Genetic Diversity and Classification of Cytoplasm of Chinese Elite Foxtail Millet [*Setaria italica* (L.) P. Beauv.] Germplasm

Zhengli Liu,* Ting Zhang, Cong Li, and Guihua Bai*

ABSTRACT

A single cytoplasmic source of foxtail millet male sterile lines has become a major limiting factor for wide utilization of heterosis in foxtail millet. To explore genetic diversity of Chinese foxtail millet cytoplasm, 23 pairs of mitochondrial DNA (mtDNA) primers that showed a high level of polymorphism among selected 14 genetically diverse foxtail millet accessions were identified after screening 34 pairs of consensus mtDNA primers that were derived from various plant species. The 23 pairs of primers were used to analyze genetic diversity of cytoplasm in 111 Chinese elite foxtail millet accessions. Genetic similarity coefficients for differentiation of cytoplasmic types were calculated based on the phylogenetic tree and female-parent-derived pedigree graph of all the tested accessions. The results show that the genetic diversity of foxtail millet cytoplasm is low, which makes Chinese foxtail millet germplasm vulnerable to the infection by cytoplasm-related diseases. The 111 tested accessions can be classified into eight cytoplasmic types: Qinyuanmujizui, Huangruangu, Riben60ri, Dahuanggu, Yingsuigu, Heizhigu, MissingI, and MissingII. The eight cytoplasmic types matched with the geographic and ecological distribution pattern of the most tested accessions. The results provided a scientific foundation for rationally utilizing these germplasm lines, diversifying cytoplasmic types in male sterile lines, and further improving hybrid foxtail millet cultivars.

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Abbreviations: mtDNA, mitochondrial DNA; OTUs, operational taxonomic units; PIC, polymorphism information content.

Heterosis has been proven to be the most important genetic factor that has attributed to increased crop yield in rice (*Oryza sativa*, L), maize (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and several other species (Wang and Zhang, 1982; Yao, 2007). In foxtail millet [*Setaria italica* (L.) P. Beauv.], heterosis has also been used in developing hybrid cultivars. Zhangzagu5, a hybrid cultivar for example, was released from Zhangjiakou Academy of Agricultural Sciences, Hebei Province, China, and yielded 12,159 kg per hectare versus conventional cultivars ranging from 4500 to 6000 kg per hectare in 2007. However, most of the currently released Chinese spring foxtail millet male sterile lines were derived from Chang10A, whose cytoplasm was contributed by Qinyuanmujizui (Liu et al., 2011; Wang et al., 1998), while most summer foxtail millet male sterile lines were derived from Huangmi1A with the cytoplasm from Dahuanggu (Liu et al., 1996; Liu et al., 2006). Thus, lack of genetic diversity in cytoplasmic sources is a serious problem for effective utilization of heterosis in foxtail millet.

A single source of cytoplasm makes hybrid cultivars vulnerable to infection by cytoplasm-related diseases, and these diseases can cause

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devastating yield losses and has become a limiting factor for production of hybrid cultivars (Cao and Rong, 1997). In the mid-20th Century, extensive use of T-type sterile lines in hybrid maize production led to a wide spread of the race 'T' of maize fungal pathogen, *Helminthosporium* (*H.*) *maydis*, and epidemics of the disease resulted in devastating losses in U.S. maize production, and led to the replacement of the T-type cytoplasm by C-type cytoplasm in hybrid maize production (Chen et al., 1979; Qin et al., 1989). Later, a new race of *Helminthosporium* (*H.*) *maydis* specific to the C-type cytoplasm again developed and attacked hybrids with the C-type cytoplasm (Liu et al., 1989; Li and Zhou, 1993).

Because of maternal inheritance of cytoplasm, using a single source of cytoplasm in male sterile lines will inevitably lead to all hybrid cultivars carrying a single source of cytoplasm. A specific cytoplasm-related pathogen race may quickly develop and become the predominant race under high selection pressure, and epidemics of the disease can cause devastating losses in hybrid crop production. Thus, utilization of novel cytoplasmic types in male sterile lines is urgently needed, and determination of cytoplasm genetic diversity and classification of foxtail millet germplasm is the foundation for discovery and deployment of novel types of cytoplasm in breeding programs.

