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Phylogenetic relationships between *Leymus* and related diploid Triticeae species revealed by ISSR markers

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Abstract: To investigate the genetic diversity and phylogenetic relationships between polyploid *Leymus* and related diploid species of the Triticeae tribe, inter-simple sequence repeats (ISSR) markers was used to analyze 41 *Leymus* accessions representing 22 species and 2 subspecies, together with *Pseudoroegneria stipifolia* (St), *Psathyrostachys fragilis* (Ns), *Australopyrum retrofractum* (W), *Hordeum bogdanii*, *H. chilense* (H) and *Lophopyrum elongatum* (E^e). A total of 376 clear and reproducible DNA fragments were amplified by 29 ISSR primers, among which 368 (97.87%) fragments were found to be polymorphic. 8-18 polymorphic bands were amplified by each polymorphic primer, with an average of 12.69 bands. The data of 376 ISSR bands were used to generate Nei's similarity coefficients and to construct a dendrogram by means of UPGMA. The similarity coefficients data suggested great genetic diversity in genus *Leymus* and related diploid Triticeae species, the genetic diversity among the different species more abundant than that of the different accessions. The dendrogram and principal coordinate analysis showed explicit interspecific relationships and demonstrated close phylogenetic relationships between *Leymus* species and *Psathyrostachys*.

Key words: *Leymus*; cluster analysis; genetic diversity; ISSR markers; phylogeny

Introduction

Leymus Hochst is an important polyploid perennial genus of the tribe *Triticeae* (Poaceae) and includes about 50 species and subspecies which are closely related with wheat, barley, cultivated rye and other Triticeae cereals. Phytogeographically, *Leymus* species are distributed in the temperate regions of Eurasia, North and South America, extending to the subtropic and the tropic alpine regions. Most species are found in the mountains of central Asia and North America (Tzvelev et al. 1976; Dewey 1984). The natural habitats of *Leymus* species range from coastal to inland areas, including saline or alkaline lands, and dry or semidry areas. Many *Leymus* species are highly adaptable to cold, drought, and to saline or alkaline severe environment. Some *Leymus* species also bear other such desirable traits such as disease and insect resistance, bigger spikes than wheat, more grains than wheat. Many *Leymus* species provide genetic material for the improvement of forage and cereal crops and can be utilized as potential contributors to genes of cold hardiness, drought and salt tolerance, and disease resistance for cereal crops in Triticeae (Dewey 1984; Dong et al. 1992).

Since Hochstetter (1848) separated *Leymus* from the traditional *Elymus* L., with *Leymus arenarius* (L.) Hochst. as the type species, most taxonomists have accepted the circumscription of *Leymus* (Pilger 1954; Löve & Löve 1961; Keng 1965; Tzvelev 1976; Melderis 1980; Barkworth & Atkins 1984), but some included *Leymus* in *Elymus* (Bowden 1964; Estes & Tyrl 1982). *Leymus* was originally defined as a genomically allopolyploid genus with two distinct subgenomes Ns and Xm. Meiotic pairing data from interspecific and intergeneric hybrids (Dewey 1984; Wang 1994) and molecular studies (Jensen et al. 1997; Sha et al. 2008) had revealed that the Ns genome of *Leymus* was originated from *Psathyrostachys*. Despite decades of intensive efforts in cytogenetic and molecular biology researches, there are still uncertainties regarding the origin of the Xm genome of *Leymus*.

Molecular markers have proved to be an effective and valuable technique to evaluate genetic diversity and relationships within species or genera. Inter-simple sequence repeats (ISSR) has unique advantages over other molecular markers: it doesn't need any genomic information of the target species, which is extremely important in a preliminary investigation; it consumes

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Table 1. The materials investigated in the present study.

