places in Asia (Collins 1920; Kuleshov 1954). Despite of the contrary opinion of some authors, the main view is that Chinese waxy maize originated from the maize introduced from the new world since maize was discovered (Zeng et al. 1981; Wikipedia: Waxy corn, http://wikipedia/org). Waxy maize is mainly used in food production in Asia and its amylopectin is also used in the textile, adhesive and paper industries.

A wide range of genetic diversity has been observed in Chinese waxy maize. At least 767 different accessions have been found in China and most of them (525) were collected from Yunnan and Guangxi Provinces (Huang and Rong 1998). Yunnan Province is an original area of many important plants and has the richest number of plant species in China. Several studies have suggested that Chinese waxy maize originated from the Yunnan–Guangxi region (Zeng et al. 1981; Zeng 1987; Cao and Xu 1990; Zeng and Liu 1994). In 1970, Zeng et al. (1981) collected a landrace (termed Four-row Wax due to only four rows in the cob) from Menghai County, Yunnan Province. The landrace has been planted by the local Dai minority since 1890 and is a primitive cultivar with many characters similar to that of wild species (Weatherwax 1955) (Fig. 1). Four-row Wax, together with other typical landraces such as Yishannuo, Qiaojiabainuo etc. from the Yunnan–Guangxi region according to the Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Science et al. (1988), provide important genetic materials for evolutionary studies on Chinese waxy maize.

The glutinous phenotype has been shown to be resulted from a dramatic reduction in synthesis of amylose because of mutations or insertions in the *Waxy* (*Wx*) gene, which encodes a granule-bound starch synthase in maize (Fedoroff et al. 1983; Klosgen et al. 1986) and other cereal crops (Sano 1984; Vrinten et al. 1999; Patron et al. 2002; Saito and Nakamura 2005). In maize, a single recessive gene (*wx*), located on the short arm of chromosome 9, is responsible for the waxy endosperm of the kernel. Because the waxy mutation is expressed in an easily identifiable nonlethal phenotype, it has been the major subject of genetic research during the last century. The DNA sequence of the wild type waxy

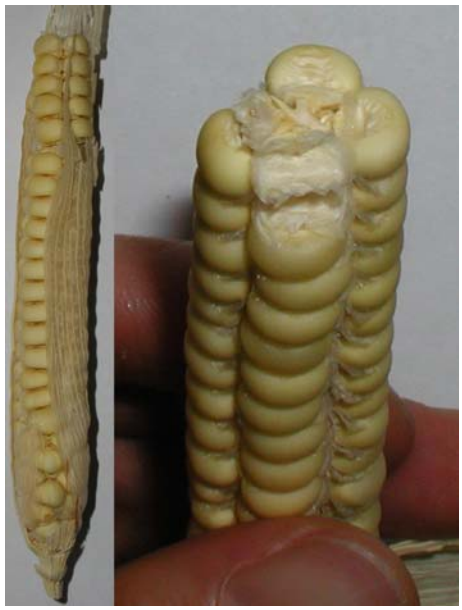

Fig. 1 Four-row Wax, a typical landrace of Chinese waxy maize collected from Yunnan Province, China

locus was determined 20 years ago in maize and is composed of 14 exons (Klosgen et al. 1986) (Fig. 2). In recent studies, *Waxy* sequences have been determined in a number of accessions from *Zea* lineage. For example, Whitt et al. (2002) sequenced the full-length DNA of *Waxy* gene in 30 maize inbred lines; Gaut's group determined the region of exons 9–13 in about 70 accessions from the cultivated maize and teosinte (Zhang et al. 2002; Tiffin and Gaut 2001).

In maize, several genes involved in the starch biosynthesis pathway have been shown to be under strong selection (such as *ae1*) but *Waxy* is not (Whitt et al. 2002). This evidence came from 30 nonglutious maize inbred lines. In rice, the origin of glutinous rice is associated with reduced genetic variation, characteristic of selection at the *Waxy* locus (Olsen and Purugganan 2002) and a selective sweep of about 250 kb in the *Waxy* genomic region was observed (Olsen et al. 2006). Meanwhile, a selective sweep was also observed in other loci in maize, for example *tb1* and *Y1* loci (Wang et al. 1999; Palaisa et al. 2004).

