Agricultural Sciences, 2022, 13, 404-428
https://www.scirp.org/journal/as
ISSN Online: 2156-8561
ISSN Print: 2156-8553

Genetic Diversity and Classification of Chinese Elite Foxtail Millet [*Setaria italica* (L.) P. Beauv.] Revealed by Acid-PAGE Prolamin

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How to cite this paper: Ma, G.X., Li, Q., Li, S.Y., Liu, Z.L., Cui, Y.J., Zhang, J. and Liu, D. (2022) Genetic Diversity and Classification of Chinese Elite Foxtail Millet [*Setaria italica* (L.) P. Beauv.] Revealed by Acid-PAGE Prolamin. *Agricultural Sciences*, **13**, 404-428.
https://doi.org/10.4236/as.2022.133028

Received: February 20, 2022
Accepted: March 14, 2022
Published: March 17, 2022

Abstract

Arid and semi-arid regions of China account for more than half of the country. Because of drought resistance and high nutritive value, elite foxtail millet (*Setaria Italica* (L.) P. Beauv.) is one of the most important cereal crops in China. Evaluation of germplasm and genetic diversity of foxtail millet is still in its infancy, but prolamin could play an important role as a protein marker. To investigate the genetic diversity and population structure of foxtail millet from different ecological zones of China, 90 accessions of foxtail millet were collected from three major ecological areas: North, Northwest, and Northeast China. The prolamin contents were examined by acid polyacrylamide gel electrophoresis (acid-PAGE). Five to twenty-two prolamin bands appeared in tested varieties, of which were polymorphic, so prolamin patterns of foxtail millet varieties can be used in variety identification and evaluation. Structure analysis identified six groups, which matches their pedigree information but not their geographic origins. This indicated a high degree (87.78%) of consistency with a phylogenetic classification based on SSR. The results showed prolamin banding patterns were an effective method for analyzing foxtail millet genetic variability.

Keywords

Foxtail Millet [*Setaria italica* (L.) P. Beauv.], Seed Storage Protein, Protein Polymorphism, Prolamin

1. Introduction

Insufficient water supply is a major issue in the world for crop production. Chi-

na has serious water shortage problems, especially in northern regions. Foxtail millet (*Setaria italica* (L.) P. Beauv.) has drought-tolerant properties that render it an important cereal crop species in these regions. Foxtail millet originated in the Yellow River Valley of China at least 8700 years ago [1]; it has a short life cycle, high photosynthetic efficiency, and is enriched in various nutrients, and thus is an important crop used extensively for food, fodder, bioenergy in arid and semi-arid areas of Asia, North Africa, South and North America [2] [3].

For foxtail millet, some high yield types [4], disease resistance types [5], and other types of preferred varieties [6] have accumulated in long-term cultivation and domestication processes, yet basic research on foxtail millet lags major cereal crops such as wheat. Evaluation of germplasm and genetic diversity of foxtail millet is still in its infancy. The genetic diversity analysis of some foxtail millet pedigrees suggests potential new cultivars based on agronomic performance [7].

In agricultural studies have focused on morphology [7] [8], cytology, physiology, and biochemistry [9]. Morphological characters are affected by the environment, which leads to challenges in genetic identification. Biochemical markers, such as isozymes and proteins, are the product of gene expression, not all show the codominant inheritance, polymorphism of produce is limited, the close genetic relationship and genetic basis of complex material are difficult to identify [9]. With the development of molecular biology and genomics, the research and application of DNA molecular markers have rapidly developed. Molecular markers, such as amplified fragment length polymorphism (AFLP) [10], random amplified polymorphic DNA (RAPD) markers [11], and simple sequence repeat (SSR) markers [12] [13] have been used in genetic diversity research in foxtail millet. However, these techniques have drawbacks. The sensitivity of RFLP to DNA polymorphism detection is not high, it requires large quantities of DNA and a DNA fragment as a probe, and employs radioisotope and nucleic acid hybridization technology which are neither safe nor easy to automate. The stability and repeatability of RAPD experiments are poor and very sensitive to reaction conditions, such as template and Mg2+ concentrations. AFLP requires high genomic purity and reaction conditions, and SSR detection relies on a series of standard primers with a high polymorphism that covers all chromosomes in the genome, and the detection and analysis depends on a large number of samples.

Knowledge of genetic diversity is used for efficient germplasm management and utilization, genetic fingerprinting, and genotype selection [14]. prolamin, a heterogeneous group of alcohol-soluble storage proteins, is encoded by highly conserved multigenic families. An original methodology for their electrophoretic separation is acid polyacrylamide gel electrophoresis (acid-PAGE) [15] that can be used to detect the complex polymorphisms of prolamins. As prolamin is genotype-specific, the entire process includes protein extraction, electrophoresis, and band analysis—simple, repeatable, relatively cheap, and independent of environmental variation [16] or stage of plant ontogenesis. Genetic polymorphisms

have been used to evaluate genetic diversity in many plants, such as wheat [17], barley [18] [19], *Leymus* [20], tall fescue cultivars [21], triticale [22], vetches [16] [23] and rice [24].

Clustering results based on prolamin banding patterns and SSR analysis are not always in agreement [17], but both methods produced similar total genetic diversity results for Chinse wheat landrace [25]. The application of prolamin in foxtail millet varieties is not as advanced as in other crops, because of a limited heterogeneity in the genetic background [26] [27] and protein content is lower than other crops. As the foxtail millet germline source is not clear, the breeding efficiency of high-yield foxtail millet is low. China has diverse ecological types with numerous foxtail millet varieties in different ecological regions. The homology of foxtail millet prolamin may reflect the evolution of different varieties and guide the selection of parents. However, there are few reports on prolamin of cultivated varieties in different ecological regions. In this work, the prolamin A-PAGE method was used to analyze genetic diversity among 90 elite foxtail millet parental lines collected from different ecological regions in China, to guide the breeding, identification, and evaluation of new foxtail millet varieties.

2. Materials and Methods

2.1. Plant Materials

A total of 100 foxtail millet accessions were collected in 2016 from all the breeding programs in the North China summer foxtail millet region and Northwest and Northeast China spring foxtail millet regions. Those materials were initially evaluated for agronomically and economically-important traits and high yield potential at the breeding observation nursery of the Institute of Foxtail Millet Crops, Shijiazhuang, Hebei, China. After initial screening, 90 accessions were selected as basic breeding materials based on their yield and adaptation performance. Most of these materials were from eight foxtail millet growing provinces: Hebei, Henan, Shandong, Liaoning, Jilin, Shanxi, Inner Mongolia, Shaanxi (**Supplemental Table 1**).

2.2. Protein Extraction and Electrophoresis

Electrophoresis of prolamin was performed based on Wrigley's method [28] with some modifications. For the analysis, 20 healthy seeds were randomly selected in each accession. prolamins were extracted from the individually milled seed by adding 200 μL sample extract solution (70% isopropanol, 15% sucrose) into 1.5 mL tubes (Eppendorf, Germany) that were then incubated at 220 rpm (60˚C) for 60 min. The extract was then centrifuged at 12000 r/min (4˚C) for 10 min. The supernatant was transferred into a new tube and 100 μL of methylene green solution (80% glycerin, 0.02% methyl green) was added for pre-staining. The solution was heated in an oven at 60˚C for 30 min, during which it was taken out and shaken every 10 min, and then put in a thermostat at 4˚C. The

A-PAGE gel formula was: 12% acrylamide, 0.4% methylene diacrylamide, 2% glacial acetic acid, 6% urea, 0.1% ascorbic acid, 0.075% glycine, and 0.004% ferrous sulfate; 2 μL hydrogen peroxide catalyst was used to prepare the gel with a thickness of 1 mm. A glycolic polymorphism was detected in a 15 μL reaction system with 0.4% glacial acetic acid and 0.04% glycine as the electrode solution at 500 V constant pressure and 15˚C for 80 min. After electrophoresis, acid-PAGE gel was stained with a 0.1% Coomassie Bright Blue R-250, 40% isopropyl alcohol, and 10% glacial acetic acid solution for 25 min. It was rinsed and decolorized with running water then photographs were taken with camera (Nikon, WJHH).

2.3. Statistical Analysis

To detect population genetic structure and assign individuals to subpopulations, the data obtained from acid-PAGE was scored based on the results of electrophoretic band spectra (**Supplemental Table 2**) for the presence or absence of the bands and entered as a binary data matrix. Population structure was determined by STRUCTURE software v2.3.4 [29] [30], which uses a Bayesian approach to identify clusters based on a fit to the Hardy-Weinberg equilibrium model and linkage equilibrium. Ten independent runs for each number of subpopulations value (k), which ranged from 3 to 13, were performed after the admixture model with 100,000 replicates for burn-in and 100,000 replicates during analysis. The optimal subgroup (k) value was determined based on 1) likelihood plots of these models, 2) stability of grouping patterns across the ten runs, and 3) information about the materials used in the study. The output was exported into Structure Harvester [31] to determine the most likely number of K clusters (K = 6 was optimum for this analysis, **Figure 1A** using Evanno's ΔK method [31]. Results from 10 independent STRUCTURE runs for the most likely K were assessed with the software CLUMPP [32] and plotted using DISTRUCT [32].