No.	Taxon	2n	Genome	Accesion No.	Origin
1	*Leymus racemosus* (Lam.) Tzvel	56	NsXm	PI502402	Russian Federation
2	*L. racemosus* (Lam.) Tzvel	56	NsXm	PI478832	Montana, United States
3	*L. angustus* (Trin.) Pilger	84	NsXm	PI440308	Kazakhstan
4	*L. angustus* (Trin.) Pilger	84	NsXm	PI499650	Xinjiang, China
5	*L. karelinii* (Trin.) Pilger	84	NsXm	PI598534	Xinjiang, China
6	*L. karelinii* (Turcz.) Tzvel	84	NsXm	PI636651	Kazakhstan
7	*L. multicaulis* (Kar. & Kir.) Tzvel	28	NsXm	PI440324	Kazakhstan
8	*L. multicaulis* (Kar. & Kir.) Tzvel	28	NsXm	PI440325	Kazakhstan
9	*L. secalinus* (Georgi) Tzvel	28	NsXm	ZY09131	Qinghai, China
10	*L. secalinus* (Georgi) Tzvel	28	NsXm	PI598757	Kazakhstan
11	*L. secalinus* (Georgi) Tzvel	28	NsXm	PI499535	Xinjiang, China
12	*L. pseudoracemosus* Yen and Yang	28	NsXm	PI531810	Qinghai, China
13	*L. pseudoracemosus* Yen and Yang	28	NsXm	ZY09148	Qinghai, China
14	*L. mollis* (Trin.) Pilger	28	NsXm	PI567896	Alaska, United States
15	*L. triticoides* (Buck.) Pilger	28	NsXm	PI578750	Washington, United States
16	*L. triticoides* (Buck.) Pilger	28	NsXm	PI531822	Nevada, United States
17	*L. tianschanicus* (Drob.) Tzvel	84	NsXm	Y2036	Xinjiang, China
18	*L. condensatus* (Presl) A. Löve	28	NsXm	PI442483	Antwerp, Belgium
19	*L. secalinus* (Georgi) Tzvel	28	NsXm	PI 639770	Mongolia
20	*L. ambiguous* (Vasey & Scribner) D.R. Dewey	56	NsXm	PI565019	Colorado, United States
21	*L. arenarius* (L.) Hochst	28	NsXm	PI294584	Sweden
22	*L. arenarius* (L.) Hochst	28	NsXm	PI494699	Kazakhstan
23	*L. ramosus* (Trin.) Tzvel	28	NsXm	PI440330	Kazakhstan
24	*L. ramosus* (Trin.) Tzvel	28	NsXm	PI499653	Xinjiang, China
25	*L. salinus* (M.E.Jones) Á. Löve	28	NsXm	PI565038	Utah, United State
26	*L. salinus* (M.E.Jones) Á. Löve	28	NsXm	PI636574	Mongolia
27	*L. innovatus* (Beal) Pilger	28	NsXm	PI236818	Canada
28	*L. paboanus* (Claus) Pilger	56	NsXm	PI531808	Estonia
29	*L.racemosus* ssp. *sabulosus* (Lam.) Tzvel	28	NsXm	PI531814	Estonia
30	*L. chinensis* (Trin.) Tzvel	28	NsXm	PI619486	Mongolia
31	*L. chinensis* (Trin.) Tzvel	28	NsXm	PI499519	Inner Mongolia, China
32	*L. chinensis* (Trin.) Tzvel	28	NsXm	PI499515	Inner Mongolia, China
33	*L. cinereus* (Trin.) Tzvel	56	NsXm	PI619543	Washington, United States
34	*L. cinereus* (Trin.) Tzvel	56	NsXm	PI469229	Saskatchewan, Canada
35	*L. duthiei* (Stapf) Y.H. Zhou et H.Q. Zhang	28	NsXm	ZY2004	Sichuan, China
36	*L. hybrid*	28	NsXm	PI537363	Nevada, United States
37	*L. akmolinensis* (Drobow) Tzvel	28	NsXm	PI440306	Russian, Federation
38	*L. alaicus* ssp. *karataviensis* (Roshev.) Tzvel	28	NsXm	PI314677	Alma-Ata, Kazakhstan
39	*L. alaicus* ssp. *karataviensis* (Roshev.) Tzvel	28	NsXm	PI314667	Alma-Ata, Kazakhstan
40	*L. qinghaicus* L.B. Cai	28	NsXm	HY0717	Sichuan, China
41	*L. qinghaicus* L.B. Cai	28	NsXm	HY0716	Sichuan, China
42	*Pseudoroegneria stipifolia* (Czern. ex Nevski) A. Löve	14	St	PI313960	Former Soviet Union
43	*Psathyrostachys fragilis* (Boiss.) Nevski	14	Ns	PI343191	Iran
44	*Australopyrum retrofractum* (Vickery) A. Löve	14	W	PI531553	Utah, United States
45	*Hordeum bogdanii* (Wilensky) A. Löve	14	H	Y1488	China
46	*H. chilense* (Roem. & Schult.) A. Löve	14	H	PI531781	Argentina
47	*Lophopyrum elongatum* (Host) A. Löve	14	E^e	PI574517	Argentina

small amount of template DNA and can be rapidly conducted, which is an efficient and rapid way in detecting genetic diversity of species (Semagn et al. 2006). Previous studies have demonstrated that ISSR markers are appropriate for analyses on genetic diversity and phylogenetic relationship in Graminaceous crops, wheat (Vaillancourt et al. 2008), rice (Reddy et al. 2009) and maize (Domenyuk et al. 2002). AFLP, RAPD and RAMP markers were previously used to assess genetic diversity and interspecific relationships among *Leymus* species (Yang et al. 2006; Yang et al. 2008; Culumber et al. 2011; Yang et al. 2011).

The objective of present study are: (1) to evaluate the efficiency of ISSR markers for discriminating the genetic diversity of *Leymus* species; (2) to examine the genetic diversity and interspecific or intraspecific relationships in *Leymus*; (3) to investigate intergeneric relationships among *Leymus*, *Psathyrostachys*, *Lophopyrum*, *Pseudoroegneria*, *Hordeum* and *Australopyrum* and explore the origins of the unknown Xm genome in *Leymus*.

Material and methods

A total of 47 accessions of Triticeae were used in this study, including 41 accessions of *Leymus*, which distributed in 22 species and 2 subspecies (NsXm); in addition, we used the relative diploid genera taxa such as *Psathyrostachys fragilis* (Ns), *Pseudoroegneria stipifolia* (St), *Australopyrum retrofractum* (W), *Hordeum bogdanii, H. chilense* (H)and *Lophopyrum elongatum* (E^e) representing five basic genomes to explore the origin of Ns and Xm genome in *Leymus* (Table 1). All seed materials were kindly provided by American National Plant Germplasm System (Pullman, Washington, USA) and Triticeae Research Institute of Sichuan Agriculture University. The plants and voucher specimens of all