The evidence for strong domestication selection in the rice *Waxy* locus and a selective sweep in its around genomic region make us to wonder whether there has been similar selection in the *Waxy* in the glutinous maize. Our results demonstrate a same strong selection and a sweep in the *Waxy* genomic

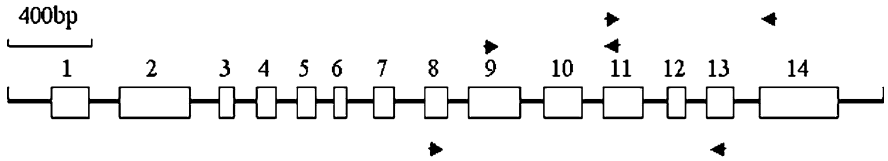

Fig. 2 Gene structure of *Waxy* in maize. Boxes represent exons numbered 1–14 from the 5′ to 3′ ends; lines between exons represent introns. Arrows indicate locations and directions of the PCR primers used in this study

region under domestication selection in Chinese waxy maize.

Materials and methods

Sampling

A set of 30 diverse accessions was selected to represent a broad range of the genetic diversity of Chinese waxy maize. The dataset included 4 landraces, 17 inbred lines and 4 hybrids of Chinese waxy maize. Of the 4 landraces, three were collected from Yunnan and Guangxi Provinces, from where Chinese waxy maize was believed to be originated. Accessions were randomly sampled from the set for determination of the sequences of the *Waxy* and other genes. A sample of *Coix lacryma-jobi* was collected from Zhejiang Province and included as an outgroup (Table 1). *Waxy* and *Adh1* genes from a wide range of nonglutinous maize accessions have been investigated (Whitt et al. 2002; Zhang et al. 2002; Klosgen et al. 1986; Tenaillon et al. 2001) and their sequences were downloaded from GenBank and used as comparisons in this study.

Amylose content determination

Apparent amylose content was determined according to the National Standards of the People's Republic of China, GB–T17891 (China Standard Press 1999).

PCR and DNA sequencing

Accessions were sequenced at *Waxy* and its three flanking loci that were located at 18–53 kb upstream or downstream of the *Waxy* locus. As a comparison, a physical independent (locating chromosome 6) and free-selection locus, *Adh1*, was also sequenced. For *Waxy*, primers for exon 9–13 (~1,300 bp) where have been sequenced in a broad range of nonglutinous maize (Zhang et al. 2002) were used in this study; meanwhile, primers for two overlapping regions for exon 9–11 (P-F: 5′-GATCCTCGAGGC CGACAGGG-3′ and P-R1: 5′-CGTACCGTTCCGT ATCGCAT-3′) and exon 11–14 (P-F1: 5′-GGATGC GATACGGAACGGTA-3′ and P-R: 5′-CTCCTTG GCGAGCGGCGCGA-3′) were designed based on conserved regions of the B73 genomic sequence (AF488416) in alignment with other sequences from GenBank and also used in this study (Fig. 2). For the three flanking genes of *Waxy*, primers were designed based on the annotated coding region in the B73 genomic segment (AF488416) (wx^{-53kb}: 8WXG-F: 5′-CCTCGGACTCAACCACCTTA-3′ and 8WXG-R: 5′-CGTCTATGTCACCTGCGTCT-3′; wx^{-43kb}: 7WXG-F: 5′-ATGAGCATCTCGGTGAACG-3′ and 5′-GCGAGGTGTAGTAGGGAGGA-3′; wx^{+18kb}: N1 G-F: 5′-CATCAACGACGAGAAGAAGC-3′ and N1 G-R: 5′-ATTGCTGTCGGTCCTCCTCT-3′). For *Adh1*, primers were designed based on the conserved regions in alignment of current sequences from GenBank (AdhF: 5′-GGCTCCCCTTGATAAAGT TTG-3′ and AdhR: 5′-TGTGCGTGATGAACTTCT CC-3′). All primers were designed using Primer3 (Rozen and Skaletsky 2000).

