Domestication and Spread of Broomcorn Millet (<i>Panicum miliaceum</i> L.) Revealed by Phylogeography of Cultivated and Weedy Populations

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Received: 7 November 2019; Accepted: 30 November 2019; Published: 3 December 2019

Abstract: Cultivated broomcorn millet (<i>Panicum miliaceum</i> L.), one of the most ancient crops, has long been an important staple food in the semiarid regions of Eurasia. Weedy broomcorn millet (<i>Panicum ruderale</i> (Kitag.) Chang comb. Nov.), the companion weed of cultivated broomcorn millet, is also widely distributed throughout Eurasia and can produce fertile offspring by crossing with cultivated broomcorn millet. The evolutionary and genetic relationships between weedy and cultivated broomcorn millets, and the explicit domestication areas and detailed spread routes of this cereal are still unclear. The genetic diversity and population structure of 200 accessions of weedy and cultivated broomcorn millets were explored to elucidate the genetic relationship between weedy and cultivated broomcorn millets, and to trace the explicit domestication areas and detailed spread routes of broomcorn millets by using 23 simple sequence repeats (SSR) markers. Our results show that the weedy populations in China may harbor the ancestral variations that gave rise to the domesticated broomcorn millet. The population structure pattern observed in the wild and domesticated broomcorn millets is consistent with the hypothesis that there may be at least two independent domestication areas in China for the cultivated broomcorn millet, the Loess Plateau and the Northeast China, with both following the westward spread routes. These two westward spread routes of cultivated broomcorn millet coincide exactly with the prehistoric Oasis Route and Steppe Route, respectively.

Keywords: broomcorn millet; <i>Panicum miliaceum</i>; domestication; simple sequence repeats

1. Introduction

Broomcorn millet (<i>Panicum miliaceum</i> L.), also known as common millet, proso millet, hog millet, or white millet, is one of the most ancient crops in the world [1]. It has long been an important staple food in the semiarid regions of Eurasia, mainly in Russia, Ukraine, China, and India, because of its short growing season and high tolerance to heat and drought [2]. Its high nutritional value has gradually attracted more and more attention worldwide.
Broomcorn millet has been suggested to be domesticated in China since substantial broomcorn millet remains were discovered in archaeological sites in northern China predating 5000 cal. BC [1,3–9]. For example, the archaeological remains of domesticated broomcorn millet were discovered at the Xinglonggou and the Baiyinchanghan sites in Northeast China [1,3], the Dadiwan site in the Loess Plateau [4], the Cishan, the Zhangmatun, the Yuezhuang, the Baligang, and the Tanghu sites in the Middle and Lower Yellow River region [5–9]. However, the explicit domestication area of this cereal in northern China is still unclear based on these studies. Both the Loess Plateau and Yellow River Basin in North China and the Western Liao River Basin in Northeast China might be the domestication areas according to these archaeological studies. Therefore, the explicit domestication area of broomcorn millet and its spread routes after its domestication remains the fundamentally controversial issue in the domestication study of this cereal. A recent genetic study based on microsatellite and granule-bound starch synthase I (GBSSI) genotype data of cultivated broomcorn millet from pan Eurasian indicated that the Loess Plateau is one of the domestication areas of broomcorn millet, which spread westward via the Inner Asia Mountain Corridor [10]. However, the results did not rule out the possibility of the existence of other domestication areas of broomcorn millet [10]. Further studies of the domestication areas and detailed spread routes of broomcorn millet are now still needed to determine the explicit domestication area of broomcorn millet and its spread routes.

Usually, the domestication area of cultivated plants might be inferred from the distribution of their wild progenitors [11]. To date, the true wild progenitor of broomcorn millet is still uncertain. Weedy broomcorn millet (Panicum ruderale (Kitag.) Chang comb. Nov.), the companion weed of cultivated broomcorn millet, is the only species in Panicum exhibiting a widespread distribution across a region spanning from northern China to western Eurasia and can produce fertile offspring by crossing with cultivated broomcorn millet. The weedy broomcorn millet is considered possibly to be the descendant of the wild ancestor or a feral type of this cereal [2,12]. SSR markers have been successfully used to study the genetic diversity of broomcorn millet in several studies [10,11,13–15]. However, the study of domestication by comparing the genetic diversity between cultivated broomcorn millet and its weedy forms using SSR markers is still lacking and the explicit domestication areas of the broomcorn millet are still unclear. In our study, the evolutionary and genetic relationships between the weedy and cultivated broomcorn millets are studied using the simple sequence repeats (SSR) markers, also known as microsatellites. Specifically, three questions were addressed: (1) Is weedy broomcorn millet the descendant of the wild ancestor of the cultivated broomcorn millet? (2) Where is the explicit domestication area of cultivated broomcorn millet in China? (3) Where are the spread routes of this cereal after being domesticated in China? Our results support our hypotheses that (1) the weedy populations in China may harbor the ancestral variations that gave rise to the domesticated broomcorn millet; (2) there may be at least two independent domestication areas of broomcorn millet in China—the cultivars domesticated in the Loess Plateau, while the cultivars domesticated in the Northeast China; and (3) the westward spread routes of broomcorn millets that we speculated in this study coincide exactly with the prehistoric Oasis Route and Steppe Route, respectively.

2. Materials and Methods

2.1. Plant Materials

A total of 50 accessions of weedy broomcorn millet from China and 150 accessions of cultivated broomcorn millet from either China (83 accessions) or six other locations outside China (67 accessions) were analyzed in the present study (Table S1). The morphological characteristics are similar between weedy and cultivated broomcorn millets, while the main differences are found in seed color and size, plant height, type of panicle, and number of branches (Table S2) [2,12,16]. Overall, the cultivated broomcorn millet shows the domesticated phenotypes, while the weedy broomcorn millet shows the wild traits. In the present study, all accessions of the weedy broomcorn millet were collected and identified by the Institute of Crop Science, Chinese Academy of Agricultural Sciences (ICSCAAS) based...
on the morphological criteria described above. Most of the accessions of the cultivated broomcorn millet were landraces selected from the core collection and were also provided by the ICSCAAS (Table S1).

