Molecular diversity and population structure of Chinese green foxtail [Setaria viridis (L.) Beauv.] revealed by microsatellite analysis

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Abstract

Green foxtail (Setaria viridis) is a new model plant for the genomic investigation of C₄ photosynthesis biology. As the ancestor of foxtail millet (Setaria italica), an ancient cereal of great importance in arid regions of the world, green foxtail is crucial for the study of domestication and evolution of this ancient crop. In the present study, 288 green foxtail accessions, which were collected from all geographical regions of China, were analysed using 77 simple sequence repeats (SSRs) that cover the whole genome. A high degree of molecular diversity was detected in these accessions, with an average of 33.5 alleles per locus. Two clusters, which were inconsistent with the distribution of eco-geographical regions in China, were inferred from STRUCTURE, Neighbor–Joining, and principal component analysis, indicating a partially mixed distribution of Chinese green foxtails. The higher subpopulation diversity was from accessions mainly collected from North China. A low level of linkage disequilibrium was observed in the green foxtail genome. Furthermore, a combined analysis of green foxtail and foxtail millet landraces was conducted, and the origin and domestication of foxtail millet was inferred in North China.

Key words: Genetic diversity, germplasm, green foxtail, Setaria viridis.

Introduction

Green foxtail, Setaria viridis (L.) Beauv., also called green millet, belongs to Setaria Beauv., a member of the grass tribe Paniceae in the subfamily Panicoideae. Green foxtail is the ancestor of cultivated foxtail millet (Setaria italica L. Beauv.), an ancient cereal of great importance to dry land agriculture that has been grown in China for >10,500 years (Yang et al., 2012) for grain and forage. Due to its small growth stature, small genome size, self-fertilization, short growing cycle, and efficient genetic transformation, green foxtail is being rapidly developed as a model plant for deciphering C₄ photosynthesis biology which was verified to be highly efficient in CO₂ fixation and has great potential for the genetic improvement of C₃ staple food crops such as rice (Brutnell et al., 2010; Li and Brutnell, 2011; Benetzen et al., 2012). Green foxtail is closely related to biofuel grasses such as switch grass (Panicum virgatum L.), proso millet (Panicum miliaceum L.), and pearl millet (Pennisetum glaucum L.). The genomic and genetic annotation of green foxtail will undoubtedly improve breeding programmes in those crops which are difficult to manipulate due to their large genomes, outcrossing breeding systems, large stature, and long growing cycles (Doust et al., 2009; Benetzen et al., 2012). Foxtail millet is also rapidly becoming a model plant for the functional genomics of grass, focusing on crop domestication, abiotic stress tolerance, and grass evolution (Doust et al., 2009; Li and Brutnell, 2011; Lata et al., 2012; Mauro-Herrera et al., 2013). The release of the draft genome sequences of foxtail millet has accelerated the development of green foxtail and foxtail millet as a novel model system (Benetzen et al., 2012; Zhang et al., 2012).
Green foxtail is an Old World species of the *Setaria* genus and it is now distributed worldwide (Austin, 2006). As a regional crop, wild germplasm collections of foxtail millet and related studies have been sparse (Li and Brutnell, 2011). Wang *et al.* (1995) investigated the genetic diversity and structure of green foxtail accessions collected from North America and Eurasia using allozyme markers, and suggested that there were similar isozymatic forms between foxtail millet and green foxtail collected from the same regions. Chinese accessions were identified as having a high level of genetic diversity using amplified fragment length polymorphism (AFLP) markers (Le Thierry d’Ennequin *et al.*, 2000), transposon display (Hirano *et al.*, 2011), and intersimple sequence repeat (ISSR) markers (Li *et al.*, 2012), and the well known A10 accession which was used as a model for C4 photosynthesis study was also from China (Brutnell *et al.*, 2010; Li and Brutnell, 2011; Bennetzen *et al.*, 2012; Caemmerer *et al.*, 2012). However, all these studies were carried out with a small sample size of no more than 40 accessions. Detailed information regarding the genetic diversity and population structure of green foxtail with a larger sample size will be useful in many related studies.

