Original Research Article

Phylogenetic relationships and diversity in *Leymus* (Poaceae, Triticeae) based on simple sequence repeats markers

ABSTRACT

Aims: The objective of the study is to investigate: (1) the Ns genome donor and elucidate the origins of the Xm genome of Leymus; (2) evaluate the phylogenetic relationships among these species.

Methodology: The CTAB (cetyltrimethylammonium bromide) procedure was used to extract total genomic DNA from fresh leaf tissue. A total of 150 pairs of SSR primers were tested to screen those produced polymorphic DNA bands to continue to further analysis with Elymus species and 13 diploid perennial species as templates. GS was used to construct a dendrogram via the unweighted pair group method with arithmetic average (UPGMA) and the SHAN (sequential, hierarchical, agglomerative, and nested clustering) routine in the NTSYS-pc program.

Results: The primers WMC475, WMC11 and QWM213 showed more expansion efficiency in the research. Here were significant diversity and polymorphism between *Leymus* and related diploid *Triticeae* species based on SSR makers. The largest GS coefficient values between Pse. stipifolia and *Elymus hystrix* indicates that the genetic distance is the closest and has a closer genetic relationship. In clade I, the *Leymus* species formed a very well supported into a small distinct groups (Ia) first. Specifically, *L. racemosus*, *L. salinus*, *L. secalinus* and *L. triticoides* were clustered closely.

Conclusion: *Psathyrostachys juncea*, *Psa. huanshanica* and *Psa. fragilis* (Ns genome) clustered together into clade I with *Leymus* species, which further illustrates *Leymus* that contains Ns genome are more closely related with *Psathyrostachys. St genome and J genome did not participated in the origin of Leymus*, and the genetic relationship and genetic distance of *Leymus* species are related to geographical distribution and environment.

Keywords: Leymus; SSR; Phylogenetic relationships; Phylogenetic analysis.

1. INTRODUCTION

Leymus Hochst. is an important polyploid perennial genus of Triticeae [1]. It includes approximately 30 species and 19 subspecies (Yen 2011). All the species in *Leymus* Hochst are polyploid with chromosome numbers ranging include tetraploid (2n=4x=28), hexaploid (2n=6x=42), octoploid (2n=8x=56), decaploid (2n=10x=70), and dodecaploid (2n=12x=84) [3,4,5,6,7]. They are distributed in a wide range of ecological habitats regions which from the coastal areas of North Sea, Central Asia, East Asia, extending to Alaska and the western areas of North America [3,4,6,7]. They are found in particularly large numbers on the mountains of Central Asia and

North America[4,8]. There are many *Leymus* species in China and it includes about 27 species, 3 subspecies and 3 varieties, which are mainly distributed in north-western, north, north-eastern and south-western regions in China [9]. Most species of *Leymus* are desirable traits as disease and insect resistance, bigger spikes, more and bigger grains and efficient photosynthesis [4]. They are growing in saline or alkaline lands, and dry or semi-dry areas and highly adaptable to coldness, dryness and saline or alkaline lands [10]. Thus, some species of *Leymus* are main components of grasslands and fine varieties of herbage. *Leymus* is an important genetic resource to improvement of Triticeae cereal crops [4,7,9,11].

The genus *Leymus* was erected and circumscribed by Hochstetter (1848) with *L. arenarius* (L.) Hochst [1]. as its model specie. *Leymus* was recognized to be a genus with the Ns and Xm genomes. The presence of the *Psathyrostachys* Ns genome in *Leymus* has been identified which based on the basis of meiotic pairing in interspecific hybrids and DNA sequences [12]. However, which species of *Psathyrostachys* potentially served as the genome donor of Ns genome in *Leymus* have not been identified. The Xm genome is one of the important basic genome in the perennial Triticeae species [13,14]. However, the origin of the Xm genome is still unknown. In addition, the origin and definition of the genus, precise taxonomic ranks and relationships among the species in the genus have been under discussion [5,6,8,15,16,17,18,19,20]. The study on genetic diversity of *Leymus* would provide theoretical foundation for using this genus plants to breed Triticeae crops and herbage.