All accessions were grown from seeds and DNA was extracted from 14d leaves using the protocol described by Sambrook and Russell (1989) with minor modifications. PCR products were purified with glass milk kit (BioDev Company, China). For cultivated maize, the purified PCR products were sequenced directly on both strands using an Applied Biosystems 3,730 sequencer with the forward and reverse primers. For landraces, hybrids and *C. lacryma-jobi*, in which there may be either homozygous or heterozygous individuals, PCR fragments were cloned into pGEM T-easy vectors (pMD19-T, TaKaRa) and sequenced using the forward and reverse primers. At least three independent clones

Table 1 Chinese waxy maize used in this study

Species	Accession	Type	AAC (%)[a]	Loci[b]					Origin[c]
				Waxy	Wx^{-53kb}	Wx^{-43kb}	Wx^{+18kb}	*Adh1*	
Zea mays									
	Four-row Wax	Landrace (Yunnan)	5.92 (0.38)	EU041692					1
	Qiaojiabainuo	Landrace (Yunnan)	6.19 (0.50)	EU041690	EU041659				2
	Yishannuo	Landrace (Guangxi)	4.61 (0.07)	EU041689	EU041658		EU041638		2
	Chiqibainuo	Landrace (Zhejiang)	4.56 (0.17)	EU041691					3
	622016-ZCN-2	Inbred	4.61 (0.07)	EU041673	EU041649	EU041639		EU041660	4
	622023-WX98-211	Inbred	4.83 (0.11)	EU041674		EU041640		EU041661	4
	622033-CN-33	Inbred	4.80 (0.02)	EU041675		EU041641			4
	622031-CN-36	Inbred	4.25 (0.07)	EU041676	EU041650	EU041643	EU041629		4
	622078-CN-78	Inbred	4.11 (0.04)	EU041677		EU041644	EU041630		4
	622105-CN-106	Inbred	4.56 (0.09)	EU041678		EU041646	EU041631	EU041662	4
	622141-CN-142	Inbred	4.04 (0.14)	EU041679			EU041632	EU041663	4
	622147-CN-148	Inbred	4.33 (0.25)	EU041680		EU041647	EU041633	EU041664	4
	622201-CN-203	Inbred	3.82 (0.11)	EU041681	EU041652			EU041666	4
	622244-CN-46	Inbred	4.40 (0.25)	EU041682	EU041653	EU041648		EU041667	4
	622219-CN-21	Inbred	4.54 (0.11)	EU041683				EU041668	4
	N23-16-2-2-1	Inbred	5.93 (0.21)	EU041684				EU041669	3
	N11-16-1-1-1-1	Inbred	5.59 (0.17)	EU041685	EU041654		EU041635	EU041670	3
	N26-1-1-2-2	Inbred	5.16 (0.07)	EU041686	EU041655		EU041636	EU041671	3
	N22-4-3-2-1	Inbred	5.33 (0.07)	EU041687	EU041656		EU041637		3
	CN9-5-1	Inbred	5.31 (0.17)	EU041688	EU041657				3
	N32-6-3-3	Inbred	5.12 (0.12)	EU041689					3
	MeA1	Inbred		EU041693					1
	622157-CN-159	Inbred	4.49 (0.25)		EU041651		EU041634	EU041665	4
	622068-CN-121	Inbred	3.85 (0.06)			EU041642	EU041627		4
	622125-CN-126	Inbred	4.01 (0.18)				EU041628		4
	622081-CN-82	Inbred	4.21 (0.03)			EU041645			4
	Shuyunuo1	Hybrid	4.68 (0.23)	EU041694					3
	Kenuo986	Hybrid	3.98 (0.50)	EU041695					3
	Zhefengnuo2	Hybrid	4.63 (0.02)	EU041696					3