3. Results

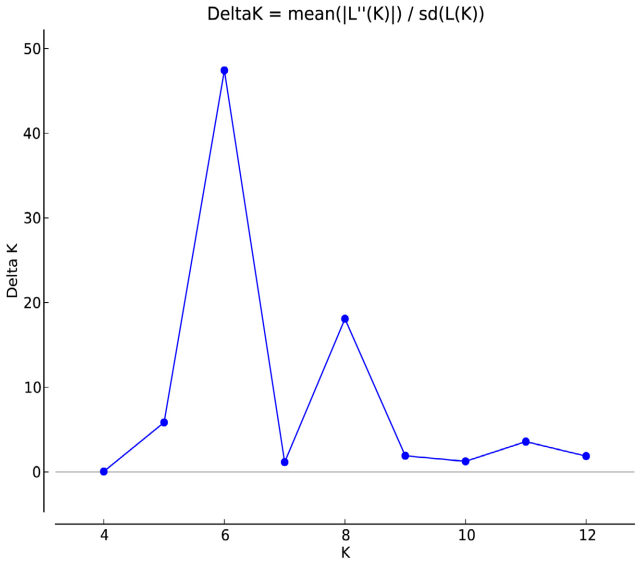
3.1. Genetic Diversity in Foxtail Millet

The prolamin contents were examined by acid-PAGE. Analysis of variance showed that 5 to 22 prolamin bands (**Supplemental Table 2**, **Figure 2**) appeared in tested varieties, of which were polymorphic. The results indicated that the genetic diversity of the breeding materials used in this study was high and should be valuable for breeding application.

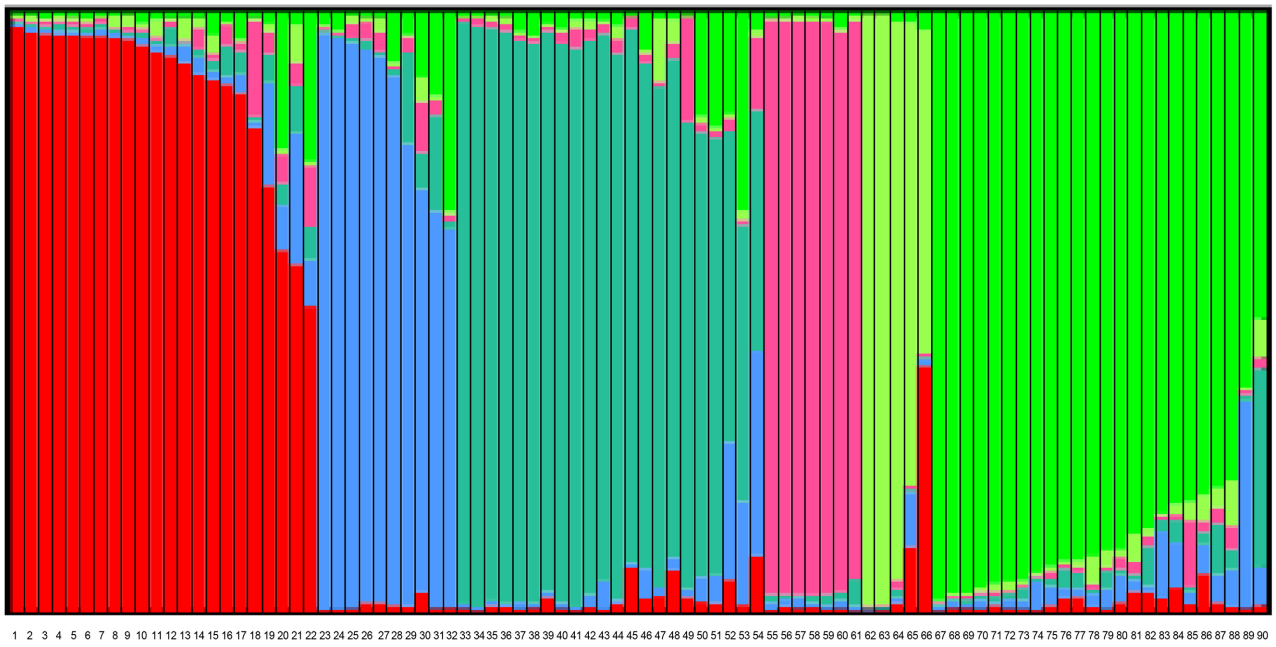
3.2. Population Structure

STRUCTURE analysis of the population structure of the 90 foxtail millet accessions showed that the most appropriate grouping was six subpopulations with a ΔK peak of 6 (**Figure 1A**, **Supplemental Table 1**). The group of foxtail millet accessions was divided into six subpopulations by the prolamin method (**Figure**

1B). Among the six subpopulations, the level of genetic diversity within pG6 (group 6 by the prolamin method) was the highest (26.67%), followed by pG1 (24.44%), pG3 (24.44), pG2 (11.11%), pG4 (7.78%), and pG5 (5.56%) (Table 1, Supplemental Table 2).



A



B

Figure 1. Population structure analysis for 90 accessions of foxtail millet. (A) Delta K values for different numbers of populations assumed (K) in the structure analysis. (B) Classification of 90 accessions into four subpopulations according to preset K value using STRUCTURE program. The distribution of the accessions to different subpopulations is indicated by color (G1: red, G2: blue; G3: Dark green; G4: purple; G5: jade-green; G6: green).

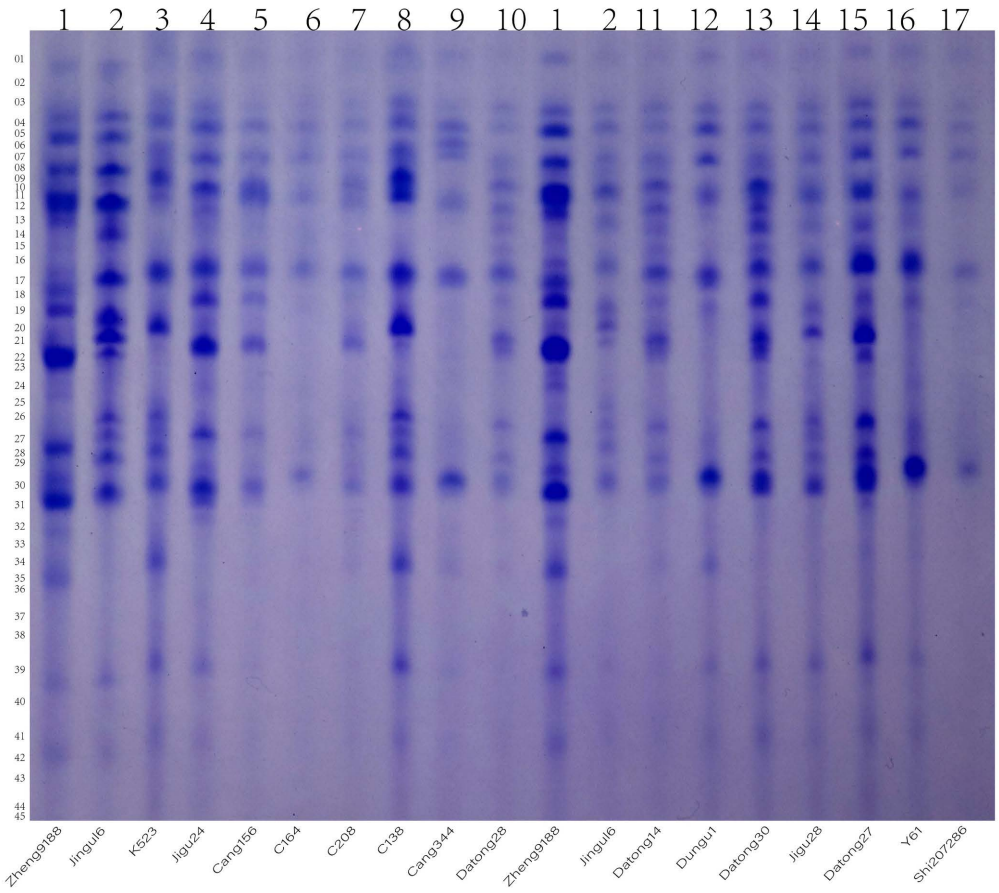


Figure 2. Prolamin patterns of some cultivars or lines. 1. V41 (Zheng9188), 2. V61 (Jingu16), 3. V9 (K523), 4. V8 (Jigu24-1), 5. V2 (Cang156), 6. V43 (C164), 7. V13 (C208), 8. V47 (C138), 9. V42 (Cang344), 10. V64 (Datong28), 11. V61 (Jingu16), 13. V62 (Datong14), 12. V35 (Dungu1), 13. V63 (Datong30), 14. V65 (Jigu28), 15. V66 (Datong27), 16. V34 (Y61), 17. V17 (Shi207286).

Table 1. Common parents, their major ancestors, geographic distribution, number of accessions, and ecotypes of six subpopulations derived from structure analysis.