Molecular marker is an effective and valuable technique to diagnose the internal gene arrangement and plant systematic to establish evolutionary relationships within or among species, subspecies, populations and genomes by directly analyzing the polymorphism of the genetic material [10,21,22]. SSR (single sequence repeats) markers were used to carry out a relationship analysis of diploid perennial species in the Triticeae [23,24]. In the present study, we carried out phylogenetic relationships using single sequence repeats for 12 *Leymus* taxa, one *Elymus* species and 13 diploid perennial species representing 7 basic genomes in Triticeae. The aims of this study were to investigate: (1) the Ns genome donor and elucidate the origins of the Xm genome of *Leymus*; (2) evaluate the phylogenetic relationships among these species.

2. MATERIAL AND METHOD

2.1 Plant materials

A total of 26 accessions of Triticeae were used in this study, including 12 accessions of *Leymus* (NsXm) and 14 relative genera taxa which contained 4 accessions of *Pseudoroegneria* (St), 3 accessions of *Psathyrostachys* (Ns), 2 accessions of *Agropyron* (P), 2 accessions of *Hordeum* (H), *Australopyrum retrofractum* (W), *Lophopyrum elongatum* (E^e) and *Elymus hystrix* (StH) (Table 1). The seed materials of Triticeae taxa with PI numbers were kindly provided by American National Plant Germplasm System (Pullmam, WA, USA); those with Y and ZY numbers were collected by the Triticeae Research Institute of Sichuan Agriculture University. The plants and voucher specimens are deposited at Triticeae Research Institute, Sichuan Agricultural University, China (SAUTI).

Table 1. The materials investigated in this present study.

No.	Species	2n	Genomo	Accession	Origin
1	*Leymus angustus*	84	NsXm	PI 271893	Kazakhstan
2	*L. arenarius*	28	NsXm	PI 272126	Kazakhstan
3	*L. chinensis*	28	NsXm	PI 499515	Inner Mongolia, China
4	*L. karelinii*	84	NsXm	PI 598529	Xinjiang, China

5	*L. multicaulis*	28	NsXm	PI 440324	Kazakhstan
6	*L. paboanus*	56	NsXm	PI 531808	Estonia
7	*L. pseudoracemosus*	28	NsXm	PI 531810	Qinghai, China
8	*L. racemosus*	56	NsXm	PI 598806	Russia
9	*L. salinus*	28	NsXm	PI 636574	United States
10	*L. secalinus*	28	NsXm	Y040	Xinjiang, China
11	*L. tianshanicus*	84	NsXm	Y2036	Xinjiang, China
12	*L. triticoides*	28	NsXm	PI 531821	United States
13	*Pseudoroegneria strigosa*	14	St	PI 531752	Estonia
14	*Pse. libanotica*	14	St	PI 228390	Iran
15	*Pse. stipifolia*	14	St	PI 313960	Russia
16	*Pse. spicata*	14	St	PI 232123	United States
17	*Hordeum bogdanii*	14	H	Y1488	Xinjiang, China
18	*H. chilense*	14	H	PI 531781	Argentina
19	*Psathyrostachys juncea*	14	Ns	PI 430871	Russia
20	*Psa. huanshanica*	14	Ns	ZY3157	Shanxi, China
21	*Psa. fragilis*	14	Ns	PI 347191	Iran
22	*Agropyron critatum*	14	P	PI 277352	Russia
23	*Ag. mongolicum*	14	P	PI 511543	Mongolia
24	*Australopyrum retrofractum*	14	W	PI 531553	Argentina
25	*Lophyrum elongatum*	14	E^e	PI 574517	Argentina
26	*Elymus hystrix*	28	StH	PI 531616	Canada

