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Research Paper

DNA barcoding of *Leymus* (Poaceae)

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ABSTRACT:- *Leymus Hochst. is a genus with 34 perennial species in the tribe Triticeae (Poaceae). Most of Leymus species are of high values in forage grass breeding and ecological restorations. Unfortunately, identification of Leymus species is extremely difficult based on morphology. Here we report our results in resolving the species using DNA barcoding method. We combined our data together with those downloaded from GenBank and evaluated the performances of six chloroplast regions, i.e. matK, atpB-rbcL, rbcL, trnL-F, rps11 and rps16, in 30 species using UPGMA methods. Unexpectedly, these regions can only resolve very few species. The rbcL and rps11 are the worst and the rps16 is relatively the best. We thus conducted analyses using different combinations of the datasets. The combination rps16+atpB-rbcL+trnL-F+matK showed the highest resolution. Addition of rbcL and rps11 did not give better results. We conclude that the barcode of Leymus remains to be discovered.*

Keywords :- *Poaceae, Leymus, DNA barcodes*

I. INTRODUCTION

Leymus Hochst is a genus with important economic value in Triticeae (Poaceae). The genus includes about 34 species around the world, which are widely distributed in Eurasia and North America, South America also has a small amount of distribution[1]. *Leymus* species have very wide adaptability, growth in coastal, desert dunes, grasslands, meadows, hillsides and undergrowth,etc[2]. Most of *Leymus* species are forage grasses for animal husbandry. Some species used as important germplasm resources for grass breeding and improving breeds of crops due to the characteristics of enduring cold, drought, and alkali[3]. With the depth of wheat crops, pasture breeding, revegetation and ecological construction, the demand for excellent plant germplasm is extremely urgent. In recent years, a new species of the genus *Leymus* continue to be found to provide more choices for practical applications[4-6]. However, due to *Leymus* is allopolyploid origin[7], complex morphological variation, species identification is very difficult, which is to rely on the use of grass germplasm great deal of trouble. With the development of science and technology, not simply rely on morphology of identification methods constantly emerging, including DNA barcode technology is one of the most influential

technology.

DNA barcode, a special DNA sequence used in species identification, has become a focus in international biodiversity research in recent years. Compared with the traditional morphological identification, the DNA barcode has characteristic not limited by material morphology and ontogeny etc[8]. The 2-locus combination of *rbcL+matK* as the plant barcode recommended by Consortium for the Barcode of Life in 2009[9]. However, Gramineae has its particularity. In the case of the Brachypodium that chloroplast *trnL-F* region showed a good ability to distinguish[10]. *rps16* discriminated between North American *Leymus* very well[11]. Furthermore, the chloroplast *atpB-rbcL* region showed a good ability to distinguish the species of the Oryzoideae and related subfamilies[12]. Therefore, we must make a concrete analysis of concrete problems. It is necessary to find a DNA barcode sequence for gramineous species. In our study, we combined our data together with those downloaded from GenBank and evaluated the performances of six chloroplast regions, i.e. *matK*, *atpB-rbcL*, *rbcL*, *trnL-F*, *rps11* and *rps16*, in which the *matK*, *rbcL* and *trnL-F* is emphatically recommended as the candidate plant barcode sequences and the *atpB-rbcL*,*rps11* and *rps16* are widely used in molecular phylogenetics of Poaceae[13-16]. By evaluating the six chloroplast regions for identification ability on the species in *Leymus*, right DNA regions were screened in *Leymus*, which would provide the scientific basis for the application of DNA barcode on identification of *Leymus*.

II. MATERIALS AND METHODS

2.1 Plant materials

The origin and accession number of 17 samples used in this study are listed in Table 1. The accessions with PI numbers were kindly provided by American National Plant Germplasm System (Pullmam, Washington, USA). All of the voucher specimens have been deposited at Herbarium of Triticeae Research Institute, Sichuan Agricultural University, China (SAUTI). GenBank database (data download dated October 18, 2013) download data are listed in Appendix 1. The sequences of all species belonging to *Leymus* available from GenBank were downloaded and combined with our data for analyses. These data cover 90% of *Leymus* species.