Table 1 continued

Species	Accession	Type	AAC (%)[a]	Loci[b]					Origin[c]
				Waxy	Wx^{-53kb}	Wx^{-43kb}	Wx^{+18kb}	*Adh1*	
	Yanhejin2000	Hybrid	3.07 (0.19)	EU041697					3
	Total			25	11	10	12	12	
Coix lacryma-jobi	Wild species	Wild (Zhejiang)		EU041672					

[a] Apparent amylose content

[b] wx^{-53kb}, wx^{-43kb} and wx^{+18kb} refer to annotated genes which are 53,311 and 42,665 bp from 5′end and 18,467 bp from 3′end to *Waxy*, respectively, according to the BAC sequence (AF488416) containing *Waxy* locus. The three genes code "putative selenium binding protein (AAQ06283)", "putative NAM (no apical meristem) protein (AAQ06284)" and "hypothetical protein (AAQ06294)", respectively. *Adh1*: alcohol dehydrogenase 1

[c] Samples used in this study were provided by (1) Institute of Genetics and Developmental Biology, Chinese Academy of Science; (2) Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Science; (3) Institute of Crop Science, Zhejiang University; (4) Institute of Crop Science, Zhejiang Academy of Agricultural Science

were sequenced. Sequence data from this article have been deposited in GenBank under accession numbers EU041627–EU041697.

Genetic diversity analysis

Sequences were aligned using ClustalW (Thompson et al. 1994) for the construction of phylogenetic trees. Neighbour-joining (NJ) phylogenies based on the Kimura 2-parameter distance matrix were generated by MEGA version 3.1 (Kumar et al. 2004). Bootstrap confidence values were obtained from 1,000 replicates. Over 75% supporting nodes were shown. 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 (Watterson 1975), the average pairwise nucleotide diversity π (Tajima 1983) and neutrality tests (Tajima's D and Li and Fu's D^* and F^*) (Tajima 1989; Fu and Li 1993) were estimated using DNASP version 4.10.2 (Rozas et al. 2003).

Results and discussion

Amylose content of Chinese waxy maize

In general, apparent amylose contents are <5% in most cultivated Chinese waxy maize (Table 1). Relatively higher values are observed in the three landraces from the Yunnan–Guangxi region, where Chinese waxy maize is originated (Zeng et al. 1981; Cao and Xu 1990). In our collection, we tried to include the main typical landraces, inbred lines and hybrids used for current production in China to show the genetic diversity of Chinese waxy maize.

Nucleotide variation at the *Waxy* in Chinese waxy maize

We examined genetic variation in a 1,300 bp region (exons 9–13) of *Waxy* in 25 Chinese glutinous maize accessions (Table 1). Glutinous and nonglutinous accessions showed an apparent difference in levels of genetic variation at the *Waxy* locus (Table 2). Average pairwise nucleotide diversity, π, in the nonglutinous maize is fourfold higher than that in the glutinous maize accessions. Similarly, θ_w is more than three times higher in the nonglutinous maize. In

Table 2 Genetic diversity in glutinous (Chinese waxy maize) and nonglutinous maize accessions

Loci[a]	Sample size (n)	π[b]	θ_w[b]	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**
Chinese waxy maize						
Waxy	25	0.00187 ± 0.00062	0.00438 ± 0.00169	−2.06*	−2.36¶	−2.65*
Wx^{-53kb}	11	0.00581 ± 0.00170	0.00984 ± 0.00431	−1.87**	−2.03¶	−2.25¶
Wx^{-43kb}	10	0.001371 ± 0.00106	0.00243 ± 0.00148	−1.67¶	−1.92¶	−2.08¶
Wx^{+18kb}	12	0.04127 ± 0.00349	0.03955 ± 0.01552	−0.02	0.17	0.14
Adh1	12	0.02050 ± 0.00265	0.01996 ± 0.00792	0.06	0.21	0.19
Nonglutinous maize						
Waxy	42	0.00859 ± 0.00120	0.01152 ± 0.00357	−1.12	−0.32	−0.72
Adh1	25	0.01298 ± 0.00181	0.01213 ± 0.00413	0.27	−0.16	−0.03