2.2 Experimental method

The CTAB (cetyltrimethylammonium bromide) procedure was used to extract total genomic DNA from fresh leaf tissue [25]. A total of 150 pairs of SSR primers were tested to screen those produced polymorphic DNA bands to continue to further analysis with *Elymus* species and 13 diploid perennial species as templates. All polymerase chain reactions (PCRs) were undertaken in a 50-μL reaction volume, containing 1 μL template DNA at the concentration of 20 ng/μL, 1.5 mmol/L MgCl2, 200 mol/L dNTPs, 1.0 mol/L of each primer, and 1.5 U ExTaq Polymerase (TaKaRa) and distilled deionized water to the final volume. PCR reaction was as follows: one cycle of 4 min at 94 ºC, 35 cycles of 30 sec at 94 ºC, 30 sec at 52 ºC, 1 min at 72 ºC, and ended with a final extension of 10 min at 72 ºC. Each sample wasdiluted 1:10 prior to injection. Finally, The PCR results were visualized by capillary electrophoresis using Qiaxcel (QIAxcel Advanced Electrophoresis System, Qiagen) and analyzed by using 35 to 1000 bp DNA size alignment marker. Then, we should screen the results of genotyping for specificity on those DNA samples, and select the best-performing for the analysis of the collection.

2.3 Data analysis.

The SSR bands were treated as dominant markers, individual band was considered as a character and were scored as present (1) or absent (0) of the same size for each primer, then entered into a binary matrix representing the SSR profile of each accession. The potential of SSR markers for estimating genetic variability was examined by measuring the marker informativeness of polymorphic loci. The loci were counted as number

of total amplified bands (TB), number of polymorphic bands (PB) and % of polymorphic bands (PPB). The data matrix was entered into the NTSYS-pc program [26]. Genetic similarities (GS) among the 26 germplasm materials were calculated based on Jaccard's coefficient using the Simqual (similarity for qualitative data) method. GS was used to construct a dendrogram via the unweighted pair group method with arithmetic average (UPGMA) and the SHAN (sequential, hierarchical, agglomerative, and nested clustering) routine in the NTSYS-pc program [26].

3. RESULTS

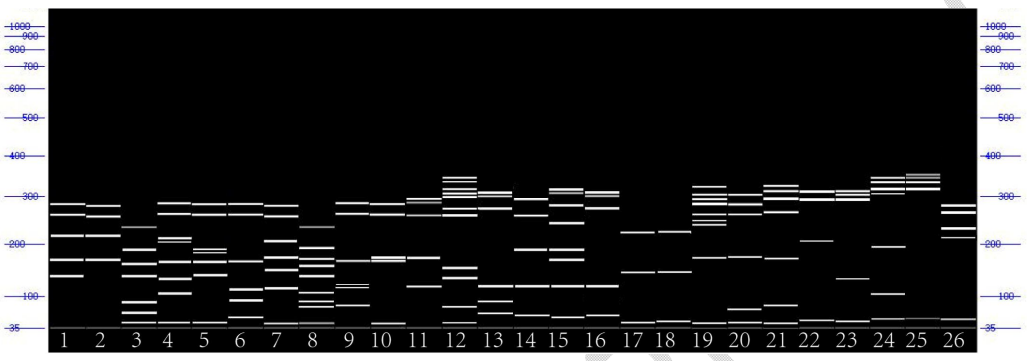

Fig 1. SSR polymorphism generated by primer WMC475 (The material order was same as in Table 1, and analyzed by using 35 to 1000 bp DNA size alignment marker).

A total of 150 pairs of primers were tested, and 23 pairs of primers were able to produce clear and stable amplified bands for the best-performing for the analysis of the collection (Table 2). The 23 pairs of primers used were amplified to obtain 438 bands with different mobility, of which 430 bands were polymorphic, accounting for 98.18%. 11-29 bands were amplified for each pair of primers, and 19.04 bands were amplified on average for each pair of primers. Among them, the primers WMC475, WMC11 and QWM213 showed more expansion efficiency in our research. Figure 1 is the PCR amplification results of primers WMC475 on 26 test materials. The results showed that there were significant diversity and polymorphism between *Leymus* and related diploid Triticeae species based on SSR makers.

Table 2. List of primers, their sequences and amplification results.