Group	pG1 (22)		pG2 (10)		pG3 (22)		pG4 (7)		pG5 (5)		pG6 (24)	
Accessions	22 (24.44%)		10 (11.11%)		22 (24.44%)		7 (7.78%)		5 (5.56%)		6 (26.67%)	
Ancestors %	Riben60ri	9 (40.91%)	Riben60ri	5 (50%)	Riben60ri	14 (63.64%)	Moligu	3 (42.86%)	Shanxidabaigu	2 (40%)	Riben60ri	4 (16.67%)
	60rihuancang	5 (22.73%)	Tulong	4 (40%)	Tulong	14 (63.64%)	Jinfen52	2 (28.57%)	Xainnong3	1 (20%)	Shuangguayin	4 (16.67%)
	Mihuanggu	4 (18.18%)	Mihuanggu	2 (20%)	Qinggouweicao	7 (31.82%)	Qitouhuang	2 (28.57%)	Huangruangu	1 (20%)	Daobaqi	3 (12.5)
	Tulong	3 (13.64%)	Xiaoliugen	1 (10%)	60rihuancang	7 (31.82%)	Riben60ri	1 (14.29%)	Zhangchunyi	1 (20%)	Huangguzi	3 (12.5)
	Maichagu	1 (4.55%)	Chaoxiangu	1 (10%)	Xiaoliugen	5 (22.73%)	Tulong	1 (14.29%)			Shaanxiheizhigu	1 (4.17%)
	Xiaoliugen	1 (4.55%)	Qinggouweicao	1 (10%)	Mihuanggu	3 (13.64%)	Xiaohuanggu	1 (14.29%)			Tulong	1 (4.17%)
	Chaoxiangu	1 (4.55%)			Kenniya	2 (9.09%)	Shuangguayin	1 (14.29%)			Jinxiangyu	1 (4.17%)
					Lvsuigu	1 (4.55%)	Qinyuanmujizui	1 (14.29%)			Meiguodatou	1 (4.17%)
					Yapoche	1 (4.55%)	Hainangu	1 (14.29%)			Yingsuigu	1 (4.17%)
					Changsuihuang	1 (4.55%)					Chaoxiangu	1 (4.17%)
											Jinfen52	1 (4.17%)
											Qitouhuang	1 (4.17%)
	Pedigree unclear	13 (59.09%)	Pedigree unclear	5 (50%)	Pedigree unclear	4 (18.18%)	Pedigree unclear	1 (14.29%)	Pedigree unclear	3 (60%)	Pedigree unclear	9 (37.5%)

PG1 was collected from the summer foxtail millet region of central and southern Hebei Province. Riben60ri and 60rihuancang can be found in the pedigrees of most of these accessions. Nine accessions have definite ancestries, thirteen accessions did not. PG2 consisted of most accessions from central and southern Hebei Province and Liaoning Province. Riben60ri and Tulong can be found in the pedigrees of most of these accessions. Five accessions had definite ancestries and five accessions did not. PG3 was collected from Hebei Province. Most of these were the derivatives Riben60ri and Tulong, such as Yugu1 and its derivatives. Riben60ri is a Japanese landrace. Eighteen accessions had definite ancestries and four accessions did not. Riben60ri and Tulong can be found in the pedigrees of most of these accessions. PG4 was from Shanxi Province and was spring foxtail millet. Three can be traced back to founder-Moligu, two can be traced back to founder-Qitouhuang, one can be traced back to founder-Hainangu and Qinyuanmujizui. Six accessions had definite ancestries and one accession did not. The phylogeny of accessions was ambiguous. PG5 was collected from Shanxi Province, and two can be traced back to founder-Shanxidabaigu and two breeding materials that did not have clear pedigree information. One was breeding material from Hebei Province without clear pedigree information. Two accessions had common ancestors and three accessions did not. PG6 had diverse pedigrees and geographic origins. They include 8 accessions from Liaoning, 6 from Hebei, 3 from Shanxi, 3 from Neimenggu, 2 from Jilin, 1 from Henan, and 1 from America. Fifteen accessions had definite ancestries and nine accessions were unclear.

3.3. Population Structure, Pedigree, and Geographic and Ecological Distributions

There was no tight association between structure and ecological group (summer or spring foxtail millet) (**Supplemental Table 1**) based on grouping results from structure analysis. No relationships among genetic diversity, geographic origin, ecological group (summer or spring foxtail millet) (**Supplemental Table 1**), and the genotypes were observed based on grouping results from structure analysis. In each structure group, both summer and spring foxtail millet types were identified. However, the majority of accessions in pG1, pG2, pG3 and pG6 were the summer type and pG4, pG5 and pG6 were spring type. PG1 had the highest proportion of summer type (90.91%) and pG4 had the highest proportion of spring type (85.71%). All six groups consisted of accessions from different ecological regions, with pG1 and pG3 having the most accessions (81.82% and 77.24%, respectively) from the central and south Hebei Province, and accessions in pG4 and pG5 (84.6% and 80%) were mainly from central and south Shanxi Province. Accessions in pG2 consisted of most accessions from the central and south Hebei Province and Liaoning Province. Accessions in pG6 were from seven different regions with diverse ecological conditions. Only a small proportion of accessions (<35%) can be traced to each region.

Basic germplasm, parent-of-origin analysis (Supplemental Table 1), and pedigree analysis (Figure 3, Supplemental Table 3) indicated the following. Thirty-three accessions were derived from Riben60ri, and these accessions belonged to pG1 (proportion, 40.91%), pG2 (50%), pG3 (63.64%), pG4 (14.29%), pG6 (16.67%). Twenty-three accessions were derived from Tulong, and these accessions belonged to pG1 (13.64%), pG2 (40%), pG3 (63.64%), pG4 (14.29%), pG6 (4.17%). Thirteen accessions were derived from 60rihuancang, and these accessions belonged to pG1 (22.73%), pG2 (10%), pG3 (31.82%). Nine accessions were derived from Qinggouweicao, and these accessions belonged to pG1 (4.55%), pG2 (10%), pG3 (31.82%). Nine accessions were derived from Mihuanggu, and these accessions belonged to pG1 (22.73%), pG2 (10%), and pG3 (13.64%). Six accessions were derived from Xiaoliugen, and these accessions belonged to pG1 (4.55%), pG2 (10%), pG3 (18.18%). Five accessions were derived from Shuangguayin, and these accessions belonged to pG4 (14.29%) and pG6 (16.67%). Nineteen accessions were derived from Yugu1, and these accessions belonged to pG1 (22.73%), pG2 (30%), pG3 (40.91%), pG4 (14.29%), and pG6 (4.17%). Nine accessions were derived from WR1, and these accessions belonged to pG1 (4.55%), pG2 (10%), and pG3 (13.64%). Eight accessions were derived from shi181-5, and these accessions belonged to pG1 (4.55%), pG2 (10%), and pG3 (27.27%). These data indicated that Riben60ri and Tulong were the major germplasm of three ecological areas, pG1, pG2, and pG3, mainly derived from Yugu1 which is a derivative of Riben60ri (Supplemental Table 1). They also showed that pG1, pG2, and pG3 were close affinities, pG4 and G6 were close affinities, and Riben60ri was the source of these five groups; pG5 is relatively independent of the other groups.

3.4. The Consistency between the SSR and A-PAGE Prolamins Methods

The group of 90 foxtail millet accessions was both divided into six subpopulations by the SSR method and the A-PAGE prolamins method. Consistency analysis indicated that the accordant rate reached 87.8% (Table 2) between the two methods. The accordant rates of groups 1-6 were 87.5%, 100.0%, 71.9%, 100%, 100%, and 100%.

In sG1 (group 1 by the SSR method), Jigu25 and Gu10A were the accessions with inconsistent groupings. The parent-of-origin of Jigu25 is WR1 × Shi181-5, and the main germplasm base of Jigu25 are Riben60ri, 60rihuancang, Tulong, and Qinggouweicao. The main germplasm base rates (Table 1, Table 2, Figure 3) of sG1 are Riben60ri (68.75%), Tulong (68.75%), and Qinggouweicao (43.75%); Riben60ri (50%), Tulong (40%), and Qinggouweicao (10%) in pG2, therefore Jigu25 belonged to sG1. Data indicated that the SSR method is more robust. In sG3, the parent-of-origin of Heng968, Datong14, Datong28, and Datong27 are Lugu5 (7112 × (male-sterile lines × Riben60ri) × Lugu2) × 91101, Xiannong3 × Jingu9 (Shanxidabaigu), (Huangruangu × Zhangchunyi) F2 × Jingu9

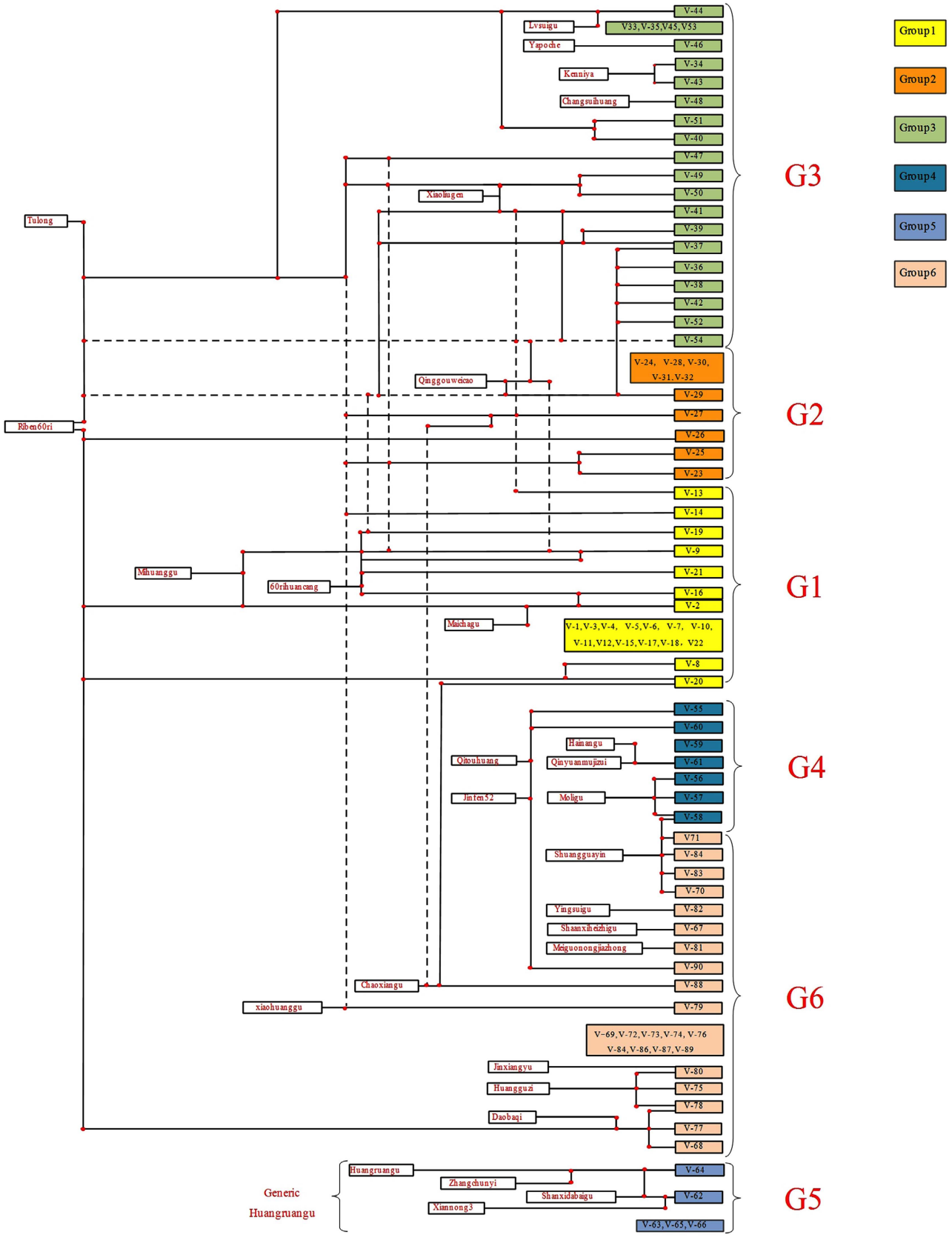


Figure 3. Pedigrees of major germplasm used in this study. G: group.