** $P < 0.01$; $^{*}P < 0.05$; $^{¶}P < 0.10$

[a] For wx^{-53kb}, wx^{-43kb} and wx^{+18kb} see Table 1. Data for nonglutinous maize from: *Waxy*: Whitt et al. (2002), Zhang et al. (2002) and Klosgen et al. (1986); *Adh1*: Tenaillon et al. (2001)

[b] π, the average pairwise nucleotide diversity (Tajima 1983); θ_w, an estimate of $4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per nucleotide (Watterson 1975)

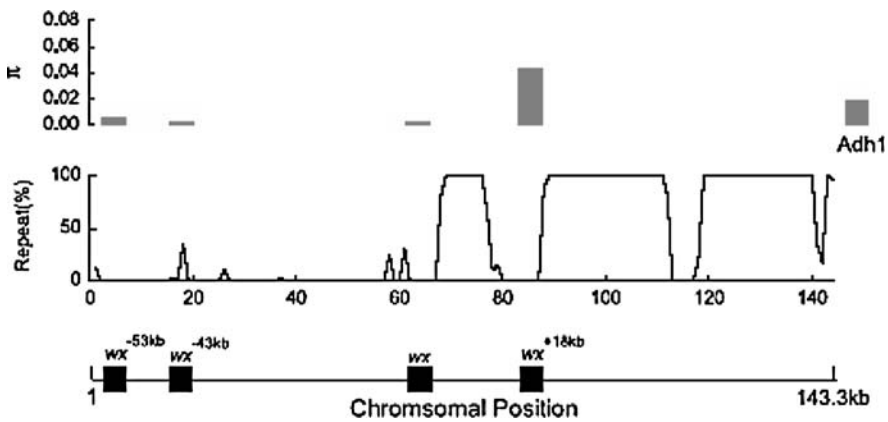

Fig. 3 Nucleotide variation (π) and repeat distribution across *Waxy* genomic region of Chinese waxy maize. The unlinked gene *Adh1* is also shown. For details of π values see Table 2

contrast, no reduction in genetic diversity from nonglutinous to glutinous accessions is observed at the unlinked gene *Adh1*. The alcohol dehydrogenase gene (*Adh*) family, usually comprising 2–3 gene members in the flowering plants, has been thoroughly studied in grasses (Poaceae) (Gaut and Clegg 1993). One locus, *Adh1*, has been identified in many cultivated maize (Tenaillon et al. 2001). No selection has been detected in this locus in several cereals, such as maize (Gaut and Clegg 1993; Tenaillon et al. 2001) and rice (Zhu et al. 2007). *Adh1* is located on chromosome 6, while *Waxy* is on chromosome 9 of maize. Thus the results suggest that a reduction in genetic diversity specific to the *Waxy* locus is associated with the rise of glutinous maize.

We also estimated genetic variation in three genes (wx^{-53k}, wx^{-43kb} and wx^{+18kb}) upstream or downstream of *Waxy* in Chinese waxy maize (Table 2). A BAC clone containing *Waxy* has been sequenced (AF488416) and provides an opportunity to investigate selective sweep in the *Waxy* genomic region in maize (see Domestication selection and selective sweep in the *Waxy* genomic region). At first, two annotated genes (wx^{-53kb} and wx^{+18kb}) at two ends of the clone were chosen for sequencing. Later, in order to confirm the result at the upstream gene wx^{-53kb}, its near gene, wx^{-43kb}, was further determined. These genes are 18–53 Kb from the *Waxy* locus (Fig. 3). The two diversity estimators, π and θ_w, are five folds higher in the downstream gene than in the two

upstream genes, which have a similar level of genetic variation to the *Waxy*.