The cultivated areas of broomcorn millet in China were divided into seven regions according to the ecotype distribution of this cereal including (1) spring-sowing area of Northeast China, (2) spring-sowing area of North China, (3) summer-sowing area of North China, (4) spring and summer-sowing area of Chinese Loess Plateau, (5) spring and summer-sowing area of Northwest China, (6) spring-sowing area of Chinese Qinghai-Tibetan Plateau, and (7) autumn and winter area of South China [17]. The factors used to distinguish these regions include the latitude, altitude, annual average temperature and precipitation, property of soil, sowing season (spring or summer), grain (size and color), plant morphology (type of panicle, height, and root system), waxiness, and resistance (drought resistance, salt and alkali resistance, moisture resistance, and antiviral capability). The 83 cultivated accessions examined in this study from China were collected from all seven regions and the 50 weedy accessions were collected from four (regions 1, 2, 4, and 5) of these seven regions, while the 67 cultivated accessions were collected from six areas outside China, including Mongolia, Russia and Ukraine, Central Asia, India, Central Europe and Turkey, and the United States. Based on these geographic locations, the weedy and cultivated accessions were further defined into three and twelve populations, respectively, excluding the single cultivated accession (XZ1) collected from spring-sowing area of Chinese Qinghai-Tibetan Plateau and the single weedy accession (WXJ1) collected from spring and summer-sowing area of Northwest China (Table 1). Geographic distributions of the cultivated and weed accessions are shown in Figure 1A,B, respectively. It is noted that we used geographical boundaries to circumscribe the populations, though a few individual populations (with detailed geographical details provided) were also named based on the political boundaries just in order to make the explanations more convenient. Specifically, for the population of Russia and Ukraine, all samples in Russia were collected from the western areas in Russia, which were very close to Ukraine (Table S1; Figure 1A). Furthermore, both Russia and Ukraine are the main areas of producing broomcorn millets and have extensive agricultural exchanges, therefore, we combined the accessions from these two regions into one population in our study.

2.2. DNA Extraction and SSR Genotyping

Seeds from each accession were planted and grown in soil containing perlite and natural fertilizer. Leaf tissue from a single seedling for each accession at 15–20 days old was collected and dried in silica gel. DNA was extracted from leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s protocol. The purity and concentration of the extracted DNA was evaluated on an ND-1000 instrument (NanoDrop Technologies Inc., Wilmington, DE, USA). The final concentration of each DNA sample was adjusted to 30 ng/µL. A total of 23 primers showing polymorphisms in a previous study [15] were used to genotype the samples in this study. PCR was carried out in 20-µL reactions each containing 2 µL template DNA (60 ng of total DNA), 2 µL of PCR buffer, 1.6 µL of 25 mM MgCl₂, 0.5 µL of 10 mM dNTPs, 0.2 µL of 10 µM solution of each primer, 0.3 µL of 5 U/µL Taq DNA polymerase, and 13.2 µL ddH₂O. Reactions were carried out as follows: denaturation at 95 °C for 5 min, followed by 39 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 50 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were separated in an 8% polyacrylamide gel and visualized using silver staining. Band sizes were determined using a 50-bp DNA ladder.
Table 1. Genetic diversity parameters for 15 representative populations of weedy and cultivated broomcorn millets. N is the number of samples analyzed, $N_A$ the average number of alleles, $N_E$ the effective number of alleles, I the Shannon’s information index, $H_O$ the observed heterozygosity, $H_E$ the expected heterozygosity, and $A_P$ the private allelic richness. These parameters were not calculated for a population of cultivated broomcorn millet with a single accession (XZ1) from the spring-sowing area of Chinese Qinghai-Tibetan Plateau and a population of weedy millet with a single accession (WXJ1) from the spring and summer-sowing area of Northwest China, which were included in the combined cultivated and weedy populations, respectively.