Table 2. The consistency analysis between SSR method and acid-PAGE prolamins method. G: Group, Note: pG1 = pG1a + pG1b, pG2 = pG2a + pG2b + pG2c, pG3 = pG3a + pG3b + pG3c, pG4 = pG4a, pG6 = pG6a + pG6b.

SSR			Consistency					Miss						
			Consistency rate and number			Prolamins		Miss rate and number			Prolamin			
G	Number of varieties	The main germplasm base	All	Pedigree clear	Pedigree unclear	G	The main germplasm base rate	All	Pedigree clear	Pedigree unclear	G	Varieties	The source of foxtail millet germplasm	The main germplasm base
sG1	16	Riben60ri, Tulong (11, 68.75%); Qinggouweicao (7, 43.75%); 60rihuancang (5, 31.25%); Xiaoliugen (3, 18.75%), Kenniya (2, 12.5%), Changsuihuang, Mihuanggu (1, 6.25%)	14 87.50%	13	1	pG3a	Riben60ri, Tulong (10, 71.43%); 60rihuangcang, Qinggouweicao (6, 42.86%); Xiaoliugen (3, 21.43); Kenniya (2, 14.29); Mihuanggu, Changlihuang (1, 7.14%)	2 12.50%	1	1	pG2b	Jigu25	WR1 × Shi181-5	Riben60ri, 60rihuancang, Tulong, Qinggouweicao
											pG6b	Gu10A	-	-
sG2	7	Moligu (3, 42.86%); Jinfen52, Qitouhuang (2, 28.57%); Riben60ri, Tulong, Shuangguayin, Xiaohuanggu, Hainangu, Qinyuanmujizui (1, 14.29%)	7 100.00%	6	1	pG4a	Moligu (3, 42.86%); Jinfen52, Qitouhuang (2, 28.57%); Riben60ri, Tulong, Shuangguayin, Xiaohuanggu, Hainangu, Qinyuanmujizui (1, 14.29%)	0 0.00%	0	0	-	-	-	-
sG3	32	Riben60ri (5, 15.63%); Shuangguayin (4, 12.5); Daobaqi, Huangguzi (3, 9.38%); Shanxidabaigu (2, 6.25%); 60rihuancang, Tulong, Chaoxiangu, Jinfen52, Qitouhuang, Huangruangu, Yingsuigu, Jinxiangyu, Meiguodatou, Shaanxiheizhigu, Xainnong3, Zhangchunyi (1, 3.13%)	23 71.88%	15	8	pG6a	Riben60ri, Shuangguayin (4, 17.39); Daobaqi, (3, 13.04%); Huangguzi (2, 8.70%); Tulong, Chaoxiangu, Jinfen52, Qitouhuang, Yingsuigu, Jinxiangyu, Meiguodatou, Shaanxiheizhigu, (1, 4.35%)	9 28.12%	3	6	pG1b	Heng968	Lugu5 (7112 × (male sterile lines × Riben60ri) × Lugu2) × 91101	Riben60ri, 60rihuancang
											pG2c	Tiedalihuang	-	-
												Jinzhougu14	Dungu1	-
											pG3c	Shi 207191	-	-
											pG5	Datong14	Xiannong3 × Jingu9 (Shanxidabaigu)	Xiannong3, Shanxidabaigu
												Datong30	-	-
												Jigu28	-	-
												Datong28	(Huangruangu × Zhangchunyi) F2 × Jingu9 (Shanxidabaigu)	Huangruangu, Zhangchunyi, Shanxidabaigu
												Datong27	(73-50 × Zao1) × Yi17	-
sG4	7	Riben60ri (4, 57.14%); Tulong (3, 42.86%); Mihuanggu (2, 28.57%); Chaoxiangu, Xaioliugen (1, 14.29%)	7 100.00%	4	3	pG2a	Riben60ri (4, 57.14%); Tulong (3, 42.86%); Mihuanggu (2, 28.57%); Chaoxiangu, Xaioliugen (1, 14.29%)	0 0.00%	0	0	-	-	-	-
sG5	7	Riben60ri, Tulong (4, 57.14%); Mihuanggu, Xiaoliugen (2, 28.57%); 60rihuancang, Qinggouweicao, Yapoche, Lvsuigu (1, 14.29%)	7 100.00%	5	2	pG3b	Riben60ri, Tulong (4, 57.14%); Mihuanggu, Xiaoliugen (2, 28.57%); 60rihuancang, Qinggouweicao, Yapoche, Lvsuigu (1, 14.29%)	0 0.00%	0	0	-	-	-	-
sG6	21	Riben60ri (8, 38.10%); 60rihuancang, Mihuanggu (4, 19.05%); Tulong (3, 14.29%); Xiaoliugen, Chaoxiangu, Maichagu (1, 4.76%)	21 100.00%	8	13	pG1a	38.10%); 60rihuancang, Mihuanggu (4, 19.05%); Tulong (3, 14.29%); Xiaoliugen, Chaoxiangu, Maichagu (1, 4.76%)	0 0.00%	0	0	-	-	-	-
All	90	-	79 87.78%	49	28		-	11 12.22%	4	7		-	-	-

(Shanxidabaigu) and (73-50 × Zao1) × **Yi17**, respectively. The main germplasm bases of Heng968, Datong14, and Datong28 are (Riben60ri, 60rihuancang), (Xiannong3, Shanxidabaigu) and (Huangruangu, Zhangchunyi, Shanxidabaigu), respectively. The main germplasm bases of pG5 are Shanxidabaigu (40%), Xainnong3 (20%), Huangruangu (20%) and Zhangchunyi (20%), whereas they were Riben60ri (15.63%); Shanxidabaigu (6.25%); 60rihuancang (3.13%), Huangruangu (3.13%), Xainnong3 (3.13%), and Zhangchunyi (3.13%) in sG3. They showed that Heng968 belonged to pG1, and Datong14, Datong28, and Datong27 belonged to pG5 by the main germplasm base identified by the two methods. These data indicated the A-PAGE prolamins method is closer to the pedigree analysis. Because the main germplasm bases of Gu10A, Tiedalihuang, Jinzhougu14, shi207191, Datong27, Datong30, and Jigu28 are not distinct, it is difficult to judge which of the two methods is preferred.

4. Discussion

4.1. Prolamin and Genetic Diversity

Prolamin is the main storage protein of plant seeds, a gene expression product at a specific stage of seed development. The number and combination of its electrophoresis bands are controlled by genes, minimally affected by environmental factors, and thus can reflect the differences in gene coding sites of different crop varieties [33] [34]. Therefore, the analysis of plant varieties by prolamin can reveal specific genetic differences among varieties (**Figure 2**). The application of prolamin to the study of plant genetic resources has the advantages of simplicity, convenience, and accuracy. Lang *et al.* [35] found the glycolic homology degree in wheat generally reflects the distance of the genetic relationship among the main popularized wheat varieties in China and can be further used to guide the selection of parents.

In this research, a high level of polymorphism was identified for prolamin across the 90 accessions. It showed that 5 to 22 prolamin bands appeared in tested varieties. Structure analysis identified six groups, which matches with their pedigree information, but not with their geographic origins. The grouping consistency was 87.78% between the SSR method and the acid-PAGE prolamin method [13]. This might be due to highly diverse accessions which were collected from three major foxtail millet ecological regions.

4.2. Genetic Diversity and Population Structure of Chinese Foxtail Millet

Because it is genotype-specific, simple, repeatable, cheap, and independent of environmental variation nature, prolamin has frequently been used as a tool to examine the dynamics of genetic differentiation in a population. For example, structure analysis can estimate the number of subpopulations and the genetic relatedness among accessions. Cluster analysis can also group the assayed accessions into different groups. In this study, structure analysis identified six groups,

which matches with their germplasm information and SSR method grouping [13]. Basic germplasm and parent-of-origin analysis (**Supplemental Table 1**) indicated that there were not associated with a particular ecological environment, and the origins of accessions would differentiate among accessions if they are all landraces.

The extensive exchange of genetic resources breaks barriers among the three major ecological areas in China. Following germplasm being introduced into a new ecological environment, it goes through continuous domestication and hybridization with local breeding lines and eventually results in new progenies. All the groups contain both summer and spring types. Riben60ri was introduced to the North China summer foxtail millet region and became major parent cultivars in this region. After that, Riben60ri and its derivatives were usually hybridized with a locally adapted parent. Breeders from different regions selected different genotypes according to their preferences. Thus, it is expected that many new cultivars are interrelated and some geographically distant accessions may also be genetically related.