Foxtail millet originated from China and has been a traditional cereal food crop since ancient times. Archaeological evidence indicated that foxtail millet had been utilized as food in northern China 10,000 yr ago (Yang et al., 2012). It requires minimum water and fertilizer input for growth due to its high photosynthesis efficiency and drought tolerance. It also contains various amino acids and nutritive minerals in its grain; therefore, it has been one of the most important cereal crops in northern China (Liu et al., 2011). In addition, its small diploid genome (490 Mbp) similar to rice (Sivaraman and Ranjekar, 1984), self-pollination nature, and close relation to bioenergy grasses such as switchgrass (*Panicum virgatum* L.), etc., make it a suitable model species for investigation of plant architecture, genome evolution, and physiology in the bioenergy grasses (Doust et al., 2009). Currently, utilization of heterosis in foxtail millet has become a major breeding objective in many breeding programs in China.

Foxtail millet is still a minor crop and mainly distributed in developing countries such as China and India; thus, progress in foxtail millet research is slow, and the assessment of genetic resources is still in its infancy. More recently, several attempts have been made on construction of evolutionary trees to investigate the evolution and genetics of foxtail millet germplasm (Jia et al., 2009a, 2009b; Wang et al., 2006), and on assembling heterosis groups through population structure analysis (Liu, 2010; Liu et al., 2011). However, these reports are mainly aimed at the nuclear genes, and analysis on cytoplasmic genetic diversity has not been reported, especially through comparative analysis of mtDNA evolutionary trees and female-parent derivative pedigree trees to classify the germplasm.

Mitochondrial DNA exhibits maternal inheritance, and is an important genetic material outside the nuclear DNA of animal and plant species. It is distinguished from nuclear DNA by many unique characteristics, including a relatively higher mutation rate, simple structure, fast evolution, and genetic independence. These characters have been extensively used in studying phylogenetic relationships (Chowdhury and Smith, 1988; Wang et al., 2000; Yang et al., 1998), exploring plant geographic distribution (Tozuka et al., 1998), and evaluating mtDNA variation and diversity (Muza et al., 1995; Terachi et al., 1990). To examine mtDNA diversity, a set of consensus primers were designed to amplify polymorphic mtDNA at noncoding regions (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Duminil et al., 2002). In foxtail millet, mtDNA has been used to identify the geographical origination and the center of diversity of mtDNA types (Fukunaga and Kato, 2003); however, classification of germplasm using the consensus primers mtDNA has not been reported.

In this study, we introduced an mtDNA molecular evolutionary tree for classification of cytoplasm types of foxtail millet. The cytoplasm genetic diversity of foxtail millet germplasm was examined by studying mtDNA polymorphism among tested breeding materials using a set of consensus mtDNA primers. Both female-parent-derived pedigree graphs and cytoplasm evolutionary trees were used to determine the cytoplasmic types. The results provided a foundation for rational use of breeding materials to create diverse cytoplasmic types of male sterile lines and improve hybrid cultivars.

MATERIALS AND METHODS

Plant Materials

A total of 3356 foxtail millet accessions were initially collected in 2008 from all of the breeding programs in the north China summer millet region, and northwest and northeast China spring millet regions. After initial screening, 128 accessions from diverse geographic origins were selected as the foundation breeding materials based on their yield and adaptation performance, but 111 accessions were further evaluated at the Institute of Millet Crops of Hebei Academy of Agricultural and Forestry Sciences due to poor seed germination in 17 accessions. These 111 accessions that were used to construct the phylogenetic tree were mainly from eight-foxtail-millet growing provinces (Hebei, Henan, Shandong, Liaoning, Jilin, Shanxi, Inner Mongolia, and Shaanxi). In addition, two accessions (Gu66A and ZA1) with known pedigree and cytoplasm type were also included for construction of the female-parent-derivative graphs (Supplemental Table 1).

Selection of Consensus Primers and Detection of MtDNA Polymorphism

The experiment was conducted in the Institute of Millet Crops of Hebei Academy of Agricultural and Forestry Sciences. Young

leaf tissue was sampled at the four-leaf stage from each accession and grinded in a mortar with liquid nitrogen. Genomic DNA was extracted using the cetyltrimethyl ammonium bromide method (Liu et al., 2011).

Fourteen foxtail millet accessions with large genetic differences in their maternal sources and population structure (Liu et al., 2011) were selected from 111 accessions (Supplemental Table 1). A set of 34 consensus primers were selected to screen either intergenic or genic regions of the mitochondrial genome (Duminil et al., 2002) of the 14 selected accessions. Primers with a high level of polymorphisms across the 14 accessions were used for further analysis of all 111 accessions.