<table>
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<th>Status</th>
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<th>Distribution Area</th>
<th>$N$</th>
<th>$N_A$</th>
<th>$N_E$</th>
<th>I</th>
<th>$H_O$</th>
<th>$H_E$</th>
<th>$A_P$</th>
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<td>19</td>
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<td>20</td>
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<td>0.539</td>
<td>0.478</td>
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<td>NCII</td>
<td>summer-sowing area of North China</td>
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<td>2.080</td>
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<td>3.217</td>
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<td>0.559</td>
<td>0.560</td>
<td>0.182</td>
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<tr>
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<td>Combined</td>
<td>All</td>
<td>50</td>
<td>4.913</td>
<td>2.790</td>
<td>1.119</td>
<td>0.511</td>
<td>0.580</td>
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<tr>
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<td>Mean</td>
<td>All</td>
<td>13.2</td>
<td>2.974</td>
<td>2.185</td>
<td>0.764</td>
<td>0.549</td>
<td>0.442</td>
<td>0.057</td>
</tr>
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</table>
The polymorphism information content (PIC) for each microsatellite locus was estimated using CERVUS 3.0 [18]. The linkage-disequilibrium (LD) for all pairs of microsatellite loci was inferred by sequential Bonferroni correction ($p < 0.05$) and was tested using GENEPOP (http://genepop.curtin.edu.au/). Genetic diversity parameters including the average number of alleles ($N_A$), effective number of alleles ($N_E$), Shannon information index ($I$), observed heterozygosity ($H_o$), unbiased expected heterozygosity ($H_E$) [19] of each population of sampled millets and of all the weedy or cultivated populations combined, respectively, were calculated using GenALEx 6.5 [20]. The private allelic richness ($A_P$) of each population of sampled millets and of all the weedy or cultivated populations combined, respectively, were estimated by sample-size corrected using ADZE [21] because the sample sizes were different among populations. Specifically, program ADZE was used to evaluate the number of alleles present in each of the set of populations but absent in the remaining populations, considering equal-sized subsamples from each population [21]. The significance of differences in the genetic diversity ($H_E$) and the private allelic richness ($A_P$) between the weedy and cultivated broomcorn millets were tested using Wilcoxon signed-rank tests. STRUCTURE 2.3.4 [22] was run with 1,000,000 Markov Chain Monte Carlo (MCMC) generations for setting the values of K from 1 to 10 and 200,000 generations of burn-in. The simulations were repeated 10 times for each K value. The optimal
number of genetic clusters was obtained using ΔK [23] performed by STRUCTURE HARVESTER (http://taylor0.biology.ucla.edu/structureHarvester/) [24]. The optimal model was selected based on the highest value of ΔK. We followed an admixture coefficient of 70% for assigning the accessions to genetic clusters, while accessions with lower admixture coefficient were assigned to the group of “mosaics.” All accessions assigned to clusters were further divided into groups according to the distributed areas, genetic cluster, and the growing status (cultivated or weedy). INSTRUCT analyses [25] was run with 1,000,000 Markov Chain Monte Carlo (MCMC) generations for setting the values of K from 1 to 10 with 200,000 generations of burn-in. Simulations were repeated 10 times for each K value. The results were compared with that of the STRUCTURE analysis. The distance-based cluster analysis for groups and for all accessions were carried out, respectively. Phylogenetic trees were constructed based on neighbor-joining method (with 1000 bootstraps) implemented in PowerMarker [26] and displayed by MEGA 7 [27]. Principal component analysis (PCA) based on Nei’s genetic distances between accessions was performed with NTSYSpc 2.1 [28].

3. Results

3.1. Microsatellite Loci Polymorphisms

A total of 122 alleles were observed in 200 accessions of broomcorn millets using 23 microsatellite loci, with an average number of alleles per locus of 5.304 and a range of 3–10 alleles per locus (the raw data not shown). The polymorphism information content (PIC) values of the 23 loci ranged from 0.158 to 0.751, with an average of 0.462. The PIC values of nine microsatellite loci were above 0.5 showing rich polymorphisms with the locus Lmx850 having the highest PIC value of 0.751, while the other 11 microsatellite loci with PIC values ranging between 0.25 and 0.5 indicating a moderate level of polymorphisms. Only three loci had PIC values under 0.25 suggesting low polymorphisms. Overall, the microsatellite loci in the present study show the levels of polymorphisms appropriate for analyzing the genetic diversity of broomcorn millets. Test of linkage disequilibrium (LD) showed no linkage-disequilibrium in 253 pairwise of microsatellite loci after Bonferroni correction implying independence of these microsatellite loci.

3.2. Genetic Diversity

Genetic diversity parameters for 15 populations of broomcorn millets (6 cultivated in China, 6 cultivated outside China, and 3 weedy) are listed in Table 1. The average number of alleles (\(N_A\)) per population ranged from 1.870 to 4.478 with an average of 2.974. The number of effective alleles (\(N_E\)) per population ranged from 1.790 to 2.791 with an average of 2.185. The range of Shannon’s information index (\(I\)) was between 0.514 and 1.091 with an average of 0.764. Among the populations, WNCI had the highest values of \(N_A\), \(N_E\), and \(I\), and the values of \(N_A\), \(N_E\), and \(I\) for all weedy broomcorn millets (4.913, 2.790, and 1.119) were all higher than those values for all cultivated broomcorn millets (4.783, 2.207, and 0.880) (Table 1). The private allelic richness (\(A_P\)) for 15 populations ranged from 0 to 0.182. The \(A_P\) of all weedy broomcorn millets (1.030) was significantly higher than that of all cultivated broomcorn millets (0.369; \(p < 0.001\)). The unbiased expected heterozygosity (\(H_E\)) for each of the weedy populations ranged from 0.549 to 0.574 and for all the weedy broomcorn millets is 0.580, while the \(H_E\) for each of the cultivated populations ranged from 0.373 to 0.539 and for all the cultivated broomcorn millets is 0.471, indicating the significantly higher genetic diversity in weedy broomcorn millets than in cultivated broomcorn millets (\(p = 0.015\)). Moreover, the observed heterozygosity (\(H_O\)) ranging from 0.487 to 0.559 in weedy populations and for all the weedy broomcorn millets is 0.511, and the \(H_O\) for each of the cultivated populations ranged from 0.533 to 0.600 and for all the cultivated broomcorn millets is 0.552, showing no significant difference between weedy and cultivated broomcorn millets (\(p = 0.313\)). Heterozygote deficiency (\(H_O < H_E\)) was revealed in most weedy populations, while the cultivated populations showed heterozygote excess (\(H_O > H_E\)).
3.3. Population Structure

Based on the SSR data, the genetic structure of weedy and cultivated broomcorn millets was investigated using the Bayesian structure analysis. The \( \Delta K \) method showed the maximal \( \Delta K \) occurred at \( K = 2 \), with the next largest peak at \( K = 3 \). Because the \( \Delta K \) method may erroneously perform under \( K = 2 \) \cite{29}, we present the results based on both \( K = 2 \) (Figure S1) and \( K = 3 \) (Figure 1C). The population structure under \( K = 3 \) separated the weedy broomcorn millets into three gene pools (pink, yellow, and blue), while the cultivated broomcorn millets were mainly attributed to pink and yellow gene pools (Figure 1C). Overall, the results showed that the pink and yellow gene pools were common in both weedy and cultivated broomcorn millets, while the blue gene pool was more frequent in the weedy but rare in cultivated broomcorn millets with only two cultivated accessions outside of China harbor (from CETUR in Poland) containing high percentage (~100%) of blue component.