Structure analysis mainly was based on the genetic relatedness among accessions. Thus, the grouping based on these two approaches may not necessarily match with their geographic origin and ecotype. In pG4, most of the accessions derived from Qitouhuang and Moligu, which originated from central and southern Shanxi Province. Most of the accessions in pG1, pG2, and pG3 were selected from crosses between Riben60ri and locally adapted materials. For example, Qingdaolao and Yugu1 were derived by crossing Riben60ri to the Chinese landrace Mihuanggu derivative Xinnong724 and Japanese landrace Tulong. Further, Yugu1 was crossed to other locally adapted materials, and cultivars were selected for their adaptation to different growth areas, which generated pG1, pG2, and pG3. Qingdaolao derivatives mainly contributed to accessions in pG1, while accessions in pG3 were mainly transition types between Yugu1 derivatives and Qingdaolao derivatives. These included Yugu1 and Qingdaolao derivatives and progenies from the cross between the two cultivars. PG5 was mainly selected from locally adapted materials, including Shanxidabaigu, which originated from central and southern Shanxi Province. PG6 was scattered in several regions, but most accessions were from Northeast China and the nearby Northwest spring foxtail millet region.

4.3. The Implications of Genetic Improvement of Foxtail Millet

Insufficient water resources seriously affected agricultural production. Foxtail millet is a highly drought-tolerant crop. The research of genetic diversity and the population structure of foxtail millet germplasm resources will accelerate the effective utilization of the limited resources for breeding. The foxtail millet materials used in this study were derived from the main foxtail millet-producing area in China. Many accessions (38.89%) had no pedigree records; this study elucidated the genetic relationship between these unknown accessions and those with

a known pedigree to determine origins.

The study indicated that when breeding foxtail millet, prolamin analysis was simple, accurate, and efficient, which will improve breeding project design and selection accuracy. In past, male-sterile parents and geographical unrelated materials were used for hybrid breeding. The results from this study indicated that accessions separated by great geographical distance may not necessarily be genetically distant. Classification of 90 accessions into six groups matched with their genetic relatedness and thus provides a good reference for designing crosses to improve hybrid-breeding efficiency. Accessions in pG4 and pG5 have unique geographic origins and pedigrees that are different from other groups; therefore, cross accessions among pG4, pG5 and other groups are more likely to obtain expected recombination for developing both conventional and hybrid cultivars. Further research may be needed to evaluate the combining results among groups to determine the combinations of accessions from different groups with the best heterosis. Although accessions in pG6 had the Riben60ri consanguinity, they have the most diverse origins and the greatest variation within the group. Accessions in pG5 were an independent group with five other groups, and crosses between accessions within pG5 and the other five groups may generate useful heterosis. Thus, further research on accessions may facilitate effective the use of germplasm in this group.

The acid-PAGE prolamin method is reliable, and the grouping consistency was 87.78% between the SSR method and the acid-PAGE method. The classification is closer to the germline source of foxtail millet. Furthermore, the acid-PAGE prolamin method is not needed for designing a large number of specific PCR primers or high-quality genomic DNA, and it is a simpler operation with a lower cost. In sum, it is an effective method for the breeding, identification, and evaluation of new varieties of foxtail millet.

5. Conclusion

In general, the acid-PAGE prolamin method is reliable and advantageous in breeding, identification, and evaluation of new varieties of foxtail millet, which has highly consistent with SSR method in group classification and is closer to the germline source of foxtail millet. In addition, the acid-PAGE prolamin method is more convenient than SSR method, don't have to design or select a large number of specific PCR primers or high-quality genomic DNA.

Acknowledgements

Funding: This work was supported by the S&T Program of Hebei (19227687D, 21326337D, and 21326302D) and the Scientific Research Fund of Tangshan Normal University (grant number 2020C01).

Author Contributions

ZL and DL contributed to the study conception and design. SL and JZ collected

the foxtail millet materials. GM, YC, and QL genotyped the accessions, and GM and DL conducted the population structure analysis and other data analysis. The first draft of the manuscript was written by GM, and ZL revised the article. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Supplemental Table 1. The grouping of foxtail millet by acid-PAGE prolamins method.

No.	Group %	Variety	Ecotypes (%)		Geographical position	No. accessions (%)	parent-of-origin	Basic germplasm
V1		Shi202242	summer		Hebei		-	-
V2		Cang156	summer		Hebei		**Heng8916** (Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)) × Lugu5 (7112 × (male sterile lines × Riben60ri) × Lugu2)) × **Maichagu**	Riben60ri, 60rihuancang, Mihuanggu, Maichagu
V3		Chaogu12	spring		Liaoning		-	-
V4		Shi207393	summer		Hebei		-	-
V5		Shi207382	summer		Hebei		-	-
V6		Shi02521	summer		Hebei		-	-
V7		Shi02399	summer		Hebei		-	-
V8		Jigu24	summer		Hebei		**R219** (Gufeng1 (474))	Riben60ri
V9		K523	summer		Hebei		**Jigu19** (Jigu12 (Yugu1 (Riben60ri × Tulong) × (Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)) × Gaolianggu223)) × Ai88 (Zheng737 (81407 (Shuilihun × Yugu1) × 81474 (Zhengai2 (Qingdaolao)))) × **Jigu25** (WR1 × Shi181-5)	Riben60ri, 60rihuancang, Tulong, Mihuanggu, Qinggouweicao
V10		Shi206058	summer		Hebei	Hebei (18, 81.82%), Shandong (2, 9.09%), Liaoning (1, 4.55%), Shanxi (1, 4.55%)	-	-
V11		Shi207286	summer	Summer (20, 90.91%); Spring (2, 9.09%)	Hebei		-	-
V12	pG1	C445	summer		Hebei		-	-
V13		C208	summer		Hebei		**Gufeng2** (95307 × Lugu10 (Yugu1 (Riben60ri × Tulong) × Bu5019 (Riben60ri × Xiaoliugen))) × **Jigu19** (Jigu12 (Yugu1 × (Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)) × Gaolianggu223)) × Ai88 (Zheng737 (81407 (Shuilihun × Yugu1) × 81474 (Zhengai2 (Qingdaolao))))	Riben60ri, Mihuanggu, Xiaoliugen
V14		Ji9409	summer		Shandong		**Jichong5** (Zheng737 (81407 (Shuilihun × Yugu1 (Riben60ri × Tulong)) × 81474 (Zhengai2 (Qingdaolao (Reben60ri × Xinnong724 (Mihuanggu)))))) × **Zheng413**	Riben60ri, Tulong
V15		Jigu26	summer		Hebei		-	-
V16		Jigu22	summer		Hebei		**Jinan8131** ((Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)) × Lugu5 (7112 × (male sterile lines × Riben60ri) × Lugu2) × **Shi92406** (Bu5019 (Riben60ri × Xiaoliugen) × Yugu1 (Riben60ri × Tulong))	Riben60ri, 60rihuancang, Mihuanggu
V17		Shi98700	summer		Hebei		-	-
V18		Jigu20	summer		Hebei		-	-
V19		Ji9403	summer		Shandong		**Lugu8** (Lugu5 (7112 × (male sterile lines × Riben60ri) × Lugu2)) × An316 (Tulong × Riben60ri)) × **Baai3**	Riben60ri, 60rihuancang, Tulong
V20		Richaogu	summer		Hebei		**Riben60ri** × **Chaoxiangu**	Riben60ri, Chaoxiangu
V21		Heng968	summer		Hebei		**Lugu5** (7112 × (male sterile lines × Riben60ri) × Lugu2) × **91101**	Riben60ri, 60rihuancang
V22		Jinfen1A	spring		Shanxi		**683A** × **81-16**	-
V23		An2491	summer		Henan		**Ai88** (Zheng737 (81407 (Shuilihun × Yugu1 (Riben60ri × Tulong)) × **An472**	Riben60ri, Tulong, Mihuanggu
V24		Jinzhougu12	spring		Liaoning		**Tiegu11**	-
V25		Shi06-439	summer	Summer (6, 60%); Spring (4, 40%)	Hebei	Hebei (4, 40%), Liaoning (4, 40%), Henan (1, 10%), Shandong (1, 10%)	**Ai88** (Zheng737 (81407 (Shuilihun × Yugu1 (Riben60ri × Tulong)) × **Chuang19** (Ai88 × 09007)	Riben60ri, Tulong, Mihuanggu
V26	pG2	Chaogu14	spring		Liaoning		Xiagupinzhong × Shenqigu	Riben60ri
V27		Lugu10	summer		Shandong		**Yugu1** (Riben60ri × Tulong) × **Bu5019** (Riben60ri × Xiaoliugen)	Riben60ri, Tulong, Xiaoliugen, Chaoxiangu
V28		Gu3A	summer		Hebei		-	-
V29		Jigu25	summer		Hebei		**WR1** × **Shi181-5**	Riben60ri, 60rihuancang, Tulong, Qinggouweicao