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

Primer	Sequence(5'→3')	Tm(℃)	TB	PB	PPB(%)
UBC807	(AG)8T	48	12	12	100
UBC808	(AG)8C	56.5	17	16	94.11
UBC809	(AG)8G	55	12	12	100
UBC810	(GA)8T	48	11	11	100
UBC815	(CT)8G	49.5	8	8	100
UBC823	(TC)8C	51.8	11	11	100
UBC824	(TC)8G	49.6	8	8	100
UBC825	(AC)8T	52.4	12	12	100
UBC826	(AC)8C	55	11	10	90.90
UBC827	(AC)8G	54.5	13	13	100
UBC828	(TG)8A	53.5	8	8	100
UBC829	(TG)8C	52	11	10	90.90
UBC834	(AG)8YT	55.5	13	13	100
UBC835	(AG)8YC	55.5	13	12	92.31
UBC836	(AG)8YA	50.5	14	12	85.71
UBC841	(GA)8YC	56.5	13	11	84.62
UBC844	(CT)8RC	50	14	14	100
UBC845	(CT)8RG	50	14	14	100
UBC847	(CA)8RC	52.8	15	15	100
UBC853	(TC)8RT	50.8	12	12	100
UBC855	(AC)8YT	52.5	11	11	100
UBC857	(AC)8YG	51.5	11	11	100
UBC873	(GACA)4	48.5	15	15	100
UBC880	(GGAGA)3	48	18	18	100
UBC881	(GGGTG)3	55	17	17	100
UBC888	BDB (CA)7	53	15	15	100
UBC889	DBD (AC)7	55.5	15	15	100
UBC890	VHV (GT)7	56	16	16	100
UBC895	(AG)2TTGGTAG(CT)2TGATC	51	16	16	100
Total	29		376	368	
Average		52.4	12.97	12.69	97.87

Abbreviations: Y = (C, T), R = (A, G), B = (non A), D = (non C), V = (non T), H = (non G). Tm: amplified annealing temperature; TB: number of total bands; PB: number of polymorphic bands; PPB: % of polymorphic bands.

the materials have been deposited at the perennial nursery and Herbarium of the Triticeae Research Institute, Sichuan Agriculture University, China (SAUTI).

Fresh young leaf tissues (3 g) were collected from 5-10 plants of each accession, frozen with liquid nitrogen and grinded into powder. Genomic DNA was extracted following a slightly modified CTAB (cetyltrimethylammonium bromide) protocol (Doyle & Doyle 1987). DNA concentration was measured by comparing with BM 5000 DNA Marker (BM® Biomed) using 1% agarose gel electrophoresis under UV light.

A total of 65 ISSR primers (UBC primer set no. 9, University of British Columbia, Canada) were tested to select those produced polymorphic DNA bands. According to the amplification efficiency and reproducibility of the band patterns, 29 ISSR primers were selected for further analysis. The final PCR volume was 25 μL and contained 1.5 μL template DNA at the concentration of 20 ng/μL, 12.5 μL 2 × Taq PCRMasterMiX (4 mM $MgCl_2$, 0.4 mM dNTPs of each nucleotide, 0.05 units/μL Taq DNA polymerase), 10 μM primer 1.0 μL and 10.0 μL ddH_2O. The amplification reactions were performed with an initial denaturing step at 94℃ for 5 min, followed by 35 cycles of 1 min denaturing at 94℃, 1 min annealing at 50℃, 2min extension at 72℃ , and a final extension step at 72℃ for 10 min on BIO-RAD S1000™ Thermal cycler. Amplified products were separated on a 1.5% agarose gel through electrophoresis in 1 × TAE buffer (pH8.0) with BM 2000 DNA Marker (BM® Biomed).

The agarose gels were photographed using Gel imaging system (BIO-RAD Gel Doc XR + Molecular Imager). The ISSR 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 ISSR profile of each accession. The potential of ISSR 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 raw data matrix was then used to calculate Nei's genetic similarity coefficients (GS) for measuring pairwise band similarities between individuals, $GS = 2N_{ij}/(N_i+N_j)$, where N_{ij} is the number of ISSR locus in common between accessions i and j, N_i and N_j are the total number locus generated by accessions i and j respectively(Nei & Li 1979). Based on the 0/1 matrix, a cluster analysis was conducted based on Nei's similarity coefficients using unweighted pair group method with arithmetic average (UPGMA) method with the SAHN module of *NTSYS-pc* 2.10e software package. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The principal coordinated analysis (PCA) was performed with the modules DCEN-

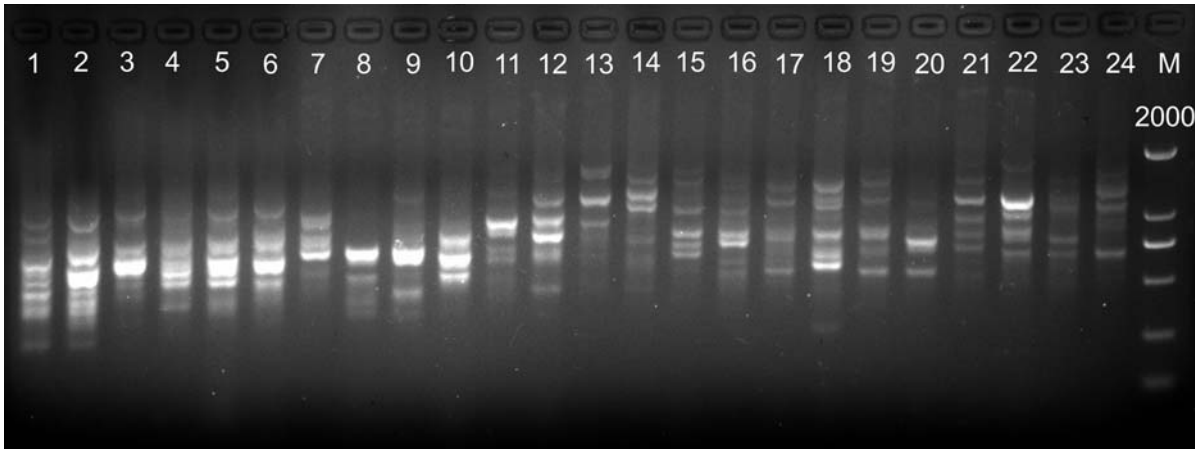

Fig. 1. ISSR polymorphism in parts of *Leymus* species amplified by primer UBC827. The accessions are described in Table 1. M-2000 bp DNA Marker.