Primer		Sequence 5'-3'	TB	PB	PPB%
GWM257	F	AGAGTGCATGGTGGGACG	25	25	100
	R	CCAAGACGATGCTGAAGTCA			
GWM247	F	GCAATCTTTTTTCTGACCACG	21	21	100
	R	ATGTGCATGTCGGACGC			
GWM304	F	AGGAAACAGAAATATCGCGG	16	16	100
	R	AGGACTGTGGGGAATGAATG			
GWM410	F	GCTTGAGACCGGCACAGT	24	22	91
	R	CGAGACCTTGAGGGTCTAGA			
GWM617	F	GATCTTGGCGCTGAGAGAGA	17	16	94
	R	CTCCGATGGATTACTCGCAC			
WMC111	F	ATTGATGTGTACGATGTGCCTG	27	27	100
	R	CATGTCAATGTCATGATGAAGC			
WMC41	F	TCCCTCTTCCAAGCGCGGATAG	19	19	100
	R	GGAGGAAGATCTCCCGGAGCAG			

WMC611	F	GGTTCGCTTTCAAGGTCCACTC	16	16	100
	R	CGGGACACTAGTGCTCGATTCT			
GWM146	F	CCAAAAAAACTGCCTGCATG	12	12	100
	R	CTCTGGCATTGCTCCTTGG			
GWM162	F	AGTGGATCGACAAGGCTCTG	14	14	100
	R	AGAAGAAGCAAAGCCTTCCC			
GWM182	F	TGATGTAGTGAGCCCATAGGC	13	11	84
	R	TTGCACACAGCCAAATAAGG			
GWM153	F	GATCTCGTCACCCGGAATTC	11	11	100
	R	TGGTAGAGAAGGACGGAGAG			
GWM458	F	TGCCTGGCTCGTTCTATCTC	15	15	100
	R	CTAGCTTAGCACTGTCGCCC			
GWM213	F	CTAATTGCAACAGGTCATGGG	26	26	100
	R	TACTTGTGTTCTGGGACAATGG			
GWM359	F	AATGGCAATTGGAAGACATAGC	23	22	95
	R	TTCGCAATGTTGATTTGGC			
GWM577	F	ATGGCATAATTTGGTGAAATTG	18	18	100
	R	TGTTTCAAGCCCAACTTCTATT			
WMC475	F	AACACATTTTCTGTCTTTCGCC	29	29	100
	R	TGTAGTTATGCCCAACCTTTCC			
GDM29	F	CTAGTTGTGCTAGGCGCTCC	11	11	100
	R	CTGGCTGCTCCCTCCTC			
GDM77	F	GACACACAATAGCCAAAGCA	25	25	100
	R	TGATGTCGGCACTATTTTGG			
GDM8	F	TTCTCCAACGCACGTTAGC	14	11	78
	R	CCCAAATGATGGCAGCTACT			
WMC10	F	GATCCGTTCTGAGGTGAGTT	10	10	100
	R	GGCAGCACCTCTATTGTCTC			
WMC128	F	CGGACAGCTACTGCTCTCCTTA	28	28	100
	R	CTGTTGCTTGCTCTGCACCCTT			
WMC349	F	ACACACACTCGATCGCAC	24	24	100
	R	GCAGTTGATCATCAAAACACA			
Total			438	430	98

All the 438 SSR bands were used to calculate the Jaccard's genetic similarity coefficient (GS) multivariate analysis using the NTSYS-pc (Table 3). The genetic similarity coefficients ranged from 0.545-0.966, with an average 0.723. The largest GS coefficient values (0.966) were between *Pse. stipifolia* and *Elymus hystrix* indicates that the genetic distance is the closest and has a closer genetic relationship. *L. triticoides* and *Psathyrostachys juncea* have the smallest GS coefficient values (0.545), indicating that their genetic relationship is far away. The GS values of *L. triticoides* and *Psa. huanshanica* were also had the smallest GS coefficient values (0.545).