The 134 accessions with an admixture coefficient of \( \geq 70\% \) were attributed to three clusters (A, B, and C), mainly harboring the pink (92 accessions), yellow (17 accessions), and blue (25 accessions) components, respectively, while the other 66 accessions with an admixture coefficient <70% were deemed “mosaics.” The geographic locations and results of the structure analysis for both the cultivated and weedy accessions are shown on Figure 1A,B. The genetic structure of cultivars in China and outside China is similar and mainly composed of Clusters A and B (Figure 1A). The accessions of Cluster A account for the largest proportion (57.3% of the cultivated accessions) and are widely distributed throughout Eurasia. In the populations distributed in central and southern regions of Eurasia, and in North America, including NCII, LPC, NWC, IND, CETUR, and US, the accessions of Cluster A are largely predominant except for a small number of “mosaics.” In addition, there were relatively few accessions of Cluster B (8.7% of the cultivated accessions) found in this study, which were limited in populations distributed in the northern regions of Eurasia including NEC, NCI, MON, and RUSUKR. It is worth noting that the accessions of Cluster C which plays a dominant role in weedy broomcorn millet were generally not found in cultivars except for two accessions of CETUR from Poland.

The accessions of Cluster A found in Chinese weedy populations WLPC and WNCI are mainly found in Gansu, Ningxia, and Southern and Northern Shanxi (Figure 1B), geographically belonging to the Loess Plateau. On the other hand, the weedy accessions of Cluster B are only found in population WNEC in the Northeast China. Both Clusters A and B have limited distribution areas. However, the extremely rare Cluster C accessions in cultivated populations had the largest proportion in weedy populations including WNEC, WNCI, and WLPC (46.0% of weedy accessions representing the portion of the individuals of the weedy broomcorn millets in Cluster C) and the widest distribution.

The results of INSTRUCT analyses were largely congruent with those derived from the STRUCTURE analyses. In comparison to the results of STRUCTURE analyses at \( K = 3 \), the output of INSTRUCT showed that the grouping status was changed for a total of 27 out of 200 accessions of both the cultivated and weedy populations between “mosaics” and either Clusters A, B, or C but not among Clusters A, B, or C. Specifically, in the cultivated populations, 11 accessions of “mosaics” identified by STRUCTURE analyses were revealed as either in Cluster A (6) or Cluster B (5) by the INSTRUCT analyses, while another four accessions of “mosaics” identified by INSTRUCT analyses were revealed as either in Cluster B (3) or Cluster A (1) by the STRUCTURE analyses. For the weedy populations, nine accessions of “mosaics” identified by the INSTRUCT analyses were revealed in Cluster C by the STRUCTURE analyses, while three accessions of “mosaics” identified by STRUCTURE analyses were realized in either Cluster A (2) or Cluster B (1) by the INSTRUCT analyses.

3.4. Phylogenetic Analysis

The relationships between genetic clusters at \( K = 3 \) was further explored by constructing a phylogenetic tree of all 200 accessions (Figure 2A). The topologies revealed by the phylogenetic tree was largely consistent with the results derived from the structure analyses. The accessions in Clusters A, B, and C revealed by the structure analyses were also identified by the phylogenetic analysis with a few exceptions. For example, three accessions in Cluster C (WNM58, WNX23, and WNX24) identified by
the structure analysis were embedded in the clade containing accessions of Cluster A. A group of seven accessions in Cluster B were also realized in the clade of Cluster A (Figure 2A). The weedy accessions of Clusters A and B were grouped with Clusters A and B of the cultivated accessions, respectively, while the weedy accessions of Cluster C were basically revealed as an independent clade with only two cultivated accessions from Poland embedded. These results showed that the overall genetic relationships among the 200 accessions are not clearly revealed by this phylogenetic tree. Therefore, it is important to analyze the phylogenetic relationships within the various populations of broomcorn millets. Specifically, we separated the samples belonging to Clusters A, B, and C from each other based on the results of the structure analyses, presumably these Clusters had different evolutionary origins and spread routes and combining all of these samples from different clusters into one group could affect the results of the genetic analyses. Therefore, to further reveal the genetic relationships among different genetic clusters from various distribution regions, the 132 of the 134 accessions of both weedy and cultivated broomcorn millets excluding the “mosaics” were divided into 22 groups based on their distributed areas, genetic clusters, and growing status (cultivated or weedy) with the single accession (LN4) of NCI assigned to Cluster B and accession (WXJ1) in Cluster A excluded.

Figure 2. Phylogenetic analysis of the weedy and cultivated broomcorn millets. (A) The neighbor-joining tree shown as a circled cladogram of 200 accessions of the weedy and cultivated broomcorn millets. Each accession with the admixture coefficient value ≥70% was attributed to Clusters A, B, and C based on the structure analysis at K = 3, and was labeled with the color of cluster to which it belongs to (Figure 1C), while accessions each with an admixture coefficient value less than 70% were deemed “mosaics” and colored as grey. (B) The neighbor-joining tree of 22 groups of broomcorn millets excluding “mosaics” constructed using distributed areas, genetic clusters (identified by Structure analysis at K = 3), and growing status (cultivated or weedy). The 22 groups of broomcorn millets were abbreviated as those given in Table 1 and hyphenated with either A, B, or C for Clusters A, B, and C, respectively. The color of the cluster for each group was indicated as in Figure 1C. The scale bars refer to the number of nucleotide substitutions per site.