Domestication selection and selective sweep in the *Waxy* genomic region

Further analysis of nucleotide diversity indicates that domestication selection for the glutinous phenotype has acted at the *Waxy* locus of Chinese waxy maize (Table 2). Tajima's *D* test (Tajima 1989) and Fu and Li's test (Fu and Li 1993) both show that the glutinous Chinese maize has a significant negative deviation from the neutral model ($D = -2.06$, $P < 0.05$ and $F^* = -2.65$, $P < 0.05$, respectively), whereas nonglutinous accessions do not. In contrast, the reference gene *Adh1* fits to neutral expectations for both classes of starch (Table 2). This difference between the two genes indicates that the observed deviation from neutrality at the *Waxy* is not due to changes in population size associated with the origin of Chinese waxy maize, because such demographic effects should be detectable at both loci. Similar evidence for domestication-related selection has also been reported in maize (e.g. *tb1* locus, Wang et al. 1999) and other crops (e.g. rice *Waxy* locus, Olsen and Purugganan 2002).

Selective sweeps have been reported in both domesticated (e.g. maize and rice) and natural systems (Olsen et al. 2006). A selective sweep is expected to reduce genetic diversity not only at the specific locus (target of selection) containing a favored mutation but also at surrounding loci, so called "selective interference" (Hill and Robertson 1966). The genetic diversity of both *Waxy* and its two upstream genes (wx^{-53kb} and wx^{-43kb}) are found to be about an order of magnitude smaller in Chinese waxy maize than other genes in nonglutinous or glutinous maize (Table 2). The results suggest that a potential selective force has acted across the genomic region. A statistical analysis of nucleotide diversity across the *Waxy* genomic region provides further evidence of selective sweep in the genomic region. In Chinese waxy maize, there are negative deviations (~ -2.0) in Tajima's *D* and Fu and Li's *D** and *F** for *Waxy* and the two upstream genes (Table 2), a pattern consistent with recent directional selection on *Waxy*. In contrast, no such pattern was observed for *Adh1* or the genes downstream of *Waxy* in Chinese waxy maize. These patterns are consistent with a recent selective sweep at the *Waxy* genomic region in Chinese waxy maize.

Our results found in Chinese waxy maize are the same as those found for the *Waxy* locus in glutinous rice (Olsen and Purugganan 2002; Olsen et al. 2006). Similarly, marked reductions in genetic diversity and a pattern of significant negative value of Tajima's *D* and Fu and Li's *D** and *F** across the *Waxy* genomic region were observed in the glutinous rice population. A sweep size of $\sim$250 Kb under domestication selection was estimated in rice (Olsen et al. 2006). In maize, two genes have been estimated to have selective sweep sizes 60–90 kb and over 600 kb, respectively (Wang et al. 1999; Palaisa et al. 2004). In our study, a genomic region of 53 kb or more, because no genome sequences in this study set a limit to the region upstream of *Waxy*, was affected by selective sweep in the glutinous maize population. As a similar pattern of domestication selection in *Waxy* locus was observed in both glutinous crops (rice and maize), the pattern of domestication selection may be common for the rise of other glutinous cereals.

No selection has been detected in the downstream gene (wx^{+18kb}) of *Waxy* in Chinese waxy maize, although it is nearer to it than the two upstream genes. Two factors may be responsible for this result: (1) The whole downstream region of *Waxy* is repeat-rich (Fig. 3), so there might be a recombination hotspot between the gene and *Waxy*. High recombination rate tends to break down linkage disequilibrium (LD) and selection sweep. (2) Potentially wrong gene annotation: wx^{+18kb} was annotated as "hypothetical protein" according to the NCBI's entry. No transcriptional sequences from this gene can be found in current nr or EST sequences from plants (2007-7-13), suggesting it may be a wrong annotation.