The genetic diversity and population structure of both the wild relatives and domesticated landraces have been widely used for crop origin studies (Spooner *et al.*, 2005; Heerwaarden *et al.*, 2011; Huang *et al.*, 2012). From studies on wild green foxtail and cultivated foxtail millet, monotopic (Fukunaga *et al.*, 2006; Hirano *et al.*, 2011; Li *et al.*, 2012) and polytopic (Jusuf and Pernes, 1985) origins of foxtail millet have been inferred, and most reports agree that China was the first site of foxtail millet domestication, if not the only one (Vavilov, 1926; Austin, 2006; Li *et al.*, 2012). This is supported not only by the earliest archeological evidence of foxtail millet identified in Northern China, which existed >10 500 years ago (Lu *et al.*, 2009; Barton *et al.*, 2009; Yang *et al.*, 2012), but also by the high level of genetic diversity of Chinese foxtail millet landraces (Le Thierry d’Ennequin *et al.*, 2000; Fukunaga *et al.*, 2006; Hirano *et al.*, 2011). The analysis of genetic diversity and population structure based on a large sample size of 250 Chinese foxtail millet landraces, which represent 1% of the foxtail millet germplasm kept in the Chinese National Gene Bank, revealed a high genetic diversity of 20.9 alleles per locus, and classified the accessions into four subpopulations, in accordance with its ecological/geographical distribution in China (Wang *et al.*, 2012). The diversity analysis also suggested that foxtail millet was first domesticated in the Yellow River drainage area (E95°53′–119°50′, N32°10′–41°50′, including Gansu, Shaanxi, Shanxi, Henan, and Hebei) and then spread to other parts of the country. Investigation of the genetic diversity and population genetics of Chinese green foxtail can be used to provide complementary information, enabling a clearer understanding of this issue.

Association mapping is an effective approach requiring information on population structure and linkage disequilibrium (LD) to detect quantitative trait loci (QTLs)/genes of great importance. Using simple sequence repeat (SSR) markers, association mapping has been successfully developed in rice (Jin *et al.*, 2010), wheat (Kruger *et al.*, 2004; Maceferri *et al.*, 2005), and barley (Mather *et al.*, 2004). However, molecular genetic investigations of the diversity, population structure, and LD patterns of green foxtail using SSR markers have not yet been performed.

In this study, a large sample size of 288 green foxtail accessions collected from all geographical regions of China were analysed using SSR markers covering the nine chromosomes of green foxtail. Genetic diversity and population structure were inferred by software simulations. LD levels in the green foxtail genome were also measured. An analysis of data on green foxtail combined with previous data on foxtail millet landraces (Wang *et al.*, 2012) was also carried out and the results were compared. The data and conclusions of this study will benefit green foxtail germplasm collection and management, genomic studies, trait association mapping, and breeding applications.

**Materials and methods**

**Green foxtail sampling**

All of the green foxtail samples were collected from China in 2010. The green foxtail samples used in this study were selected from different ecoregions, and the number of accessions sampled from each region was in proportion to the number of foxtail millet accessions stored in the Chinese National Gene Bank (CNGB) from each region. Thus, the number of green foxtail samples from Northern China, where foxtail millet has a large growing area and there are many collected accessions, was larger than that of Southern China. The aim of the sampling strategy was to assemble a more representative set of accessions of green foxtail from all the ecoregions of China. The number of samples from each province used in this study is listed in Table 1.

**Genotyping of green foxtail**

The template DNA was extracted from leaves of the sampled green foxtail accessions using the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). The 77 SSR markers described previously (Wang *et al.*, 2012) were used. All SSRs were labelled with different coloured fluorescent dyes at the 5′ end of the forward primer for PCR amplification (Applied Biosystems, USA). The PCR mixture consisted of 1× Taq reaction buffer (Takala, with Mg2+), nucleotides dATP, dGTP, dCTP, and dTTP (125 μM each), 0.1 μM primer, 1 U of *Taq* DNA polymerase, and 10 ng of template DNA. The length of the amplified fragment of DNA was measured using an ABI 3730XL analyser. Polymorphism data were analysed using GeneMapper (Version 4.0). Microchecker 2.2.3 (Oosterhout *et al.*, 2004) was used for checking mistakes due to potential primer stuttering to make sure the genotyping data were reliable.