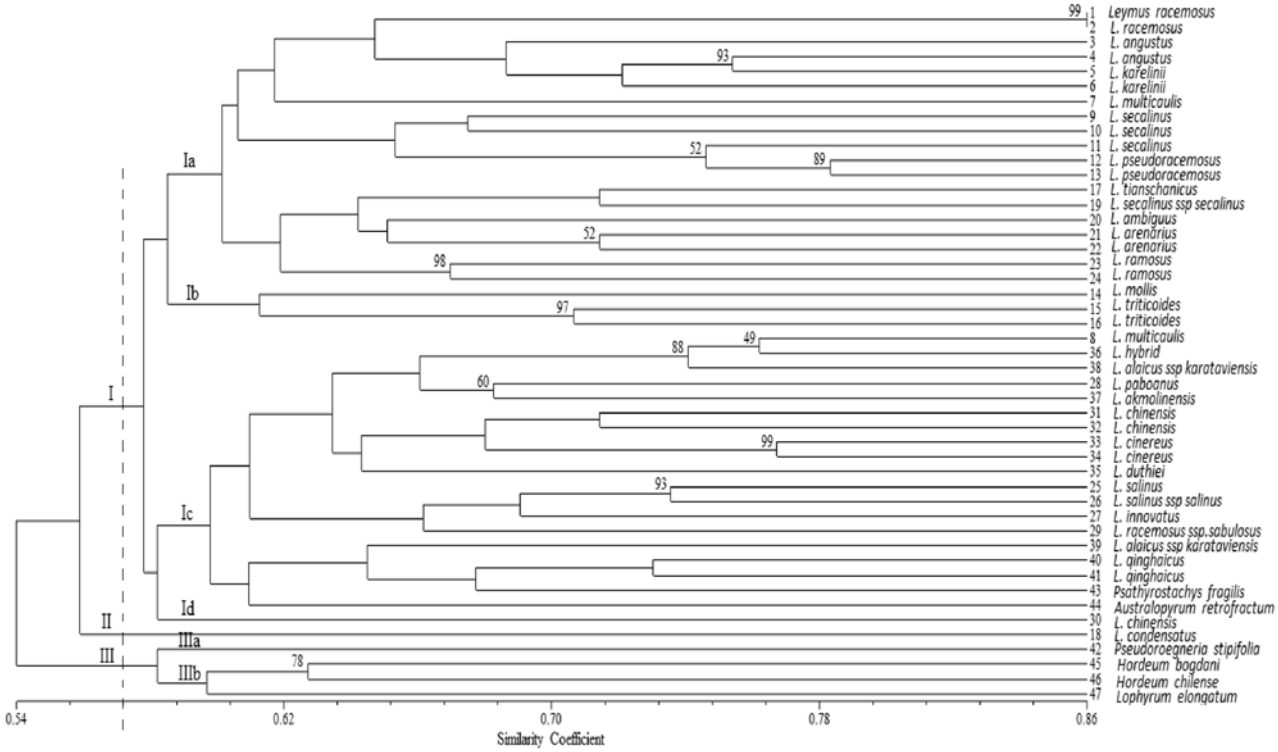

Fig. 2. UPGMA dendrogram of Nei's genetic similarity and percentage of bootstrap value (specified at selected dichotomous points) as determined related diploid Triticeae species.

TER and EIGEN implemented in *NTSYS-pc*, and the three principal coordinates were used to visualize the dispersion of accessions in a three-dimensional array of eigenvectors.

Results

Of the 65 primers tested, 29 primers were selected for further analysis, which are able to produce clear and stable amplified bands. These 29 primers produced 376 ISSR molecular fragments, among which 368 bands (97.87%) were polymorphic loci. Each primer amplified from 8 to 18 polymorphic bands, with an average of 12.97 loci, of which 12.69 were polymorphic loci. The highest (18 bands) was produced by UBC880 and the lowest (8 bands) was produced by UBC815, UBC824 and UBC828 (Table 2). Amplified product sizes ranged from 200 to 2000 bp approximately. The ISSR marker profile obtained with primer UBC827 on the basis of size comparison with BM 2000 DNA Marker (BM® Biomed) (Fig. 1).

All the 376 ISSR bands were used to calculate Nei's genetic similarity coefficients (GS) by multivariate analysis using a Simqual (similarity for qualitative data) program, which are measuring pairwise band similarities between individuals. The genetic similarity coefficients value varied from 0.460 to 0.864 with an average of 0.662 (Table 3). It indicated that there was considerable ISSR variation among species of genus *Leymus*

Table 3. Genetic similarity matrix based on ISSR polymorphism among *Leymus* and related diploid Triticeae species.