The phylogenetic tree using neighbor-joining method was constructed based on the Nei’s genetic distance among these 22 groups (Figure 2B). Clusters A, B, and C were revealed in three branches with relatively high bootstrap support, respectively. On the branch of Cluster A, weedy groups in China, cultivated groups in China, and cultivated groups outside China were largely separated from each other, with the exception that the Chinese cultivated group from Xinjiang (NWC-A) being clustered with groups outside China. On the branch of Cluster B, the only weedy group (WNEC-B) being closely related to the cultivated group from the same region (NEC-B), while the other two cultivated groups
outside China (MON-B and RUSUKR-B) are closely related to each other. On the branch of Cluster C, the only cultivated group (CETUR-C) is closely related to the weedy group WNEC-C.

### 3.5. Principle Component Analysis

PCA was performed on 200 weedy and cultivated broomcorn millet accessions based on Nei’s genetic distance (Figure 3). The accessions of Clusters A, B, and C were distinctly separated. On the first principal component (PC1), the accessions of Cluster C were separated from those of Clusters A and B. On the second principal component (PC2), the accessions of Cluster A were roughly separated from those of Cluster B. However, no clear distinction is identified between the “mosaics” and the accessions of either Clusters A, B, or C.

![Figure 3. Principal component analysis for 200 accessions of weedy and cultivated broomcorn millets.](image)

Each weedy broomcorn millet accession and cultivated broomcorn millet accession is represented by a triangle and a circle, respectively. The color of each accession is indicated by the cluster it belongs to (Figure 1C). The “mosaics” are marked as gray.

Some weedy and cultivated broomcorn millets belonging to the same cluster in the same region showed close genetic relationships (Figure 3), such as the weedy accession WSX63 from Datong, Shanxi and the cultivated accession SX31 from Pinglu, Shanxi. Both of these two accessions belong to Cluster A and are from the northern part of the Shanxi province in the spring-sowing area of North China. They are largely coincident on the PCA plot, showing the close genetic distance between these accessions. Some other weedy and cultivated accessions of Cluster B including WHLJ33 from Tailai, Heilongjiang, WHLJ34 from Duerbote, Heilongjiang, WJL12 and WJL13 from Baicheng, Jilin, and NM22 from Humengmoqi, Inner Mongolia, which are all from the junction of Heilongjiang, western Jilin, and eastern Inner Mongolia in spring-sowing area of Northeast China, also showed close genetic distance on the PCA plot. Moreover, the only two cultivated accessions of Cluster C from Poland are closely related to the weedy accessions WNM17 and WHB39 from the south-central part of spring-sowing area of North China, where there are plenty of weedy accessions of Cluster C and
a few cultivated “mosaics” with large proportion of blue component, e.g., SX28, NM45, SAX9, and NM14 with admixture coefficient of blue component of 63.1%, 46.5%, 44.1%, and 32.0%, respectively. Overall, the genetic relationships of most of the accessions do not show significant correlation with the geographical distribution.

4. Discussion

4.1. Genetic Relationship between Weedy and Cultivated Broomcorn Millets

In this study, the genetic diversity of a total of 200 weedy and cultivated broomcorn millet accessions with broad geographic distributions was investigated based on 23 SSR markers. To our knowledge, this is the first time to carry out such a comprehensive genetic study on both weedy and cultivated broomcorn millets to investigate the genetic relationship between them. The weedy broomcorn millet, as a companion weed of cultivated broomcorn millet, is broadly distributed in areas from northern China to Europe and even North America. There is no reproductive isolation between weedy and cultivated broomcorn millets. The weedy broomcorn millet is suggested to be (1) the descendant of the wild ancestor of cultivated broomcorn millet [2,30], (2) the feral types formed by the reverse mutation of cultivated broomcorn millet [12,31], or (3) the product of introgression between cultivated broomcorn millet and its wild relatives [32]. Under these three suggested conditions, the genetic information of the original type of broomcorn millet may be preserved in the weedy broomcorn millet. Therefore, the study of the genetic diversity of weedy broomcorn millet will provide important genetic information for investigating the domestication of the broomcorn millet.

The wild populations of many crops are generally considered as hybrid swarms, including both wild and feral forms. The feral forms are further categorized as either endoferal forms which are derived from a single domesticated lineage or exoferal forms which are formed by admixture among domesticated lineages or between domesticated taxa and their wild relatives. It is well-known that both the endoferal and exoferal forms are common in weedy crops [33,34].

Results of the structure analysis (Figure 1C) showed that when K = 3, the weedy and cultivated broomcorn millet accessions were classified into Clusters A, B, and C, with weedy broomcorn millet accessions being dominant in Cluster C and the cultivated broomcorn millet accessions attributing mostly to Clusters A and B. The weedy accessions of Cluster C are widely distributed in North China, while the cultivated accessions of Cluster C had only two individuals found in Poland. It seems that the weedy broomcorn millets in Cluster C have not been fully domesticated and cultivated in China, or else the Cluster C cultivars were once domesticated in China and later were gradually eliminated by the artificial selection. Therefore, the widely distributed weedy broomcorn millets in Cluster C were unlikely to be the feral type escaped or formed by reverse mutations from the cultivated broomcorn millets. On the other hand, Clusters A and B were shared by weedy and cultivated broomcorn millets. Both the results of phylogenetic analyses and PCA show the close genetic relationships between the weedy and cultivated broomcorn millets in Clusters A and B (Figures 2 and 3). However, these results are not sufficient to reveal whether the weedy broomcorn millets in Clusters A and B are either the wild or feral types. It is worth noting that the Cluster A cultivars are distributed throughout China, while the weedy broomcorn millets in Cluster A are only found in the Loess Plateau of China. Furthermore, both the Clusters A and B distributed in Northeast China, with the weedy broomcorn millet in Cluster B found only in this area, indicate that the weedy broomcorn millets in Clusters A and B cannot be explained only by feralization.