Polymerase chain reaction (PCR) amplifications were performed with a 20 μL PCR mixture containing 0.2 μL of *Taq* DNA polymerase (5U/μl), 2.0 μL of 10 × PCR buffer, 2.0 μL each of deoxynucleotide triphosphate (dNTP, 2.0 mM), 1.0 μL of forward primer (10 μM), 1.0 μL of reverse primer (10 μM), 2.0 μL of template DNA (about 60 ng), and 11.8 μL of ddH_2O. The PCR reaction was incubated at 94°C for 4 min, then continued for 35 cycles of 45 s at 94°C, 45 s at various annealing temperatures for different primers (Duminil et al., 2002), and 1 to 4 min extension at 72°C varied with different primers (Duminil et al., 2002), with 8 min at 72°C at the end of cycles (Supplemental Table 2). Ten microliters of PCR product with 1 μL of bromophenol blue was analyzed in a 1.5% agarose gel with 1×TAE buffer running at 120 V and 400 mA for 1 h. The DNA size marker was 5 μL DL2000 (Sangon, Shanghai, China). Then, the amplified products were detected by ethidium bromide (EB) staining for 5 min, then visualized and photographed under a UV transilluminator Binta 2020D (Beijing Binta Instrument Technology Co., Ltd, Beijing, China). Data were scored manually using 1 as presence of a band and 0 as absence of a band and checked twice to remove ambiguous data.

Data Analysis

Marker data were analyzed using NTSYS-pc2.1 software (Rohlf, 2000). Genetic diversity and polymorphism information content (PIC) were calculated using PowerMarker (Liu and Muse, 2005). Various association coefficients for qualitative data were computed using Similarity for Qualitative Data (SimQual). Cluster analysis was performed using an unweighted pair-group method with arithmetic means (UPGMA) of sequential agglomerative hierarchical nested (SAHN) cluster analysis. At first, two operational taxonomic units (OTUs) with minimum genetic distance were merged together to form a new OTU, and the branch node of the new OTU was located in the middle between the two OTUs; then, the average distances between the new OTU and all other OTUs were computed to identify the two OTUs with a minimum genetic distance to extend the cluster. This process was repeated until all of the OTUs were clustered to form a complete cytoplasm molecular evolutionary tree.

The materials were arranged vertically according to the order in the evolutionary tree to draw a female-parent-derivative pedigree graph. For these accessions with clearly identified maternal genealogy, they were traced to original female ancestors (landraces), and the identified relationship was drawn using solid lines in the graph; for these accessions without identifiable maternal genealogy, their estimated relationship was depicted using dashed lines according to their clustering results.

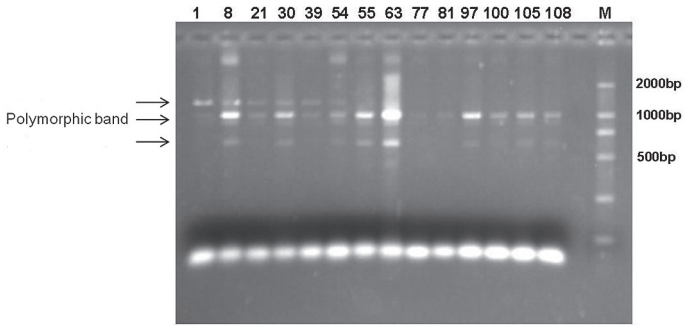

Figure 1. Amplified products of 14 selected foxtail millet samples (see Supplemental Table 1) by primer Nad7/1–2. M = DL2000 size standard marker.

By comparison between the female-parent-derivative pedigree graph and cytoplasm (mtDNA) evolutionary tree, the branch(s) that could clearly identify an individual group with the same maternal genealogy and separate it from the groups with different maternal genealogies in the evolutionary tree were classified as a cytoplasmic type. The cytoplasmic type was determined by its maternal source in the female-parent-derivative pedigree graph in the collection.

RESULTS

Cytoplasm Genetic Diversity of Foxtail Millet Accessions

A total of 34 consensus plant mtDNA primers derived from 28 different species were collected from the previously published literature (Demesure et al., 1995; Dumolin-Lapegue et al., 1997; Duminil et al., 2002). Initial screening of these primers across 14 selected foxtail millet accessions identified 23 primers that amplified foxtail millet and showed polymorphisms (Fig. 1). The rate of polymorphisms was 67.6%, implying a high level of polymorphisms of the mtDNA primers derived from different species in foxtail millet.