	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	46	47
1	1.000																																															
2	0.864	1.000																																														
3	0.683	0.654	1.000																																														
4	0.654	0.662	0.715	1.000																																												
5	0.614	0.638	0.643	0.758	1.000																																										
6	0.670	0.625	0.710	0.723	0.726	1.000																																									
7	0.611	0.614	0.609	0.595	0.609	0.680	1.000																																								
8	0.539	0.537	0.595	0.545	0.526	0.598	0.651	1.000																																							
9	0.579	0.571	0.609	0.601	0.625	0.633	0.648	0.656	1.000																																						
10	0.630	0.617	0.686	0.646	0.611	0.694	0.619	0.648	0.678	1.000																																					
11	0.571	0.553	0.611	0.598	0.606	0.646	0.640	0.638	0.672	0.707	1.000																																				
12	0.593	0.601	0.606	0.598	0.574	0.635	0.598	0.611	0.646	0.643	0.765	1.000																																			
13	0.598	0.611	0.595	0.587	0.558	0.587	0.571	0.590	0.630	0.638	0.734	0.787	1.000																																		
14	0.545	0.523	0.550	0.577	0.555	0.582	0.515	0.507	0.563	0.577	0.555	0.561	0.625	1.000																																	
15	0.558	0.545	0.577	0.585	0.550	0.590	0.569	0.603	0.606	0.614	0.561	0.593	0.619	0.627	1.000																																
16	0.614	0.590	0.606	0.603	0.606	0.641	0.598	0.574	0.593	0.617	0.627	0.606	0.606	0.603	0.710	1.000																															
17	0.640	0.632	0.648	0.667	0.638	0.651	0.582	0.579	0.598	0.638	0.611	0.617	0.627	0.587	0.640	0.675	1.000																														
18	0.577	0.553	0.537	0.539	0.547	0.555	0.550	0.537	0.571	0.569	0.547	0.574	0.521	0.574	0.518	0.574	0.617	1.000																													
19	0.625	0.590	0.627	0.662	0.648	0.646	0.582	0.553	0.577	0.638	0.611	0.622	0.611	0.574	0.630	0.627	0.718	0.595	1.000																												
20	0.553	0.555	0.593	0.595	0.614	0.606	0.574	0.625	0.611	0.625	0.582	0.582	0.593	0.582	0.579	0.603	0.609	0.571	0.656	1.000																											
21	0.627	0.625	0.598	0.643	0.619	0.601	0.558	0.507	0.569	0.614	0.593	0.619	0.603	0.598	0.590	0.587	0.651	0.603	0.710	0.648	1.000																										
22	0.595	0.598	0.640	0.617	0.630	0.622	0.569	0.566	0.611	0.672	0.582	0.587	0.598	0.555	0.590	0.587	0.625	0.587	0.619	0.659	0.718	1,000																									
23	0.582	0.590	0.638	0.571	0.595	0.587	0.566	0.590	0.593	0.585	0.558	0.585	0.547	0.566	0.566	0.595	0.627	0.569	0.627	0.640	0.609	0.630	1.000																								
24	0.617	0.587	0.598	0.569	0.571	0.606	0.553	0.507	0.553	0.609	0.582	0.603	0.614	0.569	0.563	0.625	0.646	0.593	0.630	0.579	0.611	0.617	0.672	1.000																							
25	0.617	0.603	0.582	0.627	0.582	0.585	0.579	0.539	0.585	0.571	0.571	0.555	0.550	0.539	0.563	0.587	0.609	0.561	0.630	0.601	0.590	0.558	0.523	0.558	1.000																						
26	0.595	0.593	0.603	0.680	0.625	0.606	0.574	0.593	0.611	0.614	0.566	0.598	0.603	0.569	0.611	0.593	0.635	0.561	0.635	0.632	0.622	0.601	0.561	0.563	0.739	1.000																					
27	0.571	0.553	0.595	0.603	0.601	0.593	0.582	0.579	0.545	0.579	0.558	0.547	0.521	0.526	0.571	0.569	0.590	0.585	0.617	0.625	0.582	0.555	0.563	0.577	0.688	0.699	1.000																				
28	0.539	0.563	0.585	0.603	0.526	0.577	0.571	0.627	0.598	0.606	0.601	0.590	0.601	0.577	0.603	0.542	0.595	0.558	0.558	0.598	0.545	0.561	0.563	0.523	0.577	0.662	0.611	1.000																		
29	0.603	0.595	0.638	0.598	0.611	0.619	0.582	0.606	0.603	0.595	0.585	0.579	0.617	0.539	0.582	0.579	0.622	0.547	0.606	0.609	0.587	0.587	0.569	0.577	0.656	0.662	0.675	0.606	1.000																	
30	0.534	0.547	0.563	0.545	0.563	0.587	0.529	0.553	0.555	0.563	0.542	0.574	0.505	0.577	0.497	0.521	0.489	0.526	0.558	0.582	0.582	0.550	0.505	0.539	0.571	0.593	0.606	0.574	0.627	1.000																
31	0.558	0.523	0.603	0.606	0.625	0.638	0.590	0.598	0.638	0.630	0.598	0.582	0.550	0.537	0.563	0.587	0.593	0.598	0.614	0.648	0.601	0.606	0.603	0.585	0.601	0.659	0.662	0.609	0.640	0.625	1.000														