Phylogenetics and domestication of Chinese *waxy* maize

Nucleotide sequences of genes from Chinese waxy maize accessions were first determined in this study and provide an opportunity to investigate phylogenetic relationships for Chinese waxy maize. Based on the *Waxy* sequences, a phylogenetic tree including Chinese waxy maize and other nonglutinous maize was constructed (Fig. 4). The tree indicates that Chinese waxy maize (red branches) is grouped

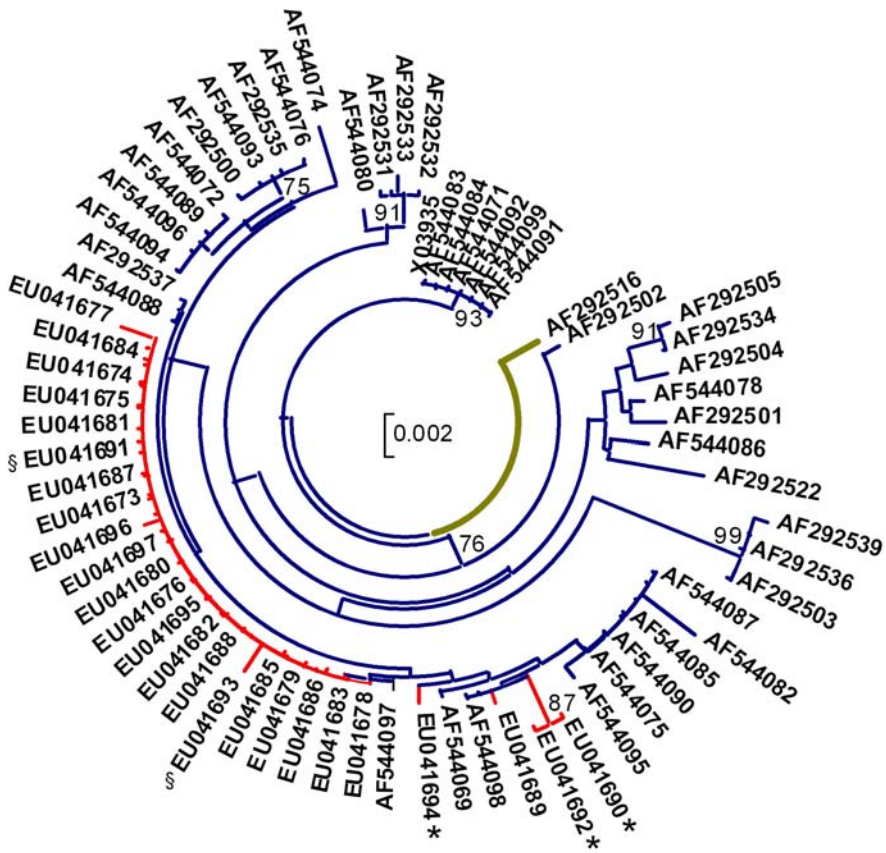

Fig. 4 Phylogenetic tree of the Chinese waxy maize based on *Waxy* loci. The Chinese waxy maize and other nonglutinous maize are shown in red and blue branches, respectively. Wild maize (*Zea mays* spp. *parviglumis*) (green branch) is as an outgroup. The lines containing the 30 bp deletion removing the last 4 bp of exon 7 (§) and the 15 bp deletion in exon 10 (*) are indicated. Bootstrap values for nodes supported in >75% of 1,000 bootstrap replicates are shown above the branches

closely with cultivated maize (*Zea mays* ssp. *mays*) (blue branches) but not with wild species (such as *Zea mays* ssp. *parviglumis*, AF292516). On the other hand, Chinese waxy maize failed to constitute an independent branch from other cultivated maize. As an early study indicated that Chinese waxy maize and *Coix* had a similar spectrum for peroxidase isoenzyme (Zeng et al. 1981), we also determined the *Waxy* sequence from *Coix*. However, the *Coix Waxy* gene was highly diverged from *Zea mays*, including Chinese waxy maize, and phylogenetic analysis indicated that Chinese waxy maize were significantly nearer *Zea mays* ssp. *parviglumis* or other wild species than *Coix* (data not shown). The results suggest that Chinese waxy maize should be regarded as post-domesticated from *Zea mays* ssp. *mays*, i.e. additional selection was acted on the *Waxy* locus in Chinese waxy maize after its initial domestication.