Continued

V30		Gu38A	summer		Hebei	-	-
V31		Tiedalihuang	spring		Liaoning	-	-
V32		Jinzhougu14	spring		Liaoning	**Dungu1**	-
V33		Bagu214	spring		Hebei	-	-
V34		Y61	summer		Hebei	(**Kang3** × **Kenniya**) × **433**	Kenniya
V35		Dungu1	spring		Shanxi	-	-
V36		K1011	summer		Hebei	**96355** × **Jigu25** (WR1 × Shi181-5)	Riben60ri, 60rihuancang, Tulong, Qinggouweicao
V37		K359	summer		Hebei	**Gufeng2** (95307 × Lugu10 (Yugu1 (Riben60ri × Tulong) × Bu5019 (Riben60ri × Xiaoliugen))) × **Jigu25** (WR1 × Shi181-5)	Riben60ri, 60rihuancang, Tulong, Qinggouweicao, Xiaoliugen
V38		K1130	summer		Hebei	**C445** × **Jigu25** (WR1 × Shi181-5)	Riben60ri, 60rihuancang, Tulong, Qinggouweicao
V39		K660	summer		Hebei	**Gufeng2** (95307 × Lugu10 (Yugu1 (Riben60ri × Tulong) × Bu5019 (Riben60ri × Xiaoliugen))) × **Jigu25** (WR1 × Shi181-5)	Riben60ri, 60rihuancang, Tulong, Qinggouweicao, Xiaoliugen
V40		An2367	summer		Henan	**Yugu1** (Riben60ri × Tulong)	Riben60ri, Tulong
V41		Zheng9188	summer		Henan	**8744** (Yugu2 × Zheng407 (Lugu2 × 7434B × Yugu1 (Riben60ri × Tulong))) × **Lugu2**	Riben60ri, 60rihuancang, Tulong, Xiaoliugen
V42	pG3	Cang344	summer	Summer (19, 86.36%); Spring (3, 13.64%)	Hebei	Hebei (17, 77.27%), Shanxi (2, 9.09%), Henan (2, 9.09%), Shandong (1, 4.55%)	**Jigu25** (WR1 × Shi181-5) × **Shi181-5**	Riben60ri, 60rihuancang, Tulong, Qinggouweicao
V43		C164	summer		Hebei		**Y61** ((Kang3 × Kenniya) × 433) × **S80** (433 × W82)	Kenniya
V44		Shi97672	summer		Hebei		**Yugu5** (An096 × Yugu1 (Riben60ri × Tulong)) × **Shi181-3**	Riben60ri, Tulong, Lvsuigu
V45		Jigu21	summer		Hebei		-	-
V46		Chenggu12	summer		Hebei		**Chao86-10** × **Chenggu3**	Yapoche
V47		C138	summer		Hebei		**Shi181-5** × **Jigu19** (Jigu12 (Yugu1 (Riben60ri × Tulong) × (Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)) × Gaolianggu223)) × Ai88 (Zheng737 (81407 (Shuilihun × Yugu1) × 81474 (Zhengai2 (Qingdaolao))))	Riben60ri, Tulong, Mihuanggu
V48		Taixuan2	spring		Shanxi		**77-32** × **Changsuihuang**	Changsuihuang
V49		06-766	summer		Hebei		**Ai88** (Zheng737 (81407 (Shuilihun × Yugu1 (Riben60ri × Tulong)) × **Lugu10** (Yugu1 (Riben60ri × Tulong) × Bu5019 (Riben60ri × Xiaoliugen))	Riben60ri, Tulong, Xiaoliugen, Mihuanggu
V50		Jinangu11	summer		Shandong		**Yugu2** × **Zheng737** (81407 (Shuilihun × Yugu1 (Riben60ri × Tulong)) × 81474 (Zhengai2 (Qingdaolao (Riben60ri × Xinnong724 (Mihuanggu)))))	Riben60ri, Tulong, Xiaoliugen, Mihuanggu
V51		Bao182	summer		Hebei		**Zheng881407** × **Bao849** (Bao842090 × Yugu1 (Riben60ri × Tulong))	Riben60ri, Tulong
V52		L70	summer		Hebei		**WR1** × **Shi181-5**	Riben60ri, 60rihuancang, Tulong, Qinggouweicao
V53		Shi207191	summer		Hebei		-	-
V54		Jigu29	summer		Hebei		**WR1** × **Yugu1** (Riben60ri × Tulong)	Riben60ri, Tulong, Qinggouweicao
V55	pG4	Changnong35	spring	Summer (1, 14.29%); Spring (6, 85.71%)	Shanxi	Shanxi (6, 85.71%), Hebei (1, 14.29%)	**Jingu2**1 (Jinfen52 × Qitouhuang) × **Ninghuang1** (Yugu1 (Riben60ri × Tulong))	Riben60ri, Tulong, Xiaohuanggu, Jingfen52, Qitouhuang

Continued

V56		Yangu12	spring		Shanxi		**Lvgu2** (Jingu6 (Moligu)) × **79-421**	Moligu
V57		Taixuan4	spring		Shanxi		**95pin10** × **Jingu20** (Jingu6 (Moligu))	Moligu
V58		Taixuan5	spring		Shanxi		**Chigu8** (80-943*Chigu5 (Zhaonong1 (Shuangguayin))) × **Jingu20** (Jingu6 (Moligu))	Moligu, Shuangguayin
V59		Gu11A	summer		Hebei		-	-
V60		Jingu35	spring		Shanxi		(**Jingu14 × Jingu21** (Jinfen52 × Qitouhuang))**F1**	Jinfen52, Qitouhuang
V61		Jingu16	spring		Shanxi-		**Changnong18** (Changnong1 (Qinyuanmujizui)) × **Hainangu**	Qinyuanmujizui, Hainangu
V62		Datong14	spring		Shanxi		**Xiannong3** × **Jingu9** (Shanxidabaigu)	Xiannong3, Shanxidabaigu
V63		Datong30	spring	Summer	Shanxi		-	-
V64	pG5	Datong28	spring	(1, 20%); Spring (4, 80%)	Shanxi	Shanxi (4, 80%), Hebei (1, 20%)	(**Huangruangu × Zhangchunyi**) **F2** × **Jingu9** (Shanxidabaigu)	Huangruangu, Zhangchunyi, Shanxidabaigu
V65		Jigu28	summer		Hebei		-	-
V66		Datong27	spring		Shanxi		(**73-50** × **Zao1**) × **Yi17**	**-**
V67		Xinggu88	spring		Shanxi		**Jingu28**	Shaanxiheizhigu
V68		Tiegu14	spring		Liaoning		**Tiegu5** (Daobaqi × Riben60ri) × **Waiyin"79127"**	Riben60ri, Daobaqi
V69		Bagu214	spring		Hebei		**Bagu214**	-
V70		Chigu4	spring		Neimenggu		**Zhaogu1** (Shuangguayin)	Shuangguayin
V71		Chaogu13	spring		Liaoning		**Zhaonong21** (Zhaogu1 (Shuangguayin)) × **Tiegu7**	Shuangguayin
V72		Datong29zi	spring		Shanxi		-	-
V73		Tie487	spring		Liaoning		-	-
V74		Gonggu68	spring		Jilin		**Gonggu62** × **80026**	-
V75		Tie8240	spring		Liaoning		**23-4** × **Huangguzi**	Huangguzi
V76		Jigu30	summer		Hebei		-	-
V77		Tiegu5	spring	Summer	Liaoning	Liaoning (8, 33.33%), Hebei (6, 25%), Shanxi (3, 12.5%), Neimenggu (3, 12.5%), Jilin (2, 8.33%), Henan (1, 4.17%), America (1, 4.17%)	**Daobaqi** × **Riben60ri**	Riben60ri, Daobaqi
V78		Tiegu8	spring	(6, 25%), Spring (17, 70.83%), Unclear (1, 4.2%)	Liaoning		**8225** ((Tiegu5 (Daobaqi × Riben60ri) × Tiegu1)) × **Tie8240** (23-4 × Huangguzi)	Riben60ri, Daobaqi, Huangguzi
V79	pG6	An9217	summer		Henan		**Yugu1** (Riben60ri × Tulong) × **Niangu**	Riben60ri, Tulong
V80		Tiegu6	spring		Liaoning		**Jinzhougu9** × **78-8** ((Jingu211 × Jinxiangyu × Huangguzi))	Huangguzi, Jinxiangyu
V81		Meiguodatou	-		American		Meiguodatou	Meiguodatou
V82		Gonggu70	spring		Jilin		**Yingsuigu** × **79127-8**	yingsuigu
V83		Chigu10	spring		Neimenggu		**Chigu8** (Chigu5 (zhaonong1 (Shuangguayin)) × 80-943) × Chigu4 (Zhaogu1 (Shuangguayin))	Shuangguayin
V84		Gu10A	summer		Hebei		-	-
V85		Chigu8	spring		Neimenggu		**Chigu5** (zhaonong1 (Shuangguayin)) × **80-943**	Shuangguayin
V86		Shi02530	summer		Hebei		-	-
V87		Jigu27	summer		Hebei		-	-
V88		Chaolv	summer		Hebei		**Chaoxiangu**	Chaoxiangu
V89		Tiegu7	spring		Liaoning		**Tiegu4** × **Xuannong7**	-
V90		Yangu13	spring		Shanxi		**Qitouhuang** × **Jingu21** (Jinfen52 × Qitouhuang)	Jinfen52, Qitouhuang

Supplemental Table 2. Prolamin patterns of 90 accessions of foxtail millet.

