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Relationships among *Leymus* species assessed by RAPD markers

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Abstract

The DNA genetic diversity of 40 accessions of genus *Leymus* was analyzed by random amplified polymorphic DNA (RAPD) markers. A total of 352 products were amplified by 34 10-mer arbitrary primers, among which 337 products (95.74 %) were found to be polymorphic. 5 - 14 polymorphic bands were amplified by each polymorphic primer, with an average of 9.91 bands. The data of 352 RAPD bands were used to generate Jaccard's similarity coefficients and to construct a dendrogram by means of UPGMA. Great genetic diversity in genus *Leymus* was observed, the genetic diversity among the different species more abundant than that of the different accessions, and the different accessions in a species or the species from the same areas were clustered together.

Additional key words: cluster analysis, genetic diversity, molecular marker, polymerase chain reaction, similarity coefficients.

Introduction

Leymus is an important perennial genus of *Triticeae* (*Poaceae*) and includes about 30 species and 19 subspecies, which are distributed in the temperate regions of Eurasia, North and South America (Tzvelev 1976, Dewey 1984), extending to the subtropic and the tropic alpine regions. Growing in saline or alkaline lands, and dry or semi-dry areas, some *Leymus* species are highly adaptable to coldness, dryness and saline or alkaline soils. Some species also bear desirable traits such as disease and insect resistance, bigger spikes, more and bigger grains. Therefore, *Leymus* species might be an important genetic source for improvement of *Triticeae* cereal crops. However, the origin and definition of the genus, precise taxonomic ranks and relationships among the species in the genus have been under discussion. Most recent European, Former Soviet Union, and Chinese taxonomists, mostly earlier, taxonomists accept *Leymus*, some North American taxonomists included *Leymus* in genus *Elymus* (Barkworth and Atkins 1984).

Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and population. The random amplified polymorphic DNA (RAPD) technique (Williams *et al*. 1990) is an increasingly popular tool in genetic studies. Developed RAPD markers provide a rapid, inexpensive and effective system for studying plant genetic relationships. Since 1990, RAPD technique has been extensively used in plant systematic studies, especially in the identification of germplasm resources such as cultivars and species, and the measurement of variation to establish evolutionary relationships within or among species, subspecies or population and genomes (Narasimhan *et al*. 2006, Padmesh *et al*. 2006, Rout 2006, Dikshit *et al*. 2007). RAPD technique has been used to produce species specific molecular markers in the *Triticeae* (Wei and Wang 1995) and to estimate the genetic diversity and to analyze genetic relationships among *Triticeae* species (Wei *et al*. 1997, Zhou *et al*. 2000). The objectives of this paper were to estimate the genetic diversity of *Leymus* species by using RAPD analysis and to evaluate the usefulness of RAPD markers in the studies of interspecific relationships.

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Abbreviations: RAPD - random amplified polymorphic DNA; UPGMA - unweighed pair-group method analysis; SHAN - sequential, hierarchical, agglomerative, and nested clustering.
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Materials and methods

Plants: Forty accessions of genus *Leymus*, which distributed in 19 species and 1 subspecies, were analyzed in this study (Table 1). All seeds were kindly provided by American National Plant Germplasm System (Pullman, Washington, USA) and all the species are currently growing in the field at the Triticeae Research Institute, Sichuan Agricultural University, China.

DNA extraction and PCR amplification: Eight to fifteen plants of each accession were examined (Table 1). Fresh leaf tissue (3 g) from two-month-old plants was frozen in liquid nitrogen, ground and used for DNA extraction. The extraction buffer and procedure were based on the phenol/chloroform protocols of Sharp *et al*. (1988). The primers used as random primers in the PCR were purchased from *Operon Technologies* (Alameda, USA). The Taq DNA polymerase, 10× PCR buffer and 30 mM $MgCl_2$ were obtained from *Hua-Mei* (Chengdu, China). The PCR volume was 0.025 cm^3 and contained 0.02 µg template DNA, 1 unit Taq DNA polymerase, 2×10^{-4} mM primer, 0.1 mM each of dNTP, 1.5 mM $MgCl_2$ and 1× PCR buffer. About 0.03 cm^3 of mineral oil was overlaid on each reaction mixture. DNA amplification was performed by using a MJ Research, Inc., PTC-200 PCR programmed for 50 cycles of 1 min at 94 °C, 1 min at 36 °Cand 2 min at 72 °C. PCR products were separated on 1.0 % agarose gels and visualized by ethidium bromide staining.