After screening 111 foxtail millet accessions with the 23 primers, 28 polymorphic bands were amplified. The mean genetic diversity was 0.29 and mean PIC was 0.23. The genetic similarity coefficients varied from 0.55 to 1.00 with an average of 0.72 among 111 accessions. These results indicated high similarity and a narrow source of the millet cytoplasm among tested accessions.

By comparing the mtDNA molecular evolutionary trees to the female-parent-derivative pedigree graph (Fig. 2), we identified a genetic similarity coefficient around 0.74 as the cutting point for separation of the groups with different cytoplasm sources. Thus, the mtDNA evolutionary tree was divided into seven branches (B1 to B7) that corresponded to seven types of cytoplasm based on their maternal genealogy.

Classification of Cytoplasm Types

All 111 accessions were classified into one of the seven cytoplasmic types. Type 1 (B1) included 12 accessions with genetic similarity coefficients ranging from 0.55 to 0.93. Nine of them were Qinyuanmujizui's derivatives, one was

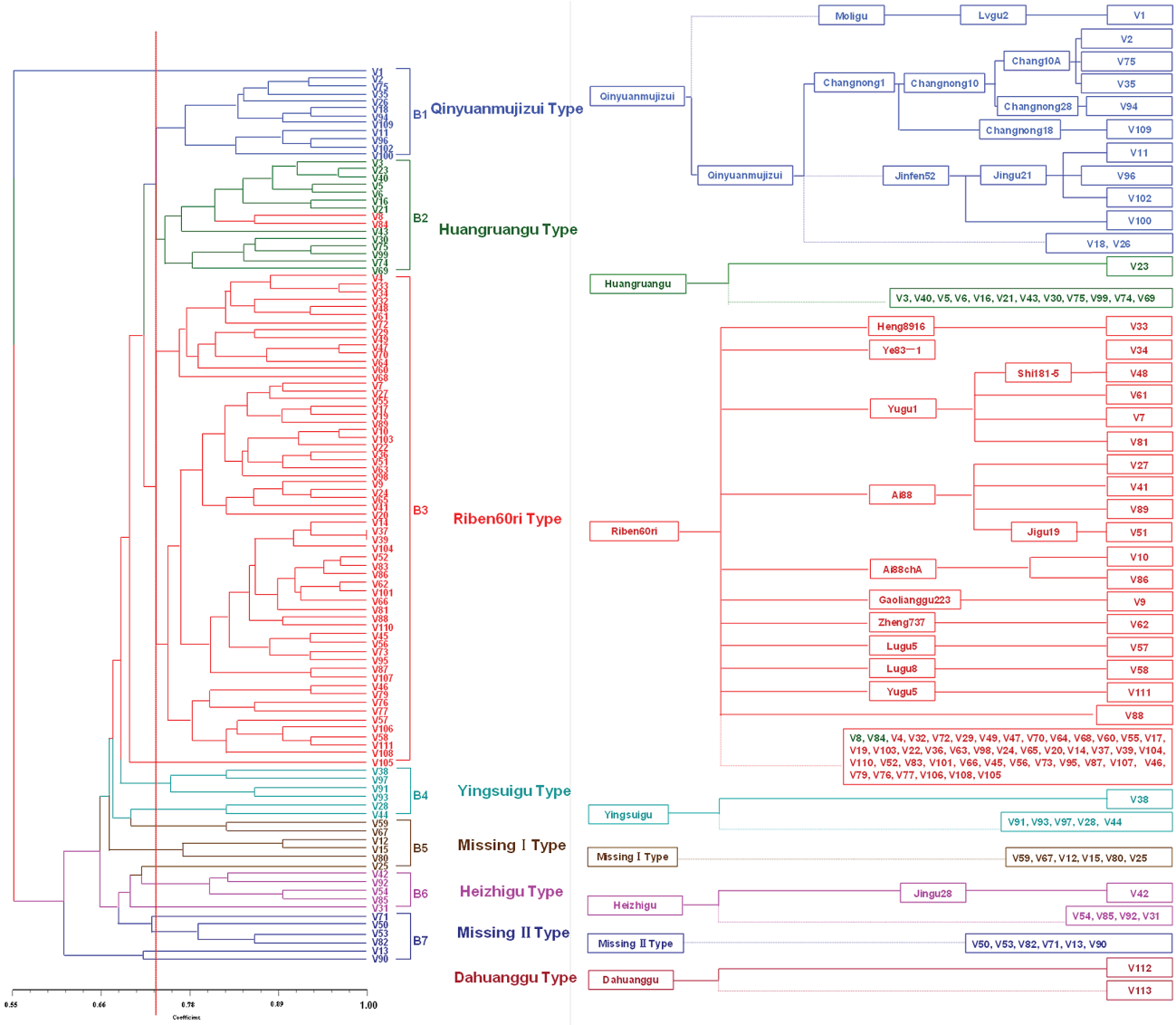

Figure 2. Classification of cytoplasm types by comparison between the mtDNA evolution tree (left) and female-parent-derivative evolutionary graph (right).