**Genetic diversity and population structure**

All summary statistics such as allele number per locus, genotype number per locus, gene diversity, PIC (polymorphism information content) values, observed homozygosity, genetic distance, and FST tests were determined using PowerMarker version 3.25 (Liu and Muse, 2005). Nei’s genetic distance (Nei and Takezaki, 1983) was calculated and used for unrooted phylogeny reconstruction based on Neighbor–Joining methods as implemented by PowerMarker software, and the tree was visualized using MEGA 5.0 (Tamura *et al.*, 2007). Principal component analysis (PCA) was carried out in GenALEX 6.4 (Peakall and Smouse, 2006). Analysis of molecular variance (AMOVA) was calculated by PowerMarker. Three levels of AMOVA were conducted for each inferred subpopulation, including the molecular variance of ‘among two SSR alleles within individuals’, ‘among individuals
within populations’, and ‘among populations’. The lengths of the amplified product of SSR markers of each accession were used as the value of microsatellite alleles for variance analysis.

The model-based software program STRUCTURE v2.3 (Pritchard et al., 2000) was used to infer population structure by a Bayesian approach using the SSR marker data set. The optimal value of K (the number of clusters) was deduced by evaluating \( K = 1 \)–14. Admixture and non-admixture models were used separately and on varying \( K \) from 1 to 14 with 20 iterations per \( K \). When \( K \) on varying \( K \) from 1 to 14, the optimal value of \( K \) was identified using both the ad hoc procedure introduced by Pritchard et al. (2000) and the method developed by Evanno et al. (2005). Genetic diversity, private allele number, and divergence estimates were calculated for the different clusters identified by the structure analysis. Substructures within each main cluster were detected by the same approach using STRUCTURE v2.3.

**Linkage disequilibrium**

LD was evaluated for each pair of SSR loci by TASSEL, both on all accessions individually and on the clusters as inferred by STRUCTURE. \( D' \) and \( r^2 \) LD measures modified for loci were used (Hedrick et al., 1987; Weir et al., 1996). Significance (\( P \)-value) of \( D' \) and \( r^2 \) for each SSR pair was determined by 100 000 permutations.

**Results**

**Genetic diversity of green foxtail**

Sixty-nine of the 77 markers were successfully amplified from the green foxtail accessions, and all the markers were polymorphic across the 288 green foxtail accessions. A total of 2312 alleles were detected, and the average allele number per locus was 33.50, ranging from 12 to 54. The average genotype number per locus was 46.37, ranging from 21 to 141. The average diversity of each locus was 0.90, ranging from 0.01 to 0.64. The average homozygosity per locus was 0.07, ranging from 0.01 to 0.64. The average homozogosity extended to 0.90, which implies that the green foxtail samples are close to inbred lines (Table 2).

**Population structure of green foxtail inferred by the admixture model**

Admixture model-based calculations were conducted based on varying \( K \) from 1 to 14 with 20 iterations per \( K \). When the STRUCTURE simulations were performed using all 288 accessions, the LnP(\( D \)) value increased with \( K \) from 1 to 14, but showed an evident inflection at \( K = 2 \) (Fig. 1A). This result indicated that there might be two divergent subpopulations. According to the second-order statistics developed for STRUCTURE (Evanno et al., 2005) to estimate the number of subpopulations, the optimal value of \( K = 2 \), at which the delta \( K \) showed a peak, was identified (Fig. 1B). This suggested that the Chinese green foxtail samples can be grouped