The genetic diversity and private allelic richness were analyzed to further explore the genetic relationships between the weedy and cultivated broomcorn millet. The weedy broomcorn millets are found to be more diverse than the cultivated broomcorn millets as indicated by the significantly higher genetic diversity (p = 0.015) and private allelic richness (p < 0.001) in the weedy populations. Higher genetic diversity or higher private allelic richness were generally found in the wild forms of many crops compared to those of their cultivated and feral forms. For example, the cultivated rice has been shown
to have a reduced subset of the total genetic variation of its wild ancestor [35], and in the feral form of rice, the nucleotide diversity is also found reduced in domestication-related genes sh4 and PROG1 compared with the wild rice, perfectly coincident with a similar diversity reduction in domesticated rice [34]. A recent study has shown an average reduction of 50% in genetic diversity in western and eastern domesticated barleys was observed relative to wild barley, while the Tibetan weedy barleys proven to be the feral forms showed lower genetic diversity than western and eastern domesticated barleys [36]. Also, the soybean was found to be genetically less diverse than its wild progenitor [37]. On the basis of these studies and our results, we speculate that at least some of the weedy broomcorn millets are the wild types which could be the representation of the ancestral inherited type of the cultivated broomcorn millet in the same genetic cluster. However, it is still possible that some of the weedy broomcorn millets are endoferal types escaped or formed by reverse mutations from cultivars. Furthermore, some weedy broomcorn millets with an admixture coefficient value less than 70% are probably the exoferal types caused by the gene flow between weedy and cultivated broomcorn millets. Lastly, the condition of $H_O > H_E$ indicating heterozygote excess in our study revealed in the cultivated broomcorn millets but not in the weedy populations is probably due to the artificial selection and the domestication bottleneck in the cultivated populations, indicating that the weedy broomcorn millets are mainly composed of wild forms. Further analysis is necessary to distinguish the wild and feral type of broomcorn millet according to the morphological and genetic characteristics. More importantly, these conclusions are also supported by the results of phylogenetic analysis (Figure 2) and PCA (Figure 3) showing that there was close genetic distance and inherited relationship among some of the weedy and cultivated broomcorn millets of the same cluster in the same area and the existence of the difference of the genetic structures among the broomcorn millets in various populations.

It is worth noting that it was reported previously that most loci might be expected to be homozygous in a highly inbred species [11]. We speculate that the higher heterozygosity in this study are probably due to the following reasons. First, excess heterozygosity was also reported previously based on SSR markers [14,15] probably because of the fixation of heterozygotes by the disomic inheritance of the heterotetraploid, leading to the high tolerance of inbreeding and selfing in the allopolyploid [38]. Second, because the 23 highly polymorphic makers used in the present study were selected based on 67 microsatellite markers identified by Liu et al. [15], therefore, it is possible for these 23 markers to show higher heterozygosity than those reported by Liu et al. [15] or others. Third, studies showed varied heterozygosity among different samples of broomcorn millets. For example, Rajput and Santra [14] reported that the number of alleles by all 100 SSR markers per genotype ranged from 41 to 317. The samples of broomcorn millets used in our study are both weedy form and landraces mostly from the core collection, indicating the possibility of having high heterozygosity.

4.2. Domestication Areas of Cultivated Broomcorn Millet in China

It is generally believed that the cultivated broomcorn millet originated from China [1,4,5,10,39], but its explicit domestication area in China is still unclear. Several geographic locations have been suggested as the possible domestication areas, including the upstream of western Liao River Basin in Northeast China [1] and the Loess Plateau area [4]. Genetic analysis of ITS and ETS DNA sequences of 199 accessions of the cultivated broomcorn millet suggested that the broomcorn millet originated in China, but the exact domestication area could not be determined yet because no significant difference in genetic structure of broomcorn millet was found in various regions of China [39]. Hunt et al. [10] indicated that the Loess Plateau is one of the possible domestication areas of broomcorn millet. Further studies are needed to explore the other possible domestication areas in addition to the Loess Plateau, if there are any.

In order to investigate the domestication areas of cultivated broomcorn millet in China, we conducted a comparative analysis of weedy and cultivated broomcorn millets in China excluding the admixed weedy individuals because of their possible feral status. We speculated that the wild forms of the current weedy broomcorn millet probably represents the inherited type of the ancestor of the
cultivated broomcorn millet in the same genetic cluster. We further assume that the distribution areas of the current populations of the weedy broomcorn millet probably cover the locations of the true wild ancestors in the same genetic cluster and may also be the indication of the domestication area from the wild ancestors to the cultivated broomcorn millet. We here further discuss the possible domestication areas of broomcorn millets.

First of all, results of the structure analysis showed that when K = 3, both weedy and cultivated broomcorn millets were each divided into three Clusters (A, B, and C) (Figure 1C). In the cultivated broomcorn millet, the Cluster A accessions accounted for the largest proportion and were widely distributed in various areas in China and outside China (Figure 1A). However, in the weedy broomcorn millet, accessions of Cluster A were located in the spring and summer-sowing area of Chinese Loess Plateau and south-central part of spring-sowing area of North China (Figure 1B). Because both areas belong to the Loess Plateau, we conclude that the cultivated broomcorn millet of Cluster A was probably domesticated in the Loess Plateau. Meanwhile, the results of PCA also showed that some weedy and cultivated accessions of Cluster A from spring-sowing area of North China (e.g., WSX63 and SX31) had extremely close geographic and genetic distances (Figure 3), suggesting that the Loess Plateau might be the domestication area of the broomcorn millet in Cluster A. Coincidently, two archaeological sites with earlier discovery of broomcorn millet in China, the Dadiwan site [4] and the Cishan site [5], are located at the west and east ends of the Loess Plateau, further indicating that the Loess Plateau might be the domestication area of the broomcorn millet in Cluster A.