Moligu's derivative, and two were from unknown maternal sources. Structure analysis (Liu et al., 2011) showed that Qinyuanmujizui's derivatives and Moligu's derivatives belonged to the same group in terms of the nuclei genome. Therefore, Moligu is more likely a derivative of Qinyuanmujizui, thus the B1 type most likely has the same cytoplasm as Qinyuanmujizui and can be designated as Qinyuanmujizui type. Type 2 (B2) included 15 accessions with the genetic similarity coefficients ranging from 0.74 to 0.96. Only Datong28 has a known pedigree and can be traced to landrace Huangruangu; all other accessions, including four early maturity varieties from the Institute of High Latitude Crops of Shanxi Academy of Agricultural Sciences, seven from the northwest China spring millet region, and nine from the spring millet region, shared the same ecotypes as Datong28 because they were all developed from the northwest China spring millet region; thus, they can be considered as Huangruangu type. Among the accessions with different ecotypes from Datong28, three have unknown pedigrees and were likely Huangruangu's derivatives; only two were different because they were Riben60ri's derivatives. Since the cytoplasm of a majority of the accessions in B2 (13/15) was from Huangruangu, their cytoplasm type is designated as Huangruangu type. The conformance rate between Huangruangu cytoplasm type and B2 type in the molecular evolutionary tree was 86.7%. Type 3 (B3) is the biggest group, including 61 accessions with genetic similarity coefficients ranging from 0.70 to 1.00. Among them, 31 were Riben60ri's derivatives, and the other 30 accessions have unknown pedigrees. Among the 30 accessions with unknown pedigrees, eight are more likely Riben60ri's derivatives but distributed in different ecological regions, six of eight were Chinese spring millet accessions, and the other two, Chaolv1 and Meiguodatou, are from North Korea and America, respectively. Meiguodatou and Chaolv1 might be Chinese cultivars that were exported to America and North Korea. The other 22

Table 1. Pedigree, geographic distribution, and ecological distribution of 113 Chinese foxtail millet accessions used in this study.

		Ecological distribution			Geographic distribution				Genetic similarity coefficient
Type	No.	Ecotype	No.	%	Origin	No.	%	Basal germplasm	
Qinyuanmujizui	12	Northwest spring	12	100.0	Shanxi	8	66.7	Moligu Qinyuanmujizui	0.55~0.93
					Shanxi	2	16.7		
					Hebei	1	8.3		
					Liaoning	1	8.3		
Huangruangu	15	Northwest spring	9	60.0	Shanxi	7	46.6	Huangruangu	0.74~0.96
		North summer	5	33.3	Hebei	6	40.0		
		Northeast spring	1	6.7	Liaoning	1	6.7		
					Jilin	1	6.7		
Riben60ri	61	North summer	50	82.0	Hebei	39	63.9	Riben60ri	0.70~1.00
		Northwest spring	9	14.7	Shandong	6	9.8		
		Others	2	3.3	Shanxi	3	4.9		
					Liaoning	5	8.2		
					Henan	5	8.2		
					Inner Mongolia	1	1.6		
					Others	2	3.3		
Yingsuigu	6	Northwest spring	3	50.0	Jilin	2	33.3	Yingsuigu	0.69~0.86
		North summer	1	16.7	Hebei	2	33.3		
		Northeast spring	2	33.3	Shanxi	2	33.3		
MissingI	6	Northwest spring	1	16.7	Liaoning	1	16.7	Unknown	0.58~0.89
		North summer	5	83.3	Hebei	5	83.3		
Heizhigu	5	Northwest spring	1	20.0	Shanxi	1	20.0	Heizhigu	0.71~0.89
		North summer	4	80.0	Hebei	4	80.0		
MissingII	6	Northwest spring	2	33.3	Henan	1	16.7	Unknown	0.65~0.86
		North summer	4	66.7	Liaoning	2	33.3		
					Hebei	3	50.0		
Dahuanggu	2	North summer	2	100.0	Hebei	2	100.0	Dahuanggu	