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**Table 1. Origin of green foxtail selected in this trial**

| Eco-regions of foxtail millet | Province | No. of accessions |
|-------------------------------|----------|------------------|
| Early spring-sowing region (ESR) | Heilongjiang | 12 |
| Spring-sowing region (SR) | Shanxi | 39 |
| | Shannxi | 22 |
| | Gansu | 18 |
| | Inner Mongolia | 5 |
| | North Hebei | 24 |
| | Tibet | 2 |
| | Xinjiang | 5 |
| | Ningxia | 6 |
| | Beijing | 6 |
| | South Hebei | 13 |
| | Henan | 35 |
| | Shandong | 9 |
| | Tianjin | 1 |
| | Jilin | 12 |
| | Liaoning | 11 |
| Southern China region (SCR) | Sichuan | 6 |
| | Hubei | 9 |
| | Guangxi | 4 |
| | Guangdong | 9 |
| | Jiangsu | 9 |
| | Zhejiang | 5 |
| | Jiangxi | 10 |
| | Guizhou | 2 |
| | Fujian | 6 |
| | Yunnan | 8 |

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**Table 2. Genetic diversity of 288 green foxtail samples as revealed by SSR markers**

| Sample | No. of alleles | No. of genotypes | Gene diversity | Heterozygosity | PIC |
|--------|----------------|-----------------|---------------|---------------|-----|
| Average | 33.50 | 46.37 | 0.90 | 0.07 | 0.90 |
| Range | 12–54 | 21–141 | 0.70–0.97 | 0.01–0.64 | 0.66–0.97 |
| SD | 11.26 | 21.14 | 0.06 | 0.10 | 0.07 |
into two populations, designated PopA and PopB. For each inferred population, substructuring under the topmost hierarchy was detected using a similar approach. PopA was divided into three (K=3) and PopB was divided into two (K=2) subgroups, with 20 iterations for each K (Supplementary Fig. S1A–D available at *JXB* online), making five subgroups in all. These were labelled as PopA-1 (63 samples), PopA-2 (107 samples), PopA-3 (51 samples), PopB-1 (51 samples), and PopB-2 (16 samples) (Fig. 1C, lower part).

A Neighbor–Joining tree of 288 accessions was constructed based on Nei’s (Nei and Takezaki, 1983) genetic distance (Fig. 2A, B), which illustrated genetic relationships that closely approximated the STRUCTURE-based membership assignment for most of the accessions. The mixed distribution of the subgroup accessions into different clusters can be seen in Fig. 2A. A PCA was conducted to assess further the population subdivisions identified using STRUCTURE (Fig. 2C). The first principal component explained 24.66%, the second principal component explained 19.71%, and the third principal component explained 16.70% of the molecular marker variation among the 288 accessions. Plotting of the first three principal components showed separation of inferred subpopulations, which was highly consistent with STRUCTURE and the Neighbor–Joining analysis above.

Relationships among subpopulations simulated from STRUCTURE were identified using pairwise genetic distance and $F_{ST}$ analysis (Table 3). The genetic distance ranged from 0.2420 between PopA-1 and PopA-2 to 0.6521 between PopA-3 and PopB-2, with an average of 0.4338. The pairwise $F_{ST}$ values for the subpopulations ranged from 0.0224...
Fig. 2. Neighbor–Joining (NJ) analysis and principal component analysis (PCA) of Chinese green foxtail accessions. (A) Unrooted NJ tree of 288 green foxtail; every coloured branch represents one accession collected from the corresponding inferred subpopulation. (B) NJ tree of inferred subgroups based on Nei’s genetic distance; the bootstrap value (out of 1000) is indicated at the branch point. (C) Differentiation of genotypes from subpopulations according to the first principal component derived from diversity analysis of 69 SSR markers.