32	0.579	0.566	0.614	0.537	0.571	0.643	0.574	0.704	0.611	0.646	0.656	0.630	0.603	0.550	0.585	0.603	0.603	0.582	0.587	0.632	0.579	0.601	0.571	0.574	0.590	0.617	0.625	0.603	0.651	0.625	0.718	1.000													
33	0.539	0.526	0.601	0.555	0.579	0.603	0.587	0.632	0.598	0.585	0.617	0.595	0.579	0.547	0.582	0.601	0.558	0.542	0.601	0.614	0.550	0.529	0.558	0.507	0.640	0.587	0.638	0.648	0.654	0.579	0.694	0.720	1.000												
34	0.555	0.553	0.558	0.566	0.601	0.587	0.550	0.622	0.603	0.569	0.606	0.595	0.569	0.531	0.534	0.601	0.569	0.569	0.595	0.598	0.561	0.545	0.579	0.555	0.614	0.587	0.648	0.611	0.622	0.585	0.683	0.635	0.771	1.000										
35	0.579	0.571	0.593	0.590	0.603	0.595	0.558	0.603	0.595	0.571	0.587	0.593	0.577	0.510	0.547	0.561	0.587	0.566	0.609	0.585	0.590	0.611	0.603	0.569	0.585	0.617	0.635	0.587	0.635	0.614	0.643	0.627	0.625	0.688	1.000									
36	0.523	0.515	0.579	0.518	0.531	0.598	0.577	0.765	0.646	0.606	0.622	0.590	0.590	0.550	0.603	0.595	0.595	0.553	0.531	0.625	0.513	0.566	0.595	0.513	0.539	0.603	0.617	0.707	0.595	0.558	0.651	0.672	0.686	0.664	0.593	1.000									
37	0.523	0.515	0.574	0.555	0.537	0.582	0.593	0.632	0.603	0.601	0.601	0.601	0.606	0.577	0.598	0.585	0.558	0.563	0.531	0.635	0.561	0.561	0.595	0.550	0.561	0.619	0.601	0.686	0.601	0.601	0.656	0.630	0.638	0.638	0.603	0.696	1.000								
38	0.526	0.492	0.587	0.537	0.507	0.606	0.579	0.731	0.601	0.598	0.609	0.577	0.577	0.537	0.611	0.561	0.582	0.555	0.571	0.654	0.542	0.595	0.625	0.553	0.558	0.627	0.651	0.667	0.635	0.561	0.664	0.686	0.651	0.630	0.648	0.757	0.651	1.000							
39	0.614	0.617	0.617	0.598	0.595	0.656	0.587	0.617	0.593	0.622	0.601	0.617	0.595	0.545	0.571	0.622	0.622	0.537	0.632	0.593	0.577	0.561	0.606	0.593	0.598	0.598	0.638	0.611	0.632	0.601	0.640	0.625	0.617	0.648	0.646	0.611	0.606	0.672	1.000						
40	0.582	0.563	0.585	0.582	0.590	0.625	0.577	0.622	0.603	0.627	0.622	0.601	0.579	0.545	0.571	0.585	0.547	0.510	0.547	0.614	0.539	0.561	0.606	0.582	0.582	0.625	0.622	0.611	0.590	0.547	0.662	0.598	0.664	0.675	0.672	0.632	0.601	0.678	0.707	1.000					
41	0.619	0.601	0.611	0.587	0.617	0.635	0.566	0.537	0.571	0.611	0.579	0.611	0.579	0.566	0.571	0.574	0.590	0.569	0.553	0.582	0.561	0.609	0.547	0.603	0.587	0.603	0.611	0.585	0.585	0.574	0.598	0.566	0.617	0.654	0.571	0.563	0.579	0.571	0.617	0.734	1.000				
42	0.566	0.547	0.542	0.497	0.500	0.529	0.507	0.574	0.545	0.521	0.505	0.515	0.521	0.550	0.539	0.558	0.515	0.547	0.500	0.523	0.497	0.539	0.500	0.518	0.561	0.566	0.531	0.537	0.537	0.569	0.545	0.566	0.510	0.590	0.582	0.579	0.595	0.550	0.574	0.563	0.553	1,000			
43	0.553	0.561	0.587	0.590	0.577	0.606	0.547	0.582	0.590	0.614	0.630	0.640	0.614	0.550	0.558	0.587	0.571	0.545	0.582	0.601	0.547	0.590	0.561	0.579	0.531	0.579	0.571	0.582	0.582	0.582	0.579	0.601	0.603	0.603	0.632	0.577	0.577	0.595	0.619	0.688	0.672	0.603	1.000		
44	0.571	0.553	0.526	0.577	0.563	0.582	0.566	0.595	0.571	0.553	0.558	0.574	0.585	0.571	0.550	0.595	0.531	0.569	0.521	0.561	0.545	0.545	0.574	0.577	0.539	0.582	0.553	0.558	0.547	0.531	0.577	0.571	0.553	0.574	0.566	0.606	0.590	0..593	0.595	0.632	0.590	0.590	0.630	1.000	
45	0.566	0.563	0.558	0.534	0.526	0.555	0.539	0.553	0.545	0.531	0.500	0.537	0.515	0.534	0.539	0.500	0.515	0.569	0.537	0.534	0.492	0.539	0.542	0.545	0.523	0.518	0.510	0.574	0.500	0.537	0.529	0.523	0.505	0.510	0.561	0.574	0.547	0.555	0.585	0.585	0.579	0.569	0.582	0.590	1.000
46	0.500	0.518	0.497	0.521	0.550	0.505	0.521	0.518	0.563	0.513	0.513	0.518	0.518	0.515	0.531	0.534	0.534	0.518	0.545	0.510	0.526	0.537	0.534	0.558	0.531	0.510	0.518	0.460	0.555	0.497	0.542	0.526	0.486	0.507	0.537	0.534	0.529	0.510	0.571	0.523	0.523	0.598	0.537	0.598	0.630	1.000
47	0.585	0.593	0.577	0.574	0.571	0.558	0.531	0.550	0.574	0.550	0.571	0.593	0.566	0.526	0.542	0.561	0.561	0.545	0.587	0.574	0.569	0.574	0.577	0.563	0.553	0.579	0.534	0.555	0.529	0.550	0.547	0.531	0.518	0.555	0.590	0.529	0.566	0.515	0.577	0.587	0.577	0.587	0.531	0.555	0.587	0.611	1.000