Second, the Chinese cultivated accessions of Cluster B are mainly distributed in the spring-sowing area of Northeast China and the eastern part of spring-sowing area of North China (Figure 1A). Similarly, the weedy accessions of Cluster B are all located in the spring-sowing area of Northeast China (Figure 1B), implying that there may be a domestication area of Cluster B broomcorn millet in Northeast China. Furthermore, some weedy and cultivated accessions of Cluster B from the central area of spring-sowing area of Northeast China, e.g., WHLJ33, WHLJ34, WJL12, WJL13, and NM22, showed extremely close genetic distance on the PCA plot (Figure 3), and the weedy group WNEC-B in Cluster B from spring-sowing area of Northeast China was closely related to the cultivated group NEC-B in Cluster B from the same area on the neighbor-joining tree (Figure 2B). These results suggested that the Northeast China might be the domestication area of broomcorn millet in Cluster B. Furthermore, large quantity of grain remains of broomcorn millet was found in Xinglonggou site [1], the early Neolithic site in the south-west end of the Northeast China, supporting the conclusion that the broomcorn millet in Cluster B was domesticated in the Northeast China.

Lastly, the weedy accessions of Cluster C are found to be distributed throughout the northern China (Figure 1B), while the cultivated accessions of Cluster C are very rare and are only found in Poland (Figure 1A). Therefore, the domestication area of Cluster C is still uncertain. The results of phylogenetic analysis showed that the only cultivated Cluster C group (CETUR-C) was closely related to the weedy groups WNEC-C and WNCI-C in Cluster C (Figure 2B), suggesting that the Cluster C broomcorn millet may be domesticated in the distribution areas of groups WNEC-C and WNCI-C, i.e., the spring-sowing area of Northeast China and the spring-sowing area of North China. The results of PCA showed that the only two accessions in Cluster C of the cultivated broomcorn millet had the closest genetic distance to the weedy accessions WNM17 and WHB39 from the spring-sowing area of North China (Figure 3), suggesting the possibility that the Cluster C cultivars might be domesticated first in this area and then spread westward to central Europe, and this type of cultivars in China may have been gradually eliminated under the artificial selection. Although the Cluster C cultivars were not found in China, high proportion of blue components was found in a few Chinese cultivars. This was possibly caused by the hybridization between the previously existing Cluster C cultivars and the cultivars in Clusters A and B. Alternatively, it might be the result of introgression of weedy broomcorn millet in Cluster C into the Clusters A and B cultivars. It is noted that we cannot yet rule out the possibilities that (1) the Cluster C broomcorn millet was not domesticated in China because of the lack
of sampling of cultivated accessions in Cluster C in China and (2) the Cluster C broomcorn millet was domesticated in Europe because of the lack of sampling of weedy accessions from Europe.

To date, there are two main viewpoints on the domestication area of broomcorn millet in China. The early archaeological findings of broomcorn millet at Dadiwan site [4] and the results of the genetic analysis of modern broomcorn millet [10] indicated that the Loess Plateau is the domestication center of broomcorn millet, while the archaeological findings of broomcorn millet at Xinglonggou site [1] suggested that the western Liao River Basin in Northeast China is the original domestication area of broomcorn millet. In our study, the genetic diversity and genetic structure of weedy and cultivated broomcorn millets based on the analysis of SSR markers showed that there may be at least two independent domestication areas of broomcorn millet in China. The cultivars in Cluster A were domesticated in the Loess Plateau, while the Cluster B cultivars were domesticated in the Northeast China. However, the domestication area of the cultivars in Cluster C remains unclear. These conclusions are consistent with the viewpoints proposed previously [1,4,10].

4.3. Spread Routes of Cultivated Broomcorn Millet

As a representative crop of dry farming, cultivated broomcorn millet is widely distributed in Asia, Europe, and even in North America and Australia. A large number of earlier archaeological sites with broomcorn millet remains have been found in northern China, Central Asia, and Europe, indicating that this cereal has spread throughout Eurasia at least 4000–5000 years ago. It is generally believed that cultivated broomcorn millet originated in northern China and then spread outside, including westward to Europe [10,11,39,40]. However, the detailed spread routes are still not clear.

We analyzed the population structure of 12 cultivated broomcorn millet populations distributed in China and outside China (Figure 1A) to show that the cultivars in Cluster A are widely distributed in Eurasia. In central and southern Eurasia including summer-sowing area of North China, spring and summer-sowing area of Chinese Loess Plateau, south-central part of spring-sowing area of North China, spring and summer-sowing area of Northwest China, as well as India, Central Asia, Turkey, and Central Europe, the Cluster A cultivars were predominantly distributed together with a few “mosaics” and two Cluster C cultivars in Poland, without any Cluster B accessions. However, in northern Eurasia including spring-sowing area of Northeast China, eastern part of spring-sowing area of North China, as well as Mongolia, Russia, and Ukraine, both the Clusters A and B cultivars account for a certain proportion. It is noted that a similar north-south differentiation of the Eurasian populations is also observed in study of Hunt et al. [10] based on the pan Eurasian dataset under K = 6. These results suggested that Clusters A and B might have different distribution patterns and spread routes. The Cluster A broomcorn millet was domesticated in the Chinese Loess Plateau, and then spread westward through Xinjiang in Northwest China, Central Asia, and West Asia to Central Europe. Simultaneously, it also spread eastward to North China, northward to Mongolia, Russia, and Ukraine, and southward to southern China. Furthermore, the limited distributions of the broomcorn millets in the Cluster B in northern Eurasia were almost completely overlapping with the distributions of yellow cluster broomcorn millets (K = 6) in the study of Hunt et al. [10], suggesting that Cluster B might be domesticated in Northeast China and spread to eastern Europe via Mongolia, Russia, and Ukraine. This spread route also coincides with the secondary phase of westward expansion suggested by Hunt et al. [10].