B / V	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
V1	−	−	+	+	−	+	+	−	−	+	−	−	+	+	+	−	+	−	+	−	−	−	−	+	+	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−	−	−	−	+
V2	+	−	−	+	+	−	+	−	−	+	+	−	−	−	−	+	−	+	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
V3	−	−	−	+	+	−	+	−	−	−	+	−	−	+	−	+	−	+	+	+	−	−	+	−	+	−	−	−	−	−	+	+	+	−	−	−	+	+	−	−	−	−	+	−	−
V4	−	−	−	+	+	−	+	−	−	−	+	−	−	+	−	+	+	+	−	+	−	−	+	+	−	−	−	−	+	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−
V5	+	−	−	−	+	−	+	−	+	+	+	−	−	+	−	+	−	−	−	+	−	−	+	+	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	+	−	−	−
V6	−	−	−	+	+	−	+	+	−	−	+	−	−	+	−	+	−	+	+	+	+	−	+	+	−	−	−	−	+	+	−	+	−	+	−	−	+	+	−	−	−	−	−	−
V7	−	−	+	+	+	−	−	−	−	−	+	−	−	+	−	+	−	+	−	+	−	−	+	−	−	−	+	+	−	−	−	+	+	−	−	−	+	−	−	−	+	+	−	+
V8	+	−	−	+	+	−	+	−	−	+	+	−	−	+	−	+	−	+	−	+	−	−	+	−	−	+	−	−	+	+	+	−	−	−	−	−	+	−	−	+	−	−	−	−
V9	+	−	−	+	+	−	+	−	+	+	+	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	−	+	−	−	+	+	+	−	−	+	+	−	+	−	+	+	−	+
V10	−	−	+	−	+	−	+	+	−	−	−	−	−	+	+	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	+	+	−	−	−	−	−	−	+
V11	+	−	−	+	+	−	+	−	+	−	+	−	−	−	−	+	−	+	−	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−
V12	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−	+	−	−	−	+	+	−	+
V13	+	−	+	−	+	−	+	−	−	+	+	−	−	−	−	+	−	+	−	+	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−
V14	−	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	+
V15	−	−	+	−	+	−	+	+	−	−	−	−	+	+	+	−	−	+	−	+	−	−	+	+	+	+	−	−	−	−	−	+	−	+	−	−	+	+	−	−	−	−	−	−	−
V16	−	−	+	−	+	+	+	−	−	−	+	−	−	+	+	+	+	+	−	+	−	−	+	+	−	−	−	−	−	+	+	+	−	+	−	−	+	+	+	−	−	+	+	−	−
V17	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	+	+	−	−	−	+	−	−	−
V18	−	−	−	+	+	−	+	−	−	−	+	−	−	−	+	+	−	+	+	−	−	−	−	+	+	−	−	−	−	+	−	+	−	+	−	−	+	+	+	−	−	+	+	−	−
V19	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−
V20	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	+	+	−	+	+	−	−	−	−	−	+	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−
V21	−	−	+	−	−	−	−	−	−	+	−	+	−	−	−	+	+	+	−	+	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−
V22	−	+	−	+	−	−	+	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	+	−	−	−	−	−	+	−	+	+	−	−	−	−	+	−	−	−	−	+	+	−
V23	−	−	+	−	+	−	−	−	−	+	−	+	−	−	−	−	+	+	+	−	−	+	−	−	−	−	−	−	−	+	+	−	+	−	+	−	−	+	−	−	−	−	−	+	−
V24	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+	+	−	−	−	−	+	−
V25	−	−	+	−	+	−	−	−	−	+	−	+	−	−	+	−	+	−	+	−	−	−	−	−	−	+	+	−	−	+	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−
V26	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	+	+	−	−	−	−	+	−
V27	−	−	−	−	+	−	−	−	−	+	−	−	+	−	+	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−	−	+	+	−	−	−	−	+	−
V28	−	+	+	−	+	−	−	+	−	+	−	−	+	−	−	−	+	+	−	−	−	+	+	−	−	+	−	−	−	+	+	−	+	−	−	−	−	+	+	−	−	−	−	+	−
V29	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−	−	−	+	−	−	−	−	−	−
V30	−	+	+	−	−	−	−	−	−	+	−	−	+	+	+	−	+	−	−	−	−	+	+	+	−	−	+	−	+	+	+	−	+	−	−	−	−	+	+	+	−	−	+	+	−
V31	−	−	+	−	−	+	−	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
V32	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−
V33	−	−	−	+	−	+	−	−	−	−	−	+	+	−	−	−	+	−	−	+	−	−	+	+	−	−	+	−	+	−	+	+	+	−	−	−	+	+	−	−	+	−	−	−	−
V34	−	+	−	+	−	+	−	−	+	−	−	+	−	+	−	−	+	−	+	−	−	−	+	+	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−
V35	−	+	−	+	−	+	−	−	+	−	−	+	−	+	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−	+	−	+	−	+	−	−	−	+	+	−	+	−	−	−	−

Continued

V36	−	−	−	+	−	+	−	+	+	−	−	+	+	+	−	−	+	+	−	+	+	−	−	+	+	−	−	−	−	+	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−
V37	−	+	−	+	−	+	−	−	+	−	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
V38	−	+	−	+	−	+	−	−	−	−	+	+	+	−	−	−	+	−	−	+	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−
V39	−	−	+	+	−	+	+	−	−	−	−	+	−	−	−	−	+	−	−	+	−	−	+	+	−	−	−	−	−	−	+	+	−	−	−	−	+	+	−	−	+	−	−	−	−
V40	−	−	−	+	−	+	−	−	+	−	+	+	+	−	+	−	+	−	−	−	−	+	+	−	−	−	+	−	+	−	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−
V41	−	+	−	+	−	+	−	−	+	−	−	+	+	−	+	−	+	+	+	+	−	+	+	+	−	−	+	−	+	+	−	+	−	+	−	−	−	−	+	−	+	−	−	−	−
V42	−	+	+	+	−	+	+	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−
V43	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
V44	−	−	−	+	−	+	−	−	+	−	−	+	+	−	−	+	−	+	+	+	+	−	−	+	−	−	−	−	−	+	+	+	−	+	−	−	+	−	−	−	+	−	−	−	−
V45	−	−	−	+	−	+	−	−	+	−	−	+	−	+	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	+	+	+	−	−
V46	−	−	−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
V47	−	+	−	+	−	+	+	−	+	−	+	+	−	−	−	−	+	−	−	+	−	+	−	−	−	+	+	−	+	+	−	−	−	+	−	−	−	+	−	+	−	−	−	−	−
V48	−	−	−	+	−	+	+	−	+	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
V49	−	−	−	+	−	+	−	−	−	−	−	+	−	+	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	−	+	+	−	−	−	+	−	−
V50	−	−	−	−	−	+	−	+	+	−	−	+	+	+	−	−	−	−	−	+	+	−	−	+	+	−	−	−	−	−	+	+	−	+	−	+	−	+	+	−	−	−	−	+	−
V51	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−	+	−	−	+	−	+	+	+	−	−	+	−	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+
V52	−	−	−	+	−	−	−	−	+	−	+	−	+	−	−	−	+	−	−	−	−	+	+	−	+	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−
V53	−	−	+	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
V54	−	−	−	+	+	−	−	−	−	−	+	−	+	−	−	−	+	−	−	−	+	+	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
V55	−	−	−	+	−	−	−	−	+	−	−	+	−	−	+	+	+	−	+	−	+	−	−	−	+	−	+	−	−	−	−	+	−	−	+	−	+	−	+	−	−	−	+	−	−
V56	−	−	−	−	+	−	+	−	−	+	−	+	−	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−
V57	−	−	−	−	+	−	+	−	−	+	−	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	+	−	−
V58	−	−	−	−	−	−	+	−	+	−	−	+	−	−	+	+	+	−	+	−	−	−	−	−	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
V59	−	+	−	+	−	−	+	−	+	−	−	+	+	−	+	+	+	−	+	−	+	−	+	+	−	−	+	−	−	+	−	+	+	−	−	−	−	−	+	−	−	−	+	−	−
V60	−	+	−	−	+	−	+	−	+	+	−	+	+	−	+	+	+	−	+	−	+	+	−	+	−	−	−	−	−	−	+	+	−	−	−	−	+	−	+	−	−	−	+	−	−
V61	−	+	−	+	−	+	+	−	+	−	−	+	−	−	+	−	+	−	+	−	+	+	−	−	+	−	+	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−
V62	−	+	+	−	+	−	+	−	+	−	−	+	+	−	+	+	−	+	−	+	+	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
V63	−	+	+	−	+	−	+	−	+	−	−	+	+	−	+	+	−	+	−	+	+	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−
V64	−	−	+	−	+	−	+	−	+	−	−	+	+	−	+	+	−	+	−	+	+	−	−	−	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
V65	−	+	+	−	+	−	+	−	−	+	−	−	+	−	−	+	−	+	−	+	−	−	−	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−
V66	−	+	+	−	+	−	+	−	−	−	+	−	+	−	−	+	−	+	−	+	+	−	+	−	+	−	+	−	+	+	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+
V67	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	−	+	−	+	−	−	+	+	−	−	−	−	−	−	+	−
V68	−	−	+	−	+	−	−	−	+	−	+	−	−	−	−	+	−	+	+	−	−	+	+	+	−	−	−	−	+	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−
V69	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−
V70	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−
V71	−	+	+	−	−	−	−	−	+	−	+	−	+	−	−	+	−	+	−	−	−	+	+	+	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−
V72	−	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	+	+	+	+	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	−	+	−

Continued

V73	−	+	+	−	−	−	−	−	+	−	+	+	−	−	−	−	−	−	−	+	−	−	+	+	−	−	−	−	+	−	+	−	+	−	−	+	−	−	−	−	−	−	−	+	−
V74	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−	−	−	+	+	−	−	−	−	+	−	+	−	+	−	+	+	−	−	−	−	−	−	−	+	−
V75	−	+	+	−	+	−	+	+	+	−	+	+	−	−	−	−	−	+	+	−	−	+	+	−	−	−	−	−	+	−	+	+	−	−	+	−	−	−	+	−	−	−	−	+	−
V76	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
V77	−	+	+	−	+	−	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	+	−	−	+	−	+	−	−	−	−	+	−	−	−	−	−	−
V78	−	+	+	−	−	−	+	−	+	+	+	+	−	−	−	+	−	+	−	−	+	+	+	−	−	−	−	−	−	−	+	−	+	−	−	−	+	−	−	+	−	−	−	+	−
V79	−	+	+	−	−	+	−	−	−	−	−	+	+	−	+	−	−	+	+	−	−	+	+	+	−	−	−	−	+	−	−	−	+	+	−	−	+	+	−	+	−	−	−	+	−
V80	−	−	+	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−	+	+	+	+	+	−	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	−
V81	−	−	+	−	−	−	+	−	−	+	+	+	−	−	−	+	−	+	+	+	+	+	+	−	−	−	−	−	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−
V82	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	+	+	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−
V83	−	−	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+	+	−	−	−	−	+	+
V84	−	+	+	+	−	−	−	−	+	−	+	+	+	−	−	+	−	+	+	−	−	+	+	−	−	+	−	−	−	−	+	+	+	−	−	−	−	+	−	+	−	−	−	+	−
V85	−	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	+	+	+	+	+	+	−	+	−	−	−	−	+	+	+	−	−	−	+	−	−	+	−	−	−	−	−	−
V86	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	−	+	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−
V87	−	−	+	−	+	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	+	+	+	−	−	−	−	−	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−
V88	−	+	+	−	−	+	+	−	−	+	+	+	+	−	+	−	−	+	+	+	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
V89	−	−	+	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−
V90	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	+	+	−	+	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−

Note: V: varietie, B: band, +: showed protein band, -: no protein bands.