and related diploid Triticeae species. The highest genetic similarity coefficient (0.864) was between *L. racemosus* (PI502402) and *L. racemosus* (PI4788 32), while the lowest genetic similarity coefficient (0.460) found between *L. paboanus* and *Hordeum chilense*. From the genetic similarity coefficients, there was clear representation that little genetic diversity among the different accessions within species, while the genetic difference among the different species is distinct.

Based on Nei's genetic similarity coefficients, cluster analysis was carried out using UPGMA method and resulted in a phylogenetic dendrogram which clearly discriminated strains from each other (Fig. 2). The 41 accessions of *Leymus* and 6 related diploid Triticeae species were divided into 3 distinct main groups at the similarity coefficient value of 0.58. Different accessions within the same species were clustered together first, then the different taxa were clustered. UPGMA cluster analysis showed clear genetic relationship among the 47 accessions and their clusters were related to known pedigree relationships.

Except for *L. condensatus*, most *Leymus* accessions clustered with *Psathyrostachys fragilis* and *Australopyrum retrofractum* in group I, which can be further divided into 4 subgroups. There are 10 *Leymus* species clustered into subgroup Ia, *L. racemosus*, *L. angustus* and *L. karelinii* were clustered together first, then they clustered with *L. multicaulis* (PI440324), further they clustered with a sister group which was formed by *L. secalinus* and *L. pseudoracemosus*. *L. tianschanicus*, *L. secalinus* (PI639770), *L. ambiguous* and *L. arenarius* were clustered together first, further they clustered with *L. ramosus*. Most of these *Leymus* accessions come from Xinjiang of China or the neighboring geographic regions, some species come from northern European or central Eurasian. Subgroup Ib comprised 2 North American species, two accessions of *L. triticoides* clustered together first,then they clustered with *L. mollis*. Subgroup Ic was formed by *Psathyrostachys fragilis*, *Australopyrum retrofractum*, 10 species and 2 subspecies of *Leymus*, including *L. multicaulis* (PI440325), *L. akmolinensis, L. hybrid*, *L. chinensis* (PI499515, PI499519), *L. cinereus*, *L. paboanus*, *L. duthiei*, *L. salinus*, *L. alaicus* ssp. *karataviensis*, *L. innovatus*, *L. racemosus* ssp. *sabulosus* and *L. qinghaicus*. *L. chinensis* (PI619486) which separate from the other two *L. chinensis* accessions clustered in subgroup Id lonely. Group II consisted of a single clade comprised by *L. condensatus* which come from Belgium of western European. Group III were divided into 2 small distinct groups which comprised four diploid species. Subgroup IIIa included *Pseudoroegneria stipifolia*, it separated into a clade. In subgroup IIIb, *Hordeum bogdanii* and *H. chilense* composed a sister group to *Lophopyrum elongatum*.

The genetic relationships among the *Leymus* and related diploid Triticeae species were also investigated using principal coordinate analysis (PCA). PCA is a multivariate approach which is more informative regarding distances among major groups (Hauser & Crovello 1982). The first three principal coordinates accounted for 7.5086%, 5.4250% and 5.1540% of the total molecular variations, respectively. A three-dimensional plot showing the dispersion of the 47 accessions was displayed in Fig. 3 and the PCA separated the 47 accessions into 3 distinct groups. The closer position indicated that they had a relatively lower level of genetic variation and a closer correlativity. PCA for ISSR data showed that it supported UPGMA clustering results, meanwhile it showed more explicit relationships among the 47 test accessions with different orientations and positions.

Discussion

ISSR marks can be effective in reflecting the relationship of *Elymus* species which have larger divergence, as an effective analysis method for remote *Elymus* species (Li et al. 2005). The high levels of polymorphism detected in this study by using ISSR, 376 molecular fragments generated by 29 ISSR primers, 368 loci (97.87%) were polymorphic. This polymorphism rate was higher than that revealed by RAPD markers of 95.74 % (Yang et al. 2008) and that detected by RAMP markers of 93.23% (Yang et al. 2006). The great quantity of polymorphic bands, together with the Nei's similarity coefficient ranging from 0.460 to 0.864, which indicated that ISSR marks have abundant genetic diversity among *Leymus* species.

The present dendrogram showed interspecific relationships in the genus *Leymus* are partly accordance with previous studies in morphological characteristics and genome homology based on meiotic pairing and intergeneric hybrids (Tzvelev 1976; Löve 1984; Barkworth & Atkins 1984; Sun et al. 1995; Zhi & Teng 2005), especially could distinguish and determine the intraspecific relationship of Leymus species accurately. ITS date showed that *L. hybrid*, *L. triticoides*, *L. ambiguous*, *L. salinus*, and *L. cinereus* which distributed in North America were grouped together with some *Leymus* species from central Asia (Sha et al. 2008).RAPD analyzing suggested that the *Leymus* species from North America were scattered in different groups and clustered with those species from central Asia (Yang et al. 2008). The present study showed that *L. ambiguus* scattered in subgroup Ia, the two accessions of *L. triticoides* and *L. mollis* clustered in subgroup Ib, meanwhile, two accessions of *L. cinereus*, two accessions of *L. salinus*, *L. hybrid* and *L. innovatus* were relatively grouped together in subgroup Ic. These *Leymus* species scattered in different subclades with high statistical support, it indicated that *Leymus* species from North America is closely related to those from central Asia and East Asia, it showed that a very high homology existed in genomes of *Leymus* species from North America and Eurasia (Most of species distributed in central Asia), which suggested that the North American Leymus species might be most likely originate from recent colonization via the Bering land bridge, the genus *Leymus* might originate from central Asia, the North American *Leymus*