Table 3. Pairwise estimates of $F_{st}$ and genetic distance among five model-based subpopulations

| Subpopulation | PopA-1 | PopA-2 | PopA-3 | PopB-1 | PopB-2 |
|---------------|--------|--------|--------|--------|--------|
| PopA-1        |        | 0.0224 | 0.0232 | 0.0568 | 0.2677 |
| PopA-2        | 0.2420 |        | 0.0287 | 0.0532 | 0.3474 |
| PopA-3        | 0.3196 | 0.2545 |        | 0.0572 | 0.2343 |
| PopB-1        | 0.3792 | 0.2877 | 0.3926 |        | 0.2122 |
| PopB-2        | 0.6518 | 0.5912 | 0.6521 | 0.5677 |        |

$F_{st}$ estimates appear above the diagonal, and pairwise genetic distance appears below the diagonal.
between PopA-1 and PopA-2 to 0.3474 between PopA-2 and B-2, with an average of 0.1301. The genetic distance was consistent with the trends of the $F_{ST}$ estimates. For instance, higher genetic distance and $F_{ST}$ were found between PopA and PopB clusters. The relationships of all five subpopulations suggested by Table 3 were also concordant with the Neighbor–Joining analysis including all 288 accessions.

To interpret the separation among the subpopulations identified above, the habitat and geographical location where each accession was collected were depicted (Fig. 3A), but this did not reveal a clear pattern of ecological differentiation corresponding to the genetic subpopulations. Instead, spreading and mixing of the subpopulations throughout the pre-defined eco-regions were inferred. The proportion of

**Fig. 3.** Spatial distribution of collected green foxtail. (A) Map of the collection locations of Chinese green foxtail accessions grouped by five subpopulations inferred in this investigation. Different coloured spots represent individuals from the various subpopulations. (B) Proportions of individuals from corresponding subgroups based on latitudes of sampled locations.
individuals collected from each subgroup according to their latitude was also calculated (Fig. 3B). It was found that PopA included more accessions collected from Northern China and PopB contained a large proportion of individuals from Southern China.

Genetic variation of subpopulations

The genetic diversity per locus was estimated for each subpopulation (Tables 4 and 5). PopA-1 had the highest gene diversity and PIC value, followed by PopA-2 and PopA-3. The highest number of population-specific alleles was found in PopA-2. Among the 2312 alleles detected in the total populations, 627 (27.12%) were subpopulation-specific or private alleles. PopB-2 had the lowest genetic diversity identified in this research. PopA-2 had the largest genetic variance (34.62%) among all the subpopulations, followed by PopA-1 (20.69%), PopA-3 (16.42%), PopB-1 (15.57%), and PopB-2 (3.38%).

LD among SSR loci in green foxtail

The extent of LD was assessed among the SSR loci pairs for all accessions, as well as for the subpopulations separately (Table 6). Across all accessions, as many as 60.24% of the total evaluated SSR pairs were in LD ($P < 0.05$) after Bonferroni correction. For these loci pairs that had significant LD, the value of $D'$ ranged from 0.237 to 0.93, with a mean of 0.581, and the value of $r^2$ ranged from 0.004 to 0.092, with a mean of 0.005. The frequency of the pairs of loci with significant LD was reduced by more than half when the LD was calculated within each subpopulation, except for PopB-2 (84.09%). The lowest percentage (11.53) of locus pairs in LD was found in PopB-1. The values of $D'$ and $r^2$ were increased when analysed within each subpopulation. PopB-2 presented the highest mean value of 0.950 for $D'$ and 0.381 for $r^2$, and the lowest mean value of $D'$ and $r^2$ of 0.673 and 0.018 were found in PopA-2.

Discussion

Molecular diversity of Chinese green foxtail and comparison with that of foxtail millet landraces

This report is the first time that the genetic diversity of Chinese wild green foxtail has been characterized by SSR markers. A majority of the markers (69 of 77) reliably amplified SSRs from the green foxtail accessions, illustrating that green foxtail and foxtail millet are very closely related. This is consistent with the theory that green foxtail is the closest wild ancestor of foxtail millet (Li et al., 1945). Green foxtail and foxtail millet were classified into the same primary gene pool of the AA genome of *Setaria* (Benabdelloua et al., 2001a, b), and they have even been considered to be subspecies of the same species in previous treatments (Dekker, 2003).