Table 1. *Leymus* species and accessions used in this study.

Number	Taxon	Accession No.	Geographic origin	*n*
1	*L. akmolinensis* (Drobow) Tzvelev	PI440306	Russian Federation	9
2	*L. alaicus* ssp. *karataviensis* (Roshev.) Tzvelev	PI314667	Alma-Ata,Kazakhstan	12
3	*L. ambiguous* (Vasey & Scribner) D.R. Dewey	PI531795	Colorado, USA	14
4	*L. angustus* (Trin.) Pilger	PI440307	Kazakhstan	11
5	*L. angustus* (Trin.) Pilger	PI440308	Kazakhstan	11
6	*L. angustus* (Trin.) Pilger	PI440317	Former Soviet Union	11
7	*L. angustus* (Trin.) Pilger	PI440318	Former Soviet Union	12
8	*L. angustus* (Trin.) Pilger	PI531797	Xinjiang, China	11
9	*L. angustus* (Trin.) Pilger	PI547357	Nei Monggol, China	12
10	*L. arenarius* (L.) Hochst.	PI272126	Alma-Ata,Kazakhstan	10
11	*L. cinereus* (Trin.) Tzvelev	PI469229	Saskatchewan, Canada	13
12	*L. cinereus* (Trin.) Tzvelev	PI478831	Montana, United States	13
13	*L. cinereus* (Trin.) Tzvelev	PI537353	Idaho, United States	11
14	*L. condensatus* (J.Presl) Á. Löve	PI442483	Antwerp, Belgium	8
15	*L. erianthus* (Phil.) Dubcovsky	W6 13826	Argentina	8
16	*L. hybrid*	PI537362	Nevada, United States	12
17	*L. hybrid*	PI537363	Nevada, United States	12
18	*L. innovatus* (Beal) Pilger	PI236818	Canada	11
19	*L. karelinii* (Turcz.) Tzvelev	PI598529	Xinjiang, China	14
20	*L. karelinii* (Turcz.) Tzvelev	PI598534	Xinjiang, China	14
21	*L. mollis* (Trin.) Pilger	PI567896	Alaska, United States	9
22	*L. multicaulis* (Kar.& Kir.) Tzvelev	PI440324	Kazakhstan	11
23	*L. multicaulis* (Kar.& Kir.) Tzvelev	PI440325	Kazakhstan	11
24	*L. multicaulis* (Kar.& Kir.) Tzvelev	PI440326	Kazakhstan	12
25	*L. multicaulis* (Kar.& Kir.) Tzvelev	PI440327	Kazakhstan	12
26	*L. multicaulis* (Kar.& Kir.) Tzvelev	PI499520	Xinjiang, China	11
27	*L. paboanus* (Claus) Pilger	PI272135	Kazakhstan	10
28	*L. paboanus* (Claus) Pilger	PI531808	Estonia	10
29	*L. pseudoracemosus* C. Yen & J.L Yang	PI531810	Qinghai, China	15
30	*L. racemosus* (Lam.) Tzvelev	PI315079	Former Soviet Union	13
31	*L. racemosus* (Lam.) Tzvelev	PI478832	Montana, United States	14
32	*L. racemosus* (Lam.) Tzvelev	PI531811	Estonia	11
33	*L. ramosus* (Trin.) Tzvelev	PI499653	Xinjiang, China	15
34	*L. ramosus* (Trin.) Tzvelev	PI502404	Russian Federation	13
35	*L. salinus* (M.E.Jones) Á. Löve	*PI531816*	Utah, United States	10
36	*L. secalinus* (Georgi) Tzvelev	PI499527	Gansu, China	14
37	*L. secalinus* (Georgi) Tzvelev	PI499535	Xinjiang, China	12
38	*L. triticoides* (Buckley) Pilger	PI516194	Oregon, United States	10
39	*L. triticoides* (Buckley) Pilger	PI531821	Nevada, United States	12
40	*L. triticoides* (Buckley) Pilger	PI537357	Nevada, United States	11

RAPD data analysis: Photographs were used to score the RAPD data. For each material × primer combination, the presence (1) or absence (0) of an amplified fragment was treated as an independent character without consideration of the quantitative aspects of the results, *i.e*. band intensity. The data matrix was entered into the *NTSYS-pc* program (Rohlf 1993). Data were analyzed using a *Simqual* (similarity for qualitative data) routine to generate Jaccard's similarity coefficients. Similarity coefficients were used to construct a dendrrogram using the unweighted pair group method with arithmetic average (UPGMA) and the SHAN (sequential, hierarchical, agglomerative, and nested clustering) routine in the *NTSYS* program.