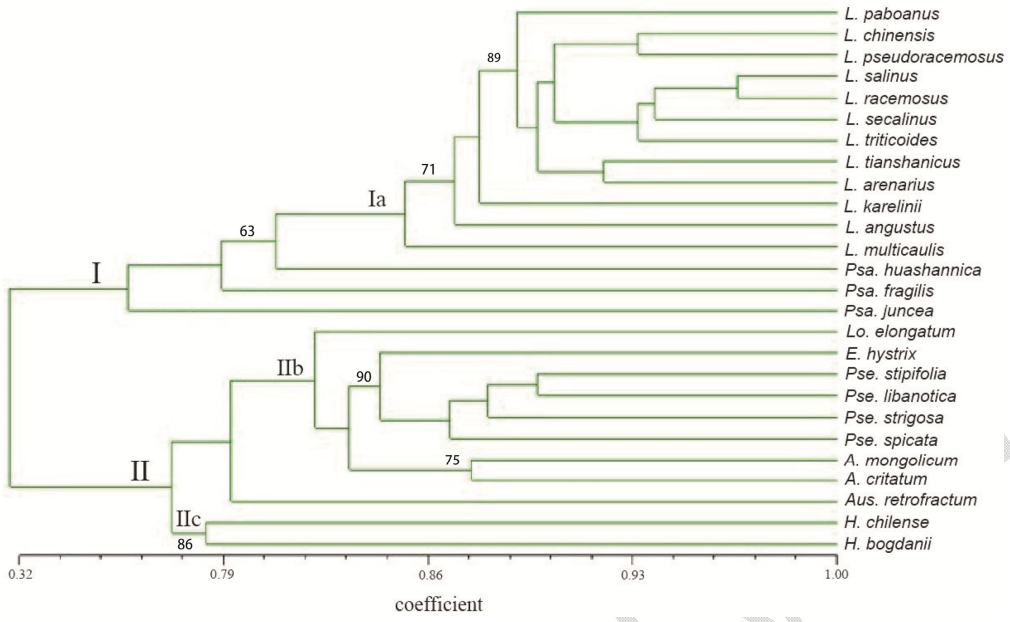

Fig 2. A dendrogram generated by using Jaccard's coefficients of similarity for the SSR.

In the SSR maker phylogenetic dendrogram, 26 species grouped into two distinct main clades, namely clade I and clade II. In clade I, the *Leymus* species formed a very well supported into a small distinct groups (Ia) first. Specifically, *L. racemosus*, *L. salinus*, *L. secalinus* and *L. triticoides* were clustered closely. Otherwise, *L. chinensis* was closer related with *L. pseudoracemosus*. Similarly, *L. tianshanicus* was closer related with *L. arenarius.* Then, all the species of *Leymus* (NsXm genome) were together with the *Psathyrostachys juncea*, *Psa. huanshanica* and *Psa. fragilis* (Ns genome). The clade II composed of 11 accessions left, of which have two subclades, *Elymus hystrix* was included in the first subclade (IIb) with *Pseudoroegneria strigosa, Pse. libanotica*, *Pse. stipifolia* and *Pse. spicata* (St genome). Meanwhile, *Hordeum bogdanii* (H genome) and *H. chilense* was separated into the secord subclade (IIc).