The average number of alleles per locus was 33.5 in the Chinese green foxtail, higher than that of 21.4 of the Chinese foxtail millet landraces, using the same set of SSR markers as in previous trials (Wang et al., 2012). This means that a large part of the genetic diversity in the wild gene pool was lost during the domestication of foxtail millet (Supplementary Table S2 at JXB online), and agrees well with previous single nucleotide polymorphism (SNP) analyses of certain genomic regions between these two species (Wang et al., 2010). A higher number of private alleles and lower allele frequencies were also observed in green foxtail compared with foxtail millet landraces (Supplementary Figs S2, S3). This observation is consistent with research conducted on other domesticated crop species (Vigouroux et al., 2002; Kuroda et al., 2006), and indicates the necessity for germplasm collection and protection of the wild relative of crops. The high genetic diversity of green foxtail could provide important genetic resources for foxtail millet improvement programmes and for functional genomics study. Foxtail millet and green foxtail are fast being developed as models of functional genomic investigations for plant morphology and physiology (Doust et al., 2009) and $C_4$ photosynthesis (Brutnell et al., 2010) owing to their small genomes, inbreeding nature, and efficient

| Subpopulations | Sample size | Genotype no./locus | Allele no./locus | Gene diversity/locus | PIC/locus | No. of population-specific alleles |
|----------------|-------------|--------------------|----------------|----------------------|----------|----------------------------------|
| Pop A-1        | 63          | 21.91              | 20.72          | 0.89                 | 0.89     | 180                              |
| Pop A-2        | 107         | 28.19              | 24.16          | 0.88                 | 0.87     | 219                              |
| Pop A-3        | 51          | 19.45              | 18.00          | 0.88                 | 0.87     | 104                              |
| Pop B-1        | 51          | 17.48              | 16.45          | 0.83                 | 0.82     | 97                               |
| Pop B-2        | 16          | 5.00               | 4.91           | 0.64                 | 0.59     | 27                               |

| Source of variations | Sum of variances | Percentage |
|----------------------|------------------|------------|
| Among individuals    |                  |            |
| PopA-1               | 6672.9412        | 0.2069     |
| PopA-2               | 11165.3743       | 0.3462     |
| PopA-3               | 5294.7049        | 0.1642     |
| PopB-1               | 5022.7740        | 0.1557     |
| PopB-2               | 1091.0731        | 0.0338     |
| Among SSR alleles    |                  |            |
| PopA-1               | 265.0000         | 0.0082     |
| PopA-2               | 501.0000         | 0.0155     |
| PopA-3               | 231.0000         | 0.0072     |
| PopB-1               | 239.0000         | 0.0074     |
| PopB-2               | 146.0000         | 0.0045     |
| Among populations    | 1621.3861        | 0.0503     |
| Total                | 32250.2537       | 1.0000     |
Genetic clusters could be due to a variety of factors, including geographical distribution of green foxtail (Darwin, 1859). The mixed geographical distribution of green foxtail accessions may result from a combination of natural and human activities. This conjecture needs more work in order to be verified. The geographical distribution structure of Chinese green foxtail landraces, although the sample size of other reports on the green foxtail population structure was too small to warrant discussion (Wang et al., 2012). In this trial, high gene diversity and PIC values of green foxtail from Northern China were found, and AMOVAs suggested that Northern China preserved a much higher diversity of green foxtail than other regions. Combining all those data and the earliest archaeological evidence found in the Yellow River region, it can be stated that Northern China is the first domestication centre of foxtail millet, if not the only one.

Understanding the population and geographical structure of both the wild and domesticated types is key in studying domestication. A typical example of this was the identification of Guangxi, China, as the place where rice was domesticated (Huang et al., 2012). The Neighbor–Joining phylogenetic tree constructed using the foxtail millet landraces and green foxtail SSR data (Supplementary Fig. S5 at JXB online) clearly divided the samples into the wild and domesticated gene pools, and four accessions of green foxtail from North China were closely related to foxtail millet, suggesting the origin of Chinese foxtail millet from the northern region. However, the exact place where it was domesticated is still ambiguous, because the four green foxtails that are closely related to foxtail millet are geographically separated. The mixed distribution of genetic clusters in the geographical eco-regions found in this study makes it difficult to answer this question. This is a similar conclusion to those reached by Bennett et al. (2012).