Results

Fifty-one 10-mer arbitrary primers were tested to select those that produced polymorphic DNA bands. Of the 51 primers tested, 45 (88.24 %) produced polymorphic fragments. Various primers produced different fragments from the same template DNA. Fig. 1 shows the results of amplification from primer OPA-07. Thirty-four primers were selected for further analysis, which are able to produce clear and stable amplified bands. These primers produced 352 bands, ranging from 5 to 15 bands per primer (Table 2), with 10.35 bands per primer. Of 352 bands, only 15 (4.26 %) fragments amplified were present in all the 40 accessions of genus *Leymus*. A total of 337 (95.74 %) polymorphic bands were obtained. The polymorphic bands produced by each primer ranged from 5 - 14, with an average of 9.91. It indicated that there was considerable RAPD variation among species of genus *Leymus*.

All the 352 bands were used to calculate Jaccard's similarity coefficients among the 40 accessions. The similarity coefficients value varied from 0.15 to 0.68

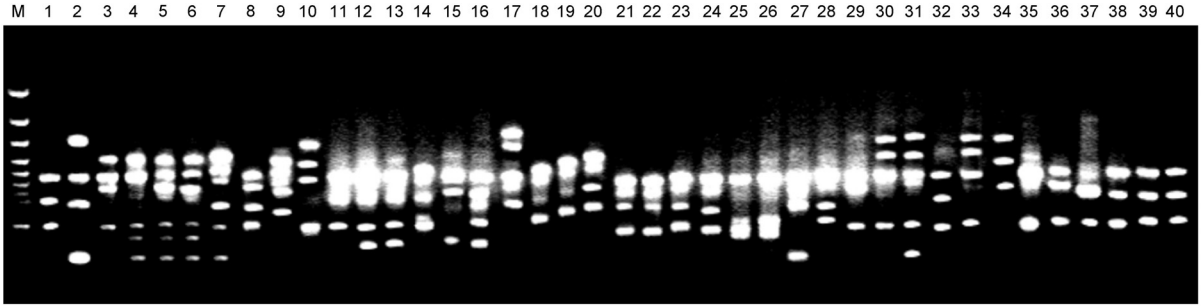

Fig. 1. RAPD polymorphism in 40 *Leymus* acessions with the primer OPA-07. The accessions 1 to 40 are described in Table 1. M - 100 bp DNA ladder (MBI).

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

Primers	Sequence (5'-3')	Total bands	Polymorphic bands	Primers	Sequence (5'-3')	Total bands	Polymorphic bands
OPA-06	GGTCCCTGAC	13	13	OPH-03	AGACGTCCAC	11	11
OPA-07	GAAACGGGTG	13	13	OPH-05	AGTCGTCCCC	8	8
OPA-08	GTGACGTAGG	8	7	OPH-06	ACGCATCGCA	11	10
OPA-09	GGGTAACGCC	6	6	OPH-07	CTGCATCGTG	12	12
OPA-10	GTGATCGCAG	7	6	OPH-08	GAAACACCCC	10	10
OPA-11	CAATCGCCGT	12	12	OPH-11	CTTCCGCAGT	14	14
OPA-12	TCGGCGATAG	14	14	OPH-12	ACGCGCATGT	8	6
OPA-16	AGCCAGCGAA	13	13	OPH-13	GACGCCACAC	11	11
OPA-19	CAAACGTCGG	14	13	OPH-14	ACCAGGTTGG	10	10
OPA-20	GTTGCGATCC	8	7	OPH-16	TCTCAGCTGG	10	9
OPB-01	GTTTCGCTCC	12	12	OPH-17	CACTCTCCTC	15	14
OPB-06	TGCTCTGCCC	11	10	OPH-19	CTGACCAGCC	9	7
OPB-07	GGTGACGCAG	7	7	OPR-02	TCGGCACGCA	13	13
OPB-10	CTGCTGGGAC	9	9	OPR-05	CCCCGGTAAC	8	7
OPB-17	AGGGAACGAG	5	5	OPR-12	AAGGGCGAGT	11	10
OPB-18	CCACAGCAGT	11	11	OPR-17	TGACCCGCCT	7	7
OPB-19	ACCCCCGAAG	9	8				
OPB-20	GGACCCTTAC	12	12	Total	34	352	337