accessions belonged to the north China summer millet type that shared the same ecotype as Riben60ri's derivatives, and most of them were developed from the Institute of Millet Crops of Hebei Academy of Agricultural and Forestry Sciences. Therefore, the B3 type almost fully matches with the Riben60ri cytoplasm type and is designated as Riben60ri Type. Similarly, the B4 in the molecular evolutionary tree matches with the Yingsuigu cytoplasm type, and the B6 matches with Heizhigu; thus, these two types are designated as Yingsuigu and Heizhigu types, respectively. The B5 and B7 in the molecular evolutionary trees correspond to cytoplasm types MissingI and MissingII, respectively. Accessions in these two groups have unknown pedigrees. The last cytoplasm type included two control cultivars, Gu66A and ZA1. Both Gu66A and ZA1 were Dahuanggu' derivatives, and thus they are Dahuanggu type. These two cultivars were not included in the 111 accessions for construction of the mtDNA evolution tree, thus all of the 113 accessions are classified into eight cytoplasmic types: Qinyuanmujizui, Huangruangu, Riben60ri, Dahuanggu, Yingsuigu, Heizhigu, MissingI, and MissingII. The match rate between the mtDNA molecular evolutionary trees and the female parent-derivative-evolutionary graph was high (98.2%), with only two exceptions, Bao182 and Jigu24, in B2. The results indicated that the consensus primer set is sufficient for classification of cytoplasmic types in Chinese germplasm.

Geographic and Ecological Distributions of Different Cytoplasmic Types

Analysis on geographic distribution of the eight cytoplasmic types (Table 1) showed that 12 accessions were Qinyuanmujizui type (B1) mainly from the South Central Shanxi Province (8 accessions, or 66.7%), only two (16.7%) were from the north Shaanxi Province, and one each was from the northeast Hebei Province and west Liaoning Province. They all belong to the northwest spring millet ecotype.

In the Huangruangu type (B2), seven accessions (46.7%) were from the northwest Shanxi Province, one (6.7%) was from the northwest Hebei Province, five (33.3%) were from the central and south Hebei Province, and one (6.7%) each was from the Liaoning and Jilin Provinces, respectively. These accessions with Huangruangu cytoplasm type were mainly grown in three different ecological regions, the northwest spring millet region (60%), northeast spring millet region (6.7%), and north summer millet region (33.3%), thus they mainly distributed in the northwest spring millet region. Among the 61 accessions in Riben60ri type (B3), 50 (82.0%) belonged to the North summer millet ecotype that were from Hebei, Shandong, and Henan Provinces, nine (14.7%) belonged to the northwest spring millet ecotype from Shanxi, West Liaoning, and East Inner Mongolia, and one each (3.3%) was from America and North

Korea. These results showed that Riben60ri type mainly distributed in the north summer millet region.

Among the six accessions of Yingsuigu type (B4), two (33.3%) were northeast spring millet type from Jilin, three (50%) were northwest spring millet type from Hebei and Shanxi, and one (16.7%) was north summer millet type from Hebei Province. In Heizhigu type (B5), four (80%) accessions were north summer millet type from Hebei Province. MissingI (B6) and MissingII (B7) types were mainly north summer millet ecotype. Dahuanggu type (B8) was the major cytoplasm type in sterile lines currently used in hybrid cultivars in the north summer millet region.