### Genetic structure of Chinese green foxtail and its geographical distribution

In previous studies of foxtail millet landraces, four subpopulations in China were described, and the genetic structure of the subpopulations was in concordance with the geographical distribution of eco-regions (Wang et al., 2012). Two clusters of green foxtail were clearly identified in this trial, but the distribution of the samples from each cluster was inconsistent with the geographical eco-regions. Nevertheless, PopA includes accessions mainly from higher latitude eco-regions in Northern China, and PopB contained a majority of lines from lower latitude eco-regions in Southern China. A lack of geographical population structure for 22 Asian and European green foxtail accessions was also reported by Le Thierry d’Ennequin et al. (2000) with AFLP markers, although the samples were collected from a much wider geographical range. Some regional geographical structures were identified by ISSR markers (Li et al., 2012), but the geographical distribution structure of 34 green foxtail accessions of worldwide origin was not clear (Li et al., 2012). The sample size of other reports on the green foxtail population structure was too small to warrant discussion (Wang et al., 2010; Hirano et al., 2011).

Why do foxtail millet landraces exist in a clear geographical population structure, while green foxtail accessions do not? Samples of both foxtail millet landraces and green foxtail were collected from the same eco-regions in China, and both kinds of samples were under the same natural environmental conditions of temperature, light, rainfall, and other factors. However, one of the main differences between wild green foxtail and domesticated foxtail millet is the human artificial selection on the cultivated forms, making it probable that human selection was the main factor which created the population structure of Chinese foxtail millet landraces, although this conjecture needs more work in order to be verified.

Wild samples from the vicinity in the same eco-regions probably share the same ancestor and are under the same natural environmental selection, so the geographical population structure in the wild species is a natural phenomenon (Darwin, 1859). The mixed geographical distribution of green foxtail genetic clusters could be due to a variety of factors, including germplasm migration induced by human and animal activities and natural factors; however, the present data are not sufficient to provide a precise cause for this phenomenon.

### Foxtail millet origin analyses using green foxtail as reference

As one of the oldest cereals in Eurasia, the origin and domestication of foxtail millet have been of great interest. Studies on morphological diversity (Li et al., 1995; Ochiai et al., 1996), isozyme type (Croullebois et al., 1989), and DNA markers of different kinds (Le Thierry d’Ennequin et al., 2000; Fukunaga et al., 2002, 2006; Li et al., 2012) have all indicated that the highest level of genetic diversity was found in Chinese samples, both of green foxtail and of foxtail millet. The earliest archaeological evidence to date is also located in Northern China (Barton et al., 2009; Lu et al., 2009; Yang et al., 2012). The genetic structure and diversity of Chinese foxtail millet landraces have been investigated, and it has been confirmed that foxtail millet probably originated in the Yellow River regions, where the highest genetic diversity of this species was preserved (Wang et al., 2012). In this trial, high gene diversity and PIC values of green foxtail from Northern China were found, and AMOVAs suggested that Northern China preserved a much higher diversity of green foxtail than other regions.

### Table 6. Percentage of SSR locus pairs in significant (P < 0.05) LD and LD statistics D’ and r2 of Chinese green foxtail populations

| Population | No. of significant marker pairs in LD | No. of marker pairs evaluated | Fraction of locus pairs (%) | Extent of LD |
|------------|-------------------------------------|-----------------------------|-----------------------------|-------------|
|            |                                     |                             |                             | D’  | r²  |
| PopA-1     | 128                                 | 520                         | 24.62                       | 0.788 | 0.025 |
| PopA-2     | 73                                  | 597                         | 12.23                       | 0.673 | 0.018 |
| PopA-3     | 72                                  | 578                         | 12.46                       | 0.809 | 0.035 |
| PopB-1     | 101                                 | 876                         | 11.53                       | 0.768 | 0.041 |
| PopB-2     | 1274                                | 1515                        | 84.09                       | 0.950 | 0.361 |
| All        | 352                                 | 586                         | 60.24                       | 0.581 | 0.005 |
by other studies (Le Thierry d’Ennequin et al., 2000; Li et al., 2012). Further genome sequence data and the identification of domestication-related genes would accelerate the understanding of this complex network of lineages of foxtail millet.