Waxy sequences of 13 landraces from the South America have been determined by Zhang et al. (2002) and provide a good genetic background for inferring the origin of Chinese waxy maize. According to the tree (Fig. 4), two Chinese landraces (Four-row Wax and Yishannuo) from Yunnan Province were clustered together first and then with a landrace from Guangxi Province and further with other Chinese waxy maize. The nearest American landraces to Chinese accessions, such as Pira (AF292537) and Chococeno (AF292500) from Colombia, are flint according to Germplasm Resources Information Network (GRIN), USDA at MaizeGDB (www.maizegdb.org). In contrast, the Chinese accessions were separated significantly from a landrace Coroico from Bolivia (Amazon Basin) (AF292502) and a group of maize accessions, including B14A (AF544071), N28Ht (AF544091), NC260 (AF544092), W153R (AF544099), IDS28 (AF544084) and I205 (AF544083) (Fig. 4). All those accessions are dent type (IDS28 is pop) according to GRIN. The results suggest that Chinese waxy maize might be domesticated from the flint maize. The result is consistent with several other observations for Chinese waxy maize (Cao and Xu 1990): (1) All maize landraces in the Yunnan–Guangxi region are

flint and no dent one was found before glutinous maize was cultivated in this area; (2) there is a high similarity in agronomic features between Chinese waxy maize and local flint landraces; and (3) there is the same spectrum of peroxidase isoenzyme for both Chinese waxy maize and flint maize, but not dent maize according to Zeng et al. (1981). Waxy maize mutated from dent maize was also found and domesticated in the USA in the 1930s (Jugenheimer 1976). Therefore, at least two independent *Waxy* mutations and following domestications have occurred in two different type of maize and resulted in the rise of a distinctive type of maize with agronomic significance in the world.

The glutinous phenotype has been shown to be resulted from a dramatic reduction in synthesis of amylose due to insertion/deletions or mutations in the *Waxy* gene. In maize, many *Waxy* mutations are caused by insertions of transposable elements and deletions (Fedoroff et al. 1983; Wessler and Varagona 1985; Klosgen et al. 1986; Wessler et al. 1990; Okagaki et al. 1991; Marillonnet and Wessler 1997), whereas deletions and even a single nucleotide substitution in noncoding (promoter and intronic regions) and coding regions reduce both *Waxy* mRNA level and amylose content in other cereals (e.g., rice, wheat and barley) (Sano 1984; Vrinten et al. 1999; Domon et al. 2002; Olsen and Purugganan 2002; Patron et al. 2002; Saito and Nakamura 2005). Comparison of the sequences of exon 9–13 from Chinese waxy maize with the published *Wx* sequence (Klosgen et al. 1986) identified several synonymous substitutions and deletions in introns and exons. Particular, a 15 bp deletion in exon 10 was observed in three Chinese accessions (Four-row Wax, Shuyunuo1 and Qiaojiabainuo; Fig. 4) but not other accessions (data not shown). Okagaki et al. (1991) reported a 30 bp deletion common to two independently derived *Waxy* mutations in maize, which removed the last 4 bp of exon 7 and is responsible for the mutant phenotype. The deletions at exon–intron junction resulting in *Waxy* mutations were also observed late in wheat (Vrinten et al. 1999). Meanwhile, a stable *Waxy* mutation due to a 5.6 kb retrotransposon insertion in *Waxy* intron 8 was reported in maize (Marillonnet and Wessler 1997). In order to determine the potential mechanisms for the rise of glutinous phenotype in Chinese waxy maize, genomic sequences of exon 6–9 of three accessions (Four-row Wax, Chiqibainuo and MeA1) from Chinese waxy maize were sequenced (EU041691–EU041693). Interestingly, the 30 bp deletion removing the last 4 bp of exon 7 was also observed in Chiqibainuo and MeA1, but not in Four-row Wax (data not shown). Further sequencing of all coding exons of *Waxy* gene in Four-row Wax did not find any other deletion (EU041692). No retrotransposon insertion was found in the intron 8 in above three Chinese accessions. The results indicated that at least two independent deletions in exons 7 (30 bp) and 10 (15 bp) might have been involved in the rise of Chinese waxy maize.

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