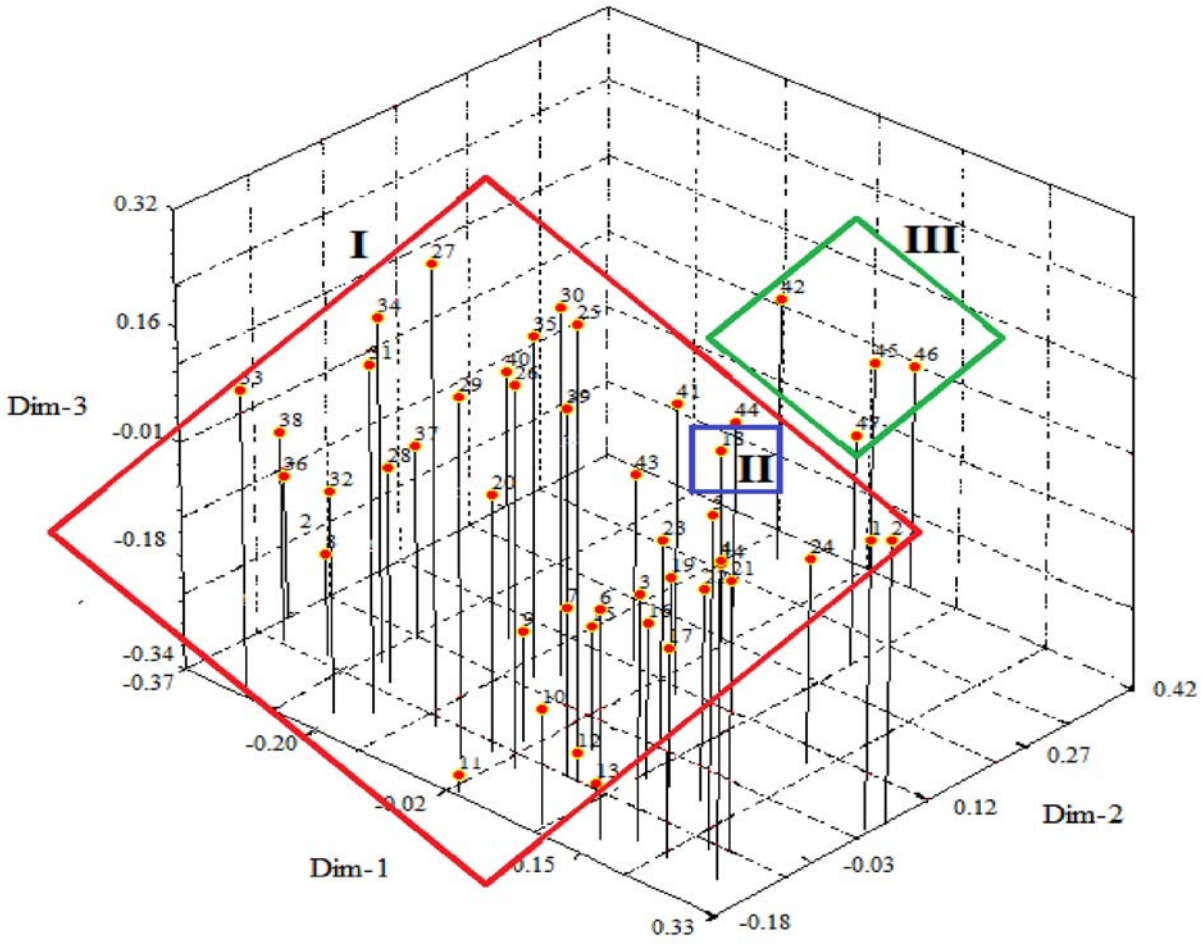

Fig. 3. 3D-scatter plot of *Leymus* and related diploid *Triticeae* species based on the first, second and third components of PCA.

and Eurasian *Leymus* species might have the same geographical origins. Based on genome in situ hybridization (GISH) and chromosome pairing, previous study found that *Hystrix duthiei* had the same genome constitution (NsXm) as *Leymus* and combined it into *Leymus* (Zhang et al. 2006). Both the present dendrogram and 3D-scatterplot showed that *L. duthiei* nested in the clade of *Leymus* and *Psathyrostachys* species. This indicated that *L. duthiei* is closely related to other *Leymus* species and is included in genus *Leymus*.

Meiotic pairing data from interspecific and intergeneric hybrids (Dewey 1984; Wang & Jensen 1994) and molecular studies (Svitashev et al. 1998; Hole et al. 1999; Bödvarsdóttir & Anamthawat-Jónsson 2003) had revealed that the Ns genome of *Leymus* (NsXm) was originated from *Psathyrostachys*. The Ns genome-specific RAPD markers were present in all the tested polyploid *Leymus* species, proving that the Ns genome in *Leymus* originated from the genus *Psathyrostachys* (Yang et al. 2011). *Psa. fragilis*, *L. alaicus* ssp. *karataviensis* and the two accessions of *L. qinghaicus* grouped together directly in subgroup Ic, suggesting a closer relationship between them. This may be related to the geographic ranges of *Psa. fragilis*, which is restrictly distributed in regions of central Asia. Except for *L. condensatus*, 40 accessions of *Leymus* clustered with *Psa. fragilis* in group I, giving strong evidence that *Psathyrostachys* is the maternal donor (Ns genome) of *Leymus* species. Meanwhile, the 3D-scatterplot of PCA demonstrated that *Psathyrostachys* is clustered closely with *Leymus* species, which also definitely indicative of their close relationship.

The genome constitution of *Leymus* was assigned NsXm, genomes E^b (Dewey 1984; Löve 1984), E^e (Sun et al. 1995), Ns (Anamthawat-Jónsson & Bödvarsdóttir, 2001) and St (Shiotani 1968) have been suggested as Xm genome. The results of the genome specific RAPD data indicated that some species of *Leymus* had close phylogenetic relationships with the St, W and H genomes (Yang et al. 2011). In the present study, *Pseudoroegneria stipifolia*, *Hordeum bogdanii*, *H. chilense* and *Lophopyrum elongatum* were clustered in Group III with no *Leymus* species, whereas *Australopyrum retrofractum* was clustered in Group I. PCA demonstrated the coincident conclusion with the UPGMA cluster analysis. This could be infer that Xm genome of *Leymus* was related to the W genome of *Australopyrum* and it hypothesized that W genome might serve as the diploid donor of Xm genome in *Leymus*.

In addition, these results from present study also proved the ISSR marker to be powerful and reliable for discriminating genotype characterization of *Leymus*

species. It can be an assisted method for molecular identification, assess genetic diversity and biosystematic relationships in *Leymus*.

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