with an average of 0.30. There is little genetic difference among the different accessions in the same species, while the genetic difference among the different species is distinct. Among the 20 taxa, *L. innovatus* is closely related to *L. karelinii*, with the highest average similarity coefficient (0.47) between *L. innovatus* and 2 accessions of *L. karelinii*. Also, *L. pseudoracemosus* is closely related to *L. racemosus.* The relationships between *L. pseudoracemosus* and *L. arenarius* is remote, with the lowest similarity coefficient (0.15).

The Jaccard's similarity coefficients were used to generate a dendrogram (Fig. 2) with UPGMA method. The results showed that RAPD markers could distinguish all the 40 accessions of genus *Leymus*. In the dendrogram, the different accessions in a species were clustered together first, then, the different taxa were clustered. At the similarity coefficient value of 0.30, the 40 accessions were divided into six groups.

In group I, there are 10 species and 1 subspecies. They are *L. akmolinensis*, *L. alaicus* ssp. *karataviensis*, *L. ambiguous*, *L. angustus*, *L. cinereus*, *L. hybrid*, *L. innovatus*, *L. karelinii*, *L. ramosus*, *L. salinus* and *L. triticoides*. *L. akmolinensis*, *L. alaicus* ssp. *karataviensis* and *L. angustus* were clustered together, and they come from Xinjiang, China or the neighboring geographical regions. *L. ambiguous*, *L. cinereus* and *L. hybrid* come from America or Canada, and they were clustered together first. *L. innovatus*, *L. karelinii* and *L. ramosus* come from America, and they have relatively close relationships. Both *L. salinus* and *L. triticoides* belong to Sect. *Anisopyrum* (Griseb.) Tzvelev, and they are from America. *L. salinus* is closely related to *L. triticoides*.

In group II, there are 4 species. They are *L. multicaulis*, *L. paboanus*, *L. pseudoracemosus* and *L. racemosus*. *L. pseudoracemosus* clustered with *L. racemosus* first, then they clustered with *L. paboanus*. Last, they clustered with *L. multicaulis*. Morphologically, *L. pseudoracemosus* differs from *L. racemosus* only in incano- pubescentibus of rachidibus, albo-villosis of lemmatibus (Yen *et al*. 1983).

L. erianthus, *L. secalinus* and *L. arenarius* were clustered in group III, IV and VI, respectively. They come from different regions, and they have distinct morphological variations. *L. condensatus* is closely related to *L. mollis*, and they were clustered into group V.