Table 3. Genetic similarities for the SSR makers

	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
1	1																									
2	0.864	1																								
3	0.864	0.864	1																							
4	0.761	0.864	0.864	1																						
5	0.795	0.761	0.761	0.761	1																					
6	0.841	0.795	0.897	0.898	0.875	1																				
7	0.875	0.84	0.807	0.801	0.841	0.861	1																			
8	0.773	0.761	0.76	0.716	0.795	0.75	0.75	1																		
9	0.715	0.807	0.795	0.886	0.750	0.83	0.852	0.807	1																	
10	0.818	0.716	0.739	0.795	0.818	0.795	0.852	0.761	0.784	1																
11	0.739	0.829	0.773	0.750	0.807	0.830	0.784	0.795	0.773	0.75	1															
12	0.761	0.773	0.795	0.761	0.807	0.795	0.841	0.727	0.830	0.830	0.852	1														
13	0.739	0.739	0.761	0.614	0.727	0.750	0.716	0.761	0.66	0.705	0.602	0.750	1													
14	0.761	0.785	0.659	0.773	0.773	0.739	0.784	0.740	0.750	0.648	0.733	0.909	0.886	1												
15	0.795	0.719	0.741	0.693	0.707	0.707	0.773	0.773	0.739	0.761	0.682	0.807	0.920	0.943	1											
16	0.75	0.773	0.795	0.648	0.761	0.761	0.727	0.773	0.693	0.761	0.636	0.761	0.875	0.920	0.932	1										
17	0.773	0.795	0.818	0.670	0.784	0.784	0.75	0.625	0.716	0.739	0.659	0.784	0.920	0.920	0.955	0.909	1									
18	0.670	0.693	0.716	0.568	0.683	0.704	0.648	0.693	0.614	0.682	0.579	0.682	0.841	0.864	0.853	0.895	0.852	1								
19	0.66	0.682	0.727	0.556	0.670	0.670	0.639	0.682	0.625	0.761	0.545	0.670	0.784	0.807	0.841	0.898	0.818	0.784	1							
20	0.682	0.682	0.704	0.602	0.648	0.670	0.659	0.727	0.602	0.670	0.545	0.693	0.807	0.807	0.818	0.898	0.795	0.739	0.773	1						
21	0.739	0.761	0.614	0.727	0.75	0.716	0.739	0.603	0.705	0.602	0.750	0.864	0.864	0.886	0.898	0.875	0.875	0.818	0.785	0.807	1					
22	0.716	0.738	0.761	0.613	0.727	0.727	0.693	0.739	0.659	0.727	0.609	0.727	0.886	0.909	0.92	0.841	0.875	0.841	0.784	0.761	0.864	1				
23	0.716	0.716	0.739	0.591	0.704	0.727	0.693	0.739	0.639	0.704	0.58	0.727	0.886	0.875	0.875	0.773	0.852	0.864	0.807	0.807	0.864	0.886	1			
24	0.784	0.807	0.807	0.682	0.795	0.795	0.761	0.784	0.727	0.727	0.695	0.795	0.864	0.866	0.898	0.852	0.92	0.864	0.807	0.784	0.864	0.886	0.841	1		
25	0.807	0.83	0.807	0.727	0.818	0.773	0.784	0.807	0.75	0.727	0.716	0.841	0.874	0.807	0.943	0.92	0.92	0.841	0.807	0.807	0.864	0.864	0.841	0.932	1	
26	0.761	0.739	0.761	0.614	0.727	0.727	0.693	0.761	0.659	0.727	0.602	0.727	0.909	0.932	0.966	0.92	0.92	0.864	0.83	0.784	0.887	0.864	0.886	0.864	0.864	1

4. DISCUSSION

Leymus Hochst. (1848) is a large polyploid perennial genus of the tribe Triticeae [1]. The genus was distinguished as a separate genus with *L. arenarius* (L.) Hochst. as the type species by Hochstetter (1848). For the ploidy levels, ranges from tetraploid (2*n*=4*x*=28) to dodecaploid (2n=12x=84), are cytologically recognized among *Leymus* species [3,4,5,6]. Species in *Leymus* contains the Ns and Xm genomes, where the presence of the *Psathyrostachys* Ns genome in *Leymus* has been repeatedly substantiated on the basis of meiotic pairing in interspecific hybrids, DNA hybridization patterns and DNA sequence data [6,7,27,28]. However, which species of *Psathyrostachys* is the donor of the Ns genome have not been identified. Meanwhile, Sequence data from the chloroplast DNA (cpDNA) *trn*L–*trn*F, *trn*H-*psb*A and mitochondrial *cox*II regions suggest that species of *Psathyrostachys* served as the maternal genome donor during the divergence of *Leymus* [29,30,31] Similarly, based on *Acc1* and *DMC*1 sequences, the results indicate that there may be multiple origins in the genus of *Leymus*, and different *Psathyrostachys* species are involved in the genus of *Leymus* polyploidization and chromosome doubling [6,28]. In this study, *Psathyrostachys juncea*, *Psa. huanshanica* and *Psa. fragilis* (Ns genome) clustered together into clade I with *Leymus* species, which further illustrates *Leymus* that contains Ns genome are more closely related with *Psathyrostachys.* Specifically, *L. multicaulis* was closer related with *Psa. huanshanica*. Thus, based on SSR maker dendrogram that the Ns genome of *L. multicaulis* maybe came from *Psa. huanshanica*. But, geographically, *Psa. huanshanica* was only distributed China, while *L. multicaulis* was located in Central Asia. However, *Leymus* species are distributed from the coastal areas of North Sea, Central Asia, East Asia, extending to Alaska and the western areas of North America [3,4,6,7]. Manwhile, *Psathyrostachys* species mainly distributed in dry, semi-dry and grasslands lands areas of Central Asia [2]. Therefore, *Leymus* species and *Psathyrostachys* species have a widely overlap in areas, which provides the possibility for their genetic exchange.