DISCUSSION

Amplification of Consensus mtDNA Primers of Plant in Foxtail Millet

The consensus mtDNA primers were originally developed based on the mtDNA sequences of *Arabidopsis thaliana* and *Beta vulgaris* and then tested in 28 plant species with successful PCR amplification rates ranging from 71 to 100% (Duminil et al., 2002). Several primers (ccb203, ccb256, cox2/1–2, cox3, nad1/2–3, nad5/1–2, nad5/4–5, nad7/2–3, nad7/3–4, and orf25) gave amplification efficiency of 100% across all 28 species, but some others (rps4, nad6, nad4/3–4, nad2/4–5, and nad2/1–2) did not amplify any PCR in both rice and maize (Duminil et al., 2002). In this study, 34 pairs of consensus mtDNA primers were selected to amplify foxtail millet, and 23 of them can amplify at least one polymorphic band across 14 selected foxtail millet accessions. This is the first study to evaluate the usefulness of mtDNA consensus primers developed from other species in studying foxtail millet cytoplasm sources. Several primers amplified multiple bands in one accession, which were unlikely due to nonspecific amplification because they were polymorphic among different accessions and repeatable in different PCR runs for a specific accession. They are likely due to the presence of multiple copies of an mtDNA gene that were derived from duplication and recombination or the movement of mtDNA to nucleus during foxtail millet evolution (Duminil et al., 2002; Bensasson et al., 2001). Natural transfer of mtDNA from mitochondria to nucleus that generates nuclear copies of mtDNA (*numts*) is an ongoing evolutionary process (Hazkani-Covo et al., 2010). *Numts* are abundant in plants, and the longest *numt* known so far is a 620-kb partially duplicated insert of the 367-kb mtDNA of *Arabidopsis thaliana* (Stupar et al., 2001). Successful amplification of foxtail millet mtDNA using these primers from other plant species (Duminil et al., 2002) in this study suggests that mtDNA are conservative across different species. Interestingly, most primers that did not amplify in rice and maize also amplified PCR in this study except primer nad6 and rps4, suggesting that the evolution of the mitochondrial genome in foxtail millet might be more conservative than rice and maize. Thus, this set of consensus primers is suitable for the study of foxtail millet cytoplasm. Identification of the consensus set of primers for mtDNA provides an essential tool for further study of mitochondria and cytoplasm of foxtail millet to improve hybrid cultivars.

Genetic Diversity of Cytoplasm Types in Foxtail Millet

Liu et al. (2011) and Wang et al. (2012) investigated the genetic diversity of Chinese foxtail millet breeding materials using simple sequence repeat (SSR) to analyze the nucleus genome and pointed out that genetic diversity was high. However, that report was focused on the nucleus genome, not including the mitochondrial genome in cytoplasm used to develop male sterile lines. In contrast to the previous study, we found the genetic similarity was high (0.72) and genetic diversity was low (0.29) for mtDNA among 111 breeding materials analyzed. These materials were all included in the previous study and are major sources of germplasm used in foxtail millet breeding programs in China. The result indicates that these breeding materials were derived from very limited sources of cytoplasm although they have a high level of genetic diversity in the nucleus genome. Thus, identifying new sources of cytoplasm is urgently needed to diversify cytoplasmic resources to protect hybrid cultivars from the attack by new cytoplasm-related pathogens.

In this study, eight cytoplasm types were identified in 113 accessions. The Riben60ri type was the largest (61 accessions, or 55% tested accessions). This cytoplasm was mainly distributed in the north China summer millet ecological region, and 85.9% of Riben60ri type was from this region. The rest of the cytoplasm types in the north China summer millet region were Heizhigu, MissingI, and MissingII, which together had only 13 accessions. The cytoplasmic type in the north China summer millet ecological region is even narrower, and male sterile lines contained only the Dahuanggu type. In the northwest China spring millet region, the main cytoplasmic types were Qinyuanmujizui and Huangruangu. The Yingsuigu type was the main cytoplasm in the northeast China spring millet region. In each ecological region, only one basic cytoplasmic type was predominant despite a total of eight cytoplasmic types identified in the collection. The results indicated that a single source of cytoplasm of male sterile lines and lack of genetic diversity of cytoplasm in each specific ecological region is a major constraint for hybrid foxtail millet breeding, especially in the north China summer millet region where development of specificity to a predominant cytoplasm by a pathogen may result in devastating disease epidemics and great losses in foxtail millet production. Thus, research and utilization of rare cytoplasm types should be emphasized, and more attention should be paid to develop and discover new types of cytoplasm to breed for male sterile lines with diversified cytoplasm in each ecological region.

Qinyuanmuzizui and Huangruangu types were mainly distributed in the northwest spring millet region and were the cytoplasms of most of the varieties used in these regions. Riben60ri, Dahuanggu, Heizhigu, MissingI, and MissingII types were mainly distributed in the north China summer millet region. However, the Dahuanggu type was the major type of cytoplasm in male sterile lines used in hybrid seed production in these regions, and the rest of the other breeding materials mainly contained the Riben60ri type of cytoplasm. In the northeast China spring millet region, Yingsuigu was the only cytoplasmic type found, which may be due to an insufficient number of varieties collected from this region. Yingsuigu, Heizhigu, MissingI, and MissingII types together had only about 23 accessions, accounting for only 20.7% of the total accessions, suggesting that these four cytoplasmic types were relatively rare types of cytoplasm in Chinese foxtail millet germplasm.