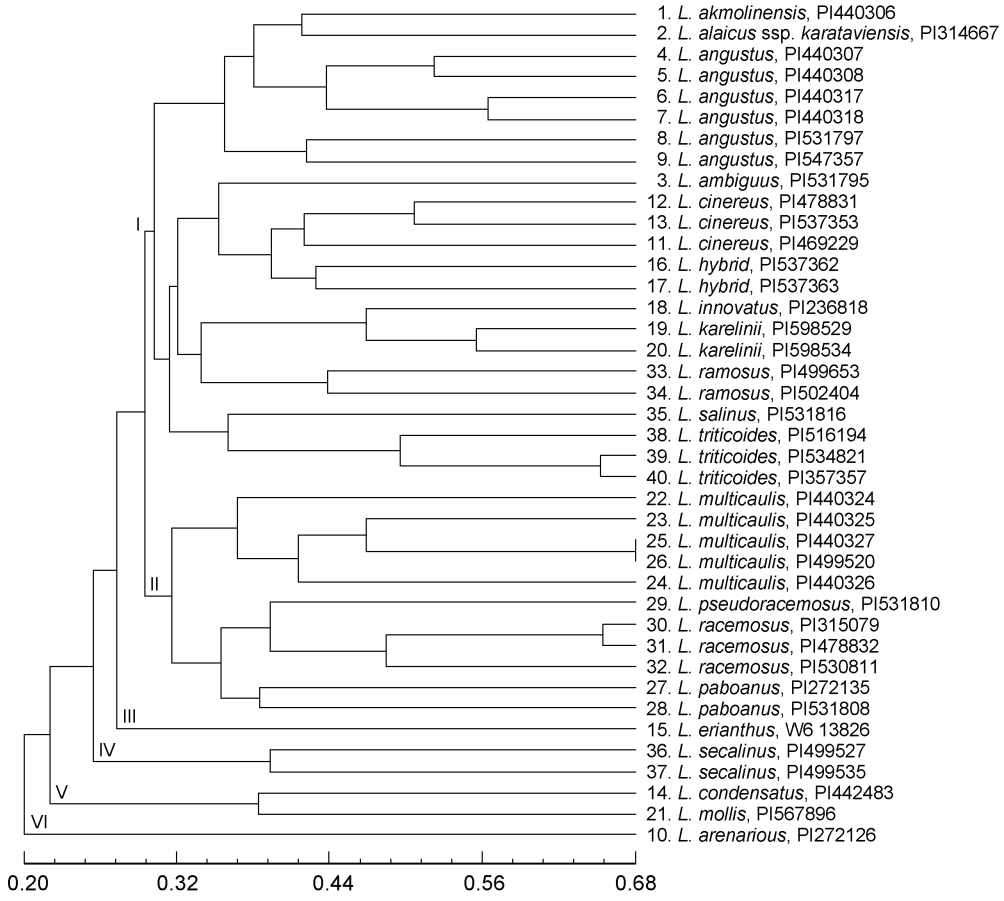

Fig. 2. A dendrogram generated from RAPD markers of 40 *Leymus* populations.

Discussion

RAPD loci were able to reveal more information than traditional morphological classification, cytological characters and other biochemical markers such as isozymes (Heun *et al*. 1995). Rout (2006) suggested that RAPD analysis reveals a high number of polymorphic loci in *Typhonium* species. Earlier studies have suggested that RAPD analysis usually reveals a high number of polymorphic loci in *Triticeae* (Wei *et al*. 1997, Zhou *et al*. 2000). The present results are concordant with these suggestions. In this study, 34 primers were employed, as many as 352 putative RAPD loci could be identified. High-level RAPD variations existed both among and within species of *Leymus*. About 95.74 % of the total 352 amplified fragments were polymorphic among and within species. This indicates that there exists rich genetic diversity in *Leymus*, which has something to do with the characters of allopolyploid and cross-pollination.

Based on the present RAPD data, the different accessions in a species and the species with similar morphological characters and the species from the same areas or neighboring geographical regions were clustered together. Five different accessions of *L. multicaulis* were first clustered together in group II. Morphologically, *L. pseudoracemosus* is similar to *L. racemosus* (Yen *et al*. 1983), and they have close genetic relationship, so they were clustered together firstly. According to morphological characters, Tzvelev (1976) divided the species of genus *Leymus* into four sections. In the present analysis, most species of sect. *Aphanoneuron* and sect. *Anisopyrum* were clustered in group I. The results are basically comparable with those obtained from studies on morphology, although the number of RAPD markers was small. *L. arenarius*, *L. racemosus* and *L. mollis* belong to sect. *Leymus*, but they were clustered in different groups. In order to study the real genetic relationships among species of genus *Leymus*, morphological, cytological and molecular data should be considered.

The RAPD marker is simpler, less expensive and less laborious than other DNA marker methodologies when studying genetic relationships of different groups of plant species (Caetano-Anolles *et al*. 1991). However, it also has some limitations. The most limiting factor is its sensibility, which affects the reproducibility of the results. But it may be overcome by eliminating variation in DNA concentration, and taking care to ensure consistent reaction conditions and thermal profile during amplification (Rafalski *et al*. 1995, Wei *et al*. 1997). In this study, when the concentrations of all reagents in RAPD reactions were optimized, and after two times each primer was tested for each individual analysis, the reproducibility was quite good. The same results were reported in *Hystrix* and *Elymus* (Zhou *et al*. 2000). Therefore, we conclude that the RAPD marker technique is an additional useful method for investigation into biosystematic relationships and genetic diversity in genus *Leymus*.

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