The Xm genome is one of the important basic genome in the perennial Triticeae species. The wheat tribe have been widely collected and recorded many wild species, but Xm-genome diploids have not been identified. At present, the *Pseudoroegneria* (St) [32], *Thinopyrum* (E^b) [3], *Psathyrostachys* (Ns) [21,32], *Lophopyrum* (E^e) [34], *Agropyron* (P) [6,29] and *Eremopyrum* (F) [6,31] have been suggested as the donate for Xm genome. However, the RAPD data indicated the Xm genome of *Leymus* may have multiple origins, and the St, W and H genomes may have taken part in the formation of some *Leymus* species [10]. Moreover, Phylogenetic analysis of single-copy nuclear *DMC*1 data suggests that the origin of the Xm genome of *Leymus* could differ among species [35]. In the present study, showed that all *Leymus* species were grouped together high statistic support with three diploid *Psathyrostachys* species. Our results are in agreement with the data by Shiotan, Yang, Wang and Jensen [13,32], it suggested that St genome and J genome did not participated in the origin of *Leymus*. Owing to no diploid species containing the Xm genome was found until now, the origin of the Xm genome in Triticeae remained a mystery. The Xm genome might origined, or may be extinct. In conclusion, the genomic constitution of *Leymus* should remain as NsXm until the source of Xm is identified.

The SSR maker dendrogram showed distinguish and determine the intraspecific relationship of *Leymus* species accurately. In this study, the results are partly accordance with previous studies in morphological characteristics and genome homology, which based on meiotic pairing and intergeneric hybrids. Furtherly, in the present study showed that *L. racemosus* (Russia), *L. salinus* (North America), *L. secalinusi* (East Asia) and *L. triticoides* (North America) were clustered together. Our results are in agreement with the ITS date and RAPD analyze showed that a very high homology existed in genomes of *Leymus* species from North America

and central Asia [8,11], which suggested that the central Asia *Leymus* species might be via the Bering land bridge, and spread to the North American and Eurasian. Furthermore, *L. tianshanicus* (Xinjiang, China) was closer related with *L. arenarius* (border between Xinjiang, China and Kazakhstan). Otherwise, *L. chinensis* (Inner Mongolia, China) was closer related with *L. pseudoracemosus* (Qinghai, China). lastly, which indicates that the genetic relationship and genetic distance of *Leymus* species are related to geographical distribution and environment.

5. CONCLUSION

The objective of the study is to investigate the Ns genome donor and elucidate the origins of the Xm genome of Leymus and evaluate the phylogenetic relationships among these species. In our study, *Psathyrostachys juncea*, *Psa. huanshanica* and *Psa. fragilis* (Ns genome) clustered together into clade I with *Leymus* species, which further illustrates *Leymus* that contains Ns genome are more closely related with *Psathyrostachys. St genome and J genome did not participated in the origin of Leymus*, and the genetic relationship and genetic distance of *Leymus* species are related to geographical distribution and environment.

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