The cytoplasmic types of foxtail millet germplasm from China follow certain geographic and ecological distribution patterns, which is different from the result derived from a study on the nucleus genome (Liu et al., 2011). With the extensive exchange of breeding materials across ecological regions, it is not unexpected that the nucleus genome of their derivatives might not follow a certain ecological distribution pattern. However, in breeding programs, locally adapted cultivars are usually used as the female parent in crosses, and introduced cultivars from other regions are usually used as male parents to contribute to the nucleus genome. Thus, new cultivars developed from one region usually contain cytoplasm from the local cultivars due to the cytoplasm nature of maternal inheritance, which attributed to the geographic distribution pattern of different cytoplasmic types. Thus, to diversify cytoplasmic types in breeding programs, introduced cultivars should also be used as female parents in breeding crosses.

Classification of Cytoplasmic Types by Integration of Cluster Analysis and Female-Parent-Derivative Pedigree Graph

In a traditional clustering analysis, researchers usually arbitrarily determine a specific similarity coefficient for separation of groups based on their experience. For example, Jia et al. (2009a) arbitrarily set similarity coefficients as 0.77 and 0.8 for group separation based on their experience and identified two groups when the similarity coefficient was set at 0.77, and five groups when the similarity coefficient was set at 0.8; Wang et al. (2006) studied 96 millet accessions of different ecological types and found inconsistent results between the cluster groups and ecological types. The classification based on experience is more subjective and may lead to significant errors that deviate from actual situations. In this study, we developed a new procedure for classification of foxtail millet cytoplasm. First, we analyzed a selected set of 14 diverse foxtail millet accessions using a set of consensus mtDNA primers from other crops to evaluate the feasibility of using the set of consensus primers in foxtail millet; second, we used the selected set of polymorphic consensus primers in foxtail millet to analyze the entire set of core breeding materials from China and constructed an mtDNA molecular evolution tree by cluster analysis of mtDNA marker data; third, instead of making artificial grouping, we grouped cytoplasmic types according to the female-parent-derivative pedigree graph built through maternal pedigree tracking. The genetic similarity coefficients that grouped materials with the same maternal genealogy source and separated materials with different maternal genealogies were used as grouping criteria to classify cytoplasmic types in the molecular evolutionary tree. This approach overcomes the subjectivity of traditional cluster analysis. The conformity between the molecular evolutionary tree and the female-parent-derivative pedigree graph was very high (98.2%); thus, the new modified approach developed in this study can be recommended for classification of cytoplasm in foxtail millet. To date, only two (Qinyuanmujizui and Dahuanggu) of these types of cytoplasm have been used in commercial production of most hybrid cultivars. However, recently, all of the eight types of cytoplasm have been used to create new male sterile lines. For example, Huangruangu cytoplasm type "Datong29" has been used to create new male sterile line "DZ2010N169A". Therefore, the cytoplasm types in male sterile lines of new hybrid cultivars will be diversified to improve resistance to cytoplasm-related diseases.

In this study, only two accessions, Bao182 and Jigu24, were found to be inconsistent between the mtDNA molecular evolutionary tree and the female-parent-derivative pedigree graph. This slight discrepancy between the two methods was expected. The number of polymorphic primers used may affect the accuracy of classification. To date, a study on foxtail millet mtDNA primers has not been reported. Thus, only limited mtDNA primers (23) were used to construct the mtDNA evolution tree in this study, and these primers were originally developed from other crop species. Although the number was enough to distinguish the different groups compared with studies on maize and rice, it may not be able to accurately separate all individual accessions. Thus, further development of more foxtail specific primers such as mtDNA simple sequence repeat primers may improve the accuracy of classification and facilitate the creation of new sterile lines with diversified cytoplasmic types for hybrid cultivars.

Importance of the Cytoplasmic Type Classification on Breeding Hybrid Cultivars of Foxtail Millet

A single source of cytoplasm makes foxtail millet vulnerable to quick development of cytoplasm-specific pathogens, and infection by the pathogens can cause complete losses in

production of hybrid cultivars that contain the same cytoplasm. Due to lack of knowledge on cytoplasmic types of male sterile lines used in commercial production in China, it has been difficult to select diverse cytoplasm types in breeding for male sterile lines of hybrid cultivars. In this study, we successfully divided Chinese foxtail millet into eight types. By comparing the geographic and ecological distributions of these cytoplasmic types, we determined predominant and rare cytoplasm types in each major ecological region, which laid a solid ground for creation of new male sterile lines with diversified cytoplasmic types. In addition, breeding conventional and hybrid cultivars using a diversified cytoplasmic types in each ecological region is recommended to avoid infection by cytoplasm-specific pathogens.

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