The results of phylogenetic analysis showed that the Cluster A groups in China and outside China are largely separated from each other. Among the Chinese groups, NWC-A, a group from spring and summer-sowing area of Northwest China (i.e., Xinjiang), clustered with groups outside China, specifically having the closest genetic relationship with IND-A from India (Figure 2B). These results further demonstrated the important role of Xinjiang in the westward spread route of Cluster A broomcorn millet. After reaching Xinjiang through the Hexi Corridor, some of the broomcorn millets continued to spread westward to Central Europe, while some others may spread southward to be the main source of Indian broomcorn millet. Previously, the study of ribosomal DNA in the broomcorn
millet remains of Xiaohe cemetery in Xinjiang also suggested that Xinjiang, as the only distribution area of the Xiaohe type, has undoubtedly become a key node on the spread route of broomcorn millet [39]. Furthermore, the results of phylogenetic analysis also showed that on the Cluster A branch, group CA-A from Central Asia did not cluster together with group NWC-A from Xinjiang, but with groups MON-A and RUSUKR-A from Mongolia, Russia, and Ukraine (Figure 2B). Because the accessions in group CA-A in the present study are all collected from northern Central Asia, suggesting that there may be a spread pathway for the Cluster A broomcorn millet between northern Central Asia and Mongolia, Russia, and Ukraine. Moreover, the group US-A from the United States showed a close genetic relationship with CA-A, MON-A, and RUSUKR-A (Figure 2B), indicating that the cultivated broomcorn millet in the United States is more likely introduced from the northern Eurasia. A number of “mosaics” found in various regions throughout Eurasia may be the result of recent hybridization between accessions from different clusters.

Archaeological studies have shown that prehistoric cultural exchanges between the East and the West may depend on two routes: One is from the Chinese Loess Plateau in the east, through the Hexi Corridor, the oasis on both sides of the Tarim Basin and the Pamirs, to the Central Asia, West Asia, and Europe in the west, known as the “Oasis Route”; the other route is from the west of the Great Xing’an Mountains, passing through the Mongolian Plateau, South Siberia, Central Asia, the north part of West Asia, to Europe in the west, which was called “Steppe Route” [41]. Along the Oasis route, the broomcorn millet spread outside China and went westward through “Inner Asia mountain corridor zone” in the third millennium BC, then first appeared in southern Central Asia in the second millennium BC [42]. By the second half of the first millennium BC, the broomcorn millet was widespread across the Europe and West Asia [43]. On the Steppe Route, the solid evidence for the spread of agricultural goods, especially broomcorn millet, into the Altai Mountains and southern Siberia was not available prior to the late second and early first millennia BC [44]. The spread routes of the Clusters A and B broomcorn millets we speculated in this study coincide exactly with the prehistoric Oasis Route and Steppe Route, respectively. Previous studies had shown that the spread of wheat from the West to the East also occurred on these two routes [41], further supporting the viewpoints that these two routes serve as the main pathways for the spread of broomcorn millet and the exchange of agricultural cultures between the East and the West in the prehistoric period.

5. Conclusions

We investigated the evolutionary and genetic relationships between weedy and cultivated broomcorn millets, and the explicit domestication areas and detailed spread routes of this cereal using SSR markers. We conclude that (1) the weedy populations in China may harbor the ancestral variations that gave rise to the domesticated broomcorn millet; (2) there may be at least two independent domestication areas of broomcorn millet in China—the cultivars in Cluster A were domesticated in the Loess Plateau, while the cultivars in Cluster B were domesticated in the Northeast China; and (3) the westward spread routes of the Clusters A and B broomcorn millets that we speculated in this study coincide exactly with the prehistoric Oasis Route and Steppe Route, respectively.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/12/835/s1. Table S1: Detailed information of the 150 accessions of cultivated and 50 accessions of weedy broomcorn millets. Table S2: The main morphological differences between weedy and cultivated broomcorn millets. Figure S1: Results of the structure analysis (K = 2) for the weedy and cultivated broomcorn millets in China and outside China.

Author Contributions: Conceptualization, Y.X. and M.L.; methodology, P.L., C.L., F.M., F.W., X.Z. (Xingyu Zhu), and X.Z. (Xin Zhao); validation, Y.X., M.L., F.S., X.Z. (Xinyu Zhao), and M.H.; formal analysis, Y.X., M.L., F.S., X.Z. (Xinyu Zhao), and M.H.; investigation, Y.X., M.L., and F.S.; resources, Y.X. and M.L.; data curation, Y.X.; writing—original draft preparation, Y.X., F.S., P.L., H.Z., M.L., C.L., F.M., X.Z. (Xinyu Zhao), M.H., F.W., X.Z. (Xingyu Zhu), and X.Z. (Xin Zhao); writing—review and editing, Y.X. and F.S.; visualization, Y.X.; supervision, Y.X.; project administration, Y.X. and F.S.; funding acquisition, Y.X.

Funding: This research was funded by the National Natural Science Foundation of China (grant number 31300279), the Fundamental Research Funds for the Central Universities, JLU, Ministry of Education, China, and the China
Agriculture Research System (CARS-06-13.5-A2). The APC was funded by Y.X. F.S. was supported by the Educational and Professional Leave from Georgia Gwinnett College when he participated in this work.

Acknowledgments: We thank the Institute of Crop Science, Chinese Academy of Agricultural Sciences (ICSCAAS) for providing the samples of the broomcorn millets.

Conflicts of Interest: The authors declare no conflict of interest.

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