Supplemental Table 3. 90 foxtail millet accessions used in this study.

Entry	Name	G1	G2	G3	G4	G5	G6
V1	0.9759	0.0053	0.004	0.0037	0.0067	0.0044	0.9759
V2	0.9658	0.0095	0.0056	0.004	0.0099	0.0052	0.9658
V3	0.9642	0.0066	0.006	0.0092	0.0033	0.0107	0.9642
V4	0.9619	0.0072	0.0116	0.0056	0.0074	0.0063	0.9619
V5	0.9613	0.0071	0.0082	0.009	0.007	0.0074	0.9613
V6	0.9601	0.005	0.0094	0.0083	0.0066	0.0106	0.9601
V7	0.9601	0.0115	0.0093	0.0081	0.0041	0.0069	0.9601
V8	0.9565	0.0092	0.005	0.003	0.0202	0.0061	0.9565
V9	0.955	0.005	0.0067	0.0087	0.0206	0.004	0.955
V10	0.945	0.0111	0.0105	0.0065	0.0081	0.0188	0.945
V11	0.9333	0.0124	0.0071	0.0065	0.0286	0.0121	0.9333
V12	0.927	0.0161	0.0315	0.0108	0.0048	0.0098	0.927
V13	0.9153	0.0297	0.0049	0.004	0.0368	0.0093	0.9153
V14	0.896	0.0283	0.0138	0.0355	0.0166	0.0098	0.896

Continued

V15	0.8867	0.0148	0.0187	0.011	0.0303	0.0385	0.8867
V16	0.8798	0.0132	0.051	0.0354	0.0044	0.0162	0.8798
V17	0.8651	0.0314	0.0382	0.0154	0.0044	0.0455	0.8651
V18	0.8072	0.0103	0.0084	0.1599	0.005	0.0092	0.8072
V19	0.711	0.1712	0.0478	0.037	0.0132	0.0197	0.711
V20	0.6037	0.0751	0.0353	0.0496	0.0085	0.2278	0.6037
V21	0.5799	0.2184	0.079	0.0386	0.0632	0.021	0.5799
V22	0.5107	0.0767	0.055	0.1023	0.0062	0.2492	0.5107
V23	0.0042	0.9603	0.005	0.005	0.0042	0.0213	0.0042
V24	0.0052	0.9515	0.0049	0.004	0.003	0.0314	0.0052
V25	0.0067	0.9425	0.0073	0.024	0.014	0.0055	0.0067
V26	0.017	0.9206	0.016	0.0304	0.0079	0.0081	0.017
V27	0.0168	0.9095	0.0076	0.0325	0.0245	0.0091	0.0168
V28	0.0126	0.8815	0.0107	0.0047	0.0079	0.0826	0.0126
V29	0.0104	0.7698	0.1525	0.0257	0.0035	0.038	0.0104
V30	0.0335	0.6717	0.0608	0.0847	0.0404	0.1089	0.0335
V31	0.0074	0.661	0.1602	0.0245	0.0076	0.1393	0.0074
V32	0.0083	0.6299	0.0133	0.011	0.0068	0.3307	0.0083
V33	0.0073	0.0071	0.9696	0.005	0.0039	0.0071	0.0073
V34	0.0036	0.0059	0.9666	0.0133	0.0039	0.0067	0.0036
V35	0.0114	0.0078	0.9546	0.011	0.0101	0.0051	0.0114
V36	0.0102	0.0049	0.9526	0.012	0.0124	0.0079	0.0102
V37	0.0055	0.0125	0.9342	0.0089	0.0061	0.0328	0.0055
V38	0.008	0.0074	0.933	0.0083	0.0032	0.0401	0.008
V39	0.0259	0.0092	0.9325	0.0077	0.005	0.0197	0.0259
V40	0.0068	0.0099	0.9307	0.0192	0.005	0.0284	0.0068
V41	0.004	0.0056	0.9302	0.0334	0.0143	0.0125	0.004
V42	0.009	0.0209	0.9224	0.0184	0.0166	0.0127	0.009
V43	0.005	0.0473	0.9119	0.0163	0.0048	0.0147	0.005
V44	0.0151	0.0065	0.9094	0.0243	0.0216	0.0231	0.0151
V45	0.0751	0.0075	0.8892	0.0203	0.0022	0.0057	0.0751
V46	0.0235	0.0504	0.8413	0.0154	0.0065	0.0629	0.0235
V47	0.0279	0.0144	0.8338	0.008	0.1048	0.0111	0.0279
V48	0.071	0.0212	0.8303	0.0262	0.0394	0.0119	0.071

Continued

V49	0.0261	0.0132	0.7773	0.1744	0.005	0.004	0.0261
V50	0.0185	0.0412	0.7403	0.015	0.0118	0.1732	0.0185
V51	0.0156	0.0462	0.7295	0.0102	0.0067	0.1918	0.0156
V52	0.0551	0.2294	0.5172	0.0217	0.007	0.1696	0.0551
V53	0.0135	0.1717	0.4603	0.0089	0.0155	0.3302	0.0135
V54	0.0946	0.3411	0.4015	0.121	0.0116	0.0302	0.0946
V55	0.0043	0.013	0.0129	0.9571	0.004	0.0087	0.0043
V56	0.0104	0.0125	0.0074	0.9559	0.0058	0.008	0.0104
V57	0.008	0.0179	0.0039	0.9555	0.0097	0.005	0.008
V58	0.009	0.0077	0.0123	0.9553	0.0087	0.007	0.009
V59	0.0056	0.0072	0.0176	0.9538	0.0065	0.0093	0.0056
V60	0.0069	0.0138	0.0131	0.9335	0.0058	0.0269	0.0069
V61	0.003	0.0083	0.0447	0.9292	0.0087	0.0061	0.003
V62	0.0022	0.0026	0.003	0.004	0.9852	0.003	0.0022
V63	0.0037	0.003	0.003	0.003	0.9843	0.003	0.0037
V64	0.0155	0.0095	0.0136	0.016	0.9295	0.0159	0.0155
V65	0.1099	0.0905	0.0051	0.0054	0.7723	0.0168	0.1099
V66	0.4112	0.0105	0.0039	0.0059	0.5383	0.0302	0.4112
V67	0.003	0.0078	0.0044	0.003	0.002	0.9798	0.003
V68	0.0079	0.0121	0.004	0.004	0.0041	0.9679	0.0079
V69	0.0076	0.0067	0.0085	0.0056	0.0042	0.9674	0.0076
V70	0.006	0.0161	0.0077	0.005	0.0072	0.958	0.006
V71	0.0125	0.0046	0.0106	0.0079	0.0125	0.9519	0.0125
V72	0.0068	0.0159	0.0098	0.004	0.0154	0.9481	0.0068
V73	0.0061	0.0151	0.0203	0.0049	0.007	0.9466	0.0061
V74	0.004	0.0474	0.007	0.004	0.0038	0.9338	0.004
V75	0.0123	0.0318	0.012	0.0129	0.0075	0.9235	0.0123
V76	0.0242	0.0172	0.0323	0.006	0.0063	0.9139	0.0242
V77	0.0265	0.0143	0.0255	0.0096	0.0115	0.9126	0.0265
V78	0.0098	0.0223	0.0058	0.01	0.0463	0.9058	0.0098
V79	0.006	0.0335	0.0303	0.0061	0.0276	0.8965	0.006
V80	0.0175	0.0447	0.0101	0.0208	0.0112	0.8957	0.0175
V81	0.035	0.02	0.0088	0.0211	0.0462	0.8689	0.035
V82	0.0336	0.011	0.0664	0.0163	0.0129	0.8598	0.0336

Continued

V83	0.0237	0.112	0.0188	0.0068	0.0033	0.8354	0.0237
V84	0.0419	0.075	0.0374	0.0092	0.0176	0.819	0.0419
V85	0.0144	0.0197	0.0087	0.1111	0.0309	0.8152	0.0144
V86	0.0647	0.0486	0.0211	0.0186	0.043	0.8039	0.0647
V87	0.017	0.046	0.0852	0.0265	0.0328	0.7925	0.017
V88	0.0099	0.0619	0.0334	0.039	0.0762	0.7796	0.0099
V89	0.0073	0.3449	0.0104	0.008	0.004	0.6254	0.0073
V90	0.0127	0.0626	0.3313	0.0186	0.0639	0.5109	0.0127