Population diversification and gene flow between green foxtail and foxtail millet

Although previous studies on intraspecific hybridization between green foxtail and foxtail millet have indicated repeated genetic introgression (Darmency et al., 1987; Jarvis and Hodgkin, 1999; Wang et al., 1995; Wang et al., 2010), the Neighbor–Joining phylogenetic tree of Chinese green foxtail and foxtail millet showed a clear division between the two gene pools (Supplementary Fig. S5 at JXB online), which suggests that the genetic introgression between the two gene pools is not so frequent. To investigate gene migrations between green foxtail and foxtail millet, $Nm$ was calculated (Statkin et al., 1989) by conducting classical $F_{ST}$ analyses using previously published data (Wang et al., 2012) (Supplementary Fig. S4). The highest level of gene flow was identified within the green foxtail subpopulations, and the lowest level was characterized between the green foxtail and the foxtail millet landraces (Supplementary Fig. S4). This is consistent with the relatively high level of allele heterozygosity of 0.076 (0.0111–0.6469) identified in this trial for green foxtail (Table 2), while that of foxtail millet was close to zero (Wang et al., 2012). The relatively high level of genetic introgression within the green foxtail millet subclusters may be one of the reasons for the mixed geographical population structure found in this report. The homozygosity of wild green foxtail is lower than that of domesticated foxtail millet, but it is more homozygous than wild rice (Gao et al., 2002; Zhou et al., 2003).

An $F$-test between green foxtail and foxtail millet revealed 24 SSR loci that had significantly (>97.5%) diversified between these two gene pools, owing to the long period of environmental adaptation or morphological selection. Five loci were localized in the gene-coding regions (Supplementary Table S3 at JXB online), which are potentially important genes of diverged metabolic pathways or have played vital roles in foxtail millet domestication.

Low level of LD of green foxtail

Lower LD was detected in this trial of wild green foxtail compared with a previous study on foxtail millet using the same approach (Wang et al., 2012). This was consistent with research comparing genomic regions of green foxtail and foxtail millet (Wang et al., 2010), and was also similar to analyses of wild and cultivated soybeans (Lam et al., 2010). Based on the present data, the LD level within each subpopulation was higher than the LD value of the total accessions, suggesting that population structure does exist in wild green foxtail. The lower level of LD in green foxtail than in foxtail millet may result from a higher rate of cross-pollination found in the homozygosity analysis of the sampled accessions. Rapid decay of LD also provides more opportunities for identification of potential markers/genes in trait association mapping, which can control important agronomical traits in green foxtail.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Determinations of value of $K$ for substructuring. (A and B) Optimal $K$ identified by LnP($D$) and delta $K$ of PopA. (C and D) Optimal $K$ identified by LnP($D$) and delta $K$ of PopB.

Figure S2. Shared and specific alleles of wild green foxtail and domesticated foxtail millet detected using SSRs.

Figure S3.Allele frequencies of SSR loci in wild green foxtail and cultivated foxtail millet landraces.

Figure S4. Gene flow estimated by $Nm$ inferred from classical $F$-test within and between green foxtail and foxtail millet.

Figure S5. Unrooted Neighbor–Joining tree of Chinese S. viridis (blue) and S. italica (green). Four accessions of S. viridis (red) from north China (Chaoyang, Wuhan, Changli, and Dingxi) were genetically closer to domesticated S. italica.

Table S1. Genetic diversities identified using SSRs in 288 green foxtail accessions.

Table S2. Comparisons of genetic diversity between wild green foxtail and cultivated foxtail millet.

Table S3. List of annotated genes co-localized with SSR loci detected as genomic regions under selection.

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