2.2 DNA extraction, amplification and sequencing

Total DNA was extracted from the quickly drought leaves using the mCTAB method[17]. The primers for *matK* were designed (*matK*F: AAGCAAGAAGATTGTTTACGAAGAA and *matK*R: TCTAGAAGACCACGACTGATC). The primers for trn*L-F* used in reference to previous studies[18]. The polymerase chain reaction (PCR) amplification mixture contained 1 × PCR buffer, 0.2μmol/L each dNTP, 1.25 μmol/L each primer, 1.25 units *Taq* polymerase, and 25 ng DNA in a total volume of 25 μL. The template DNA was initially denatured at 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 s, 50℃ for 40 s, and 72℃ for 1 min, with a final extension at 72℃ for 10 min. The PCR products were purified with PEG8000 and sequenced using ABI PRISM ® BigDye^{TM} Terminator Cycle Sequencing Kits v. 3. 1 on ABI 3730 x l DNA Analyzer (Life Technologies, 5791 Van Allen Way, PO Box 6482, Carlsbad, California 92008) following manufacturer's instructions.

2.3 Data analysis

Sequences were evaluated and assembled using Sequencher v. 4. 7 (Gene codes Corporation, Ann Arbor, Michigan, U.S.A.). The resulting data sets together with those downloaded from GenBank were aligned with Clustal 2.0 and manually adjusted with SE-Al 2.0 [19-20].

Intraspecific and interspecific genetic distance and the average distance of genetic sequences using MEGA 5 in Kimura 2-Parameter model were calculated. Barcoding gap is an indicator of DNA barcode research, the ideal barcode is interspecific genetic variation should be significantly greater than intraspecific genetic variation[21].

In this study, comparison of sequences between interspecific and intraspecific variation using Perl language and take the Wilcoxon rank sum test to test result. Evaluation of the barcoding gap of DNA barcode candidate sequences. Finally, we examine the sequence identification success rate using PUAP 4.0b 10 of UPGMA [22-23].

Table1 A list of samples in this study.

Taxon	Source	Accession No.
Leymus alaicus* ssp. *karataviensis	Former Soviet Union	PI 314667
Leymus angustus	Canada,Saskatchewan	PI 271893
Leymus arenarius	Former Soviet Union	PI 316233
Leymus chinensis	China	PI 499515
Leymus cinereus	United States,Montana	PI 478831
Leymus condensatus	Belgium	PI 442483
Leymus karelinii	Xinjiang, China	PI 598535
Leymus mollis	United States,Alaska	PI 567896
Leymus multicaulis	Kazakhstan	PI 440325
Leymus paboanus	Former Soviet Union	PI 316234
Leymus pseudoracemosus	China	PI 531810
Leymus racemosus	Russian Federation	PI 598806
Leymus ramosus	China	PI 499653
Leymus crassiusculus		ZY 07024
Leymus secalinus	China	ZY 07026
Leymus tianshanicus	Altay	Y 1465
Leymus triticoides	United States,Nevada	PI 537357

III. RESULTS AND DISCUSSION

3.1 Variability of individual chloroplast region in *Leymus*

The lengths of six chloroplast regions are shown in the Table 2. Each region length ranging from 702~2114bp, the shortest of which *atpB-rbcL*, *matK* longest (partial sequence contains *trnk*). The *matK* including 45 variable and 32 parsimony informative sites, the average genetic distance is 0.003; The *rps16* including 31 variable and 23 parsimony informative sites, the average genetic distance is 0.010, and this value is the largest of the six chloroplast regions .

Table2 The length and variability of six chloroplast regions in *Leymus*.

maker	Aligned length	GC content	Variable site	Parsimony informative site	Average genetic distance
matK	2114	32.8	45	32	0.003
trnL-F	1083	30.9	42	15	0.005
rbcL	1742	42	25	8	0.002

atpB-rbcL	836	30.4	27	7	0.005
rps11	1441	34.7	7	3	0.001
rps16	847	30.8	31	23	0.01

3.2 Analysis of genetic distance between intraspecific and interspecific species

The genetic distance of the intraspecific and interspecific species analysis revealed that the greatest interspecific variation in *rps16* and the lowest in *rps11*. The intraspecific variation got the same result. We test results of the above calculation using Wilcoxon rank sum test, it proves that these results are correct (Table 4~5). (Table 3).

Table3 Intra- and inter-species distances of six chloroplast regions in *Leymus*.

region	Intraspecific genetic distance	Interspecific genetic distance
matK	0.0009±0.0022	0.0037±0.0034
trnL-F	0.0016±0.0029	0.0046±0.0039
rbcL	0.0011±0.0015	0.0023±0.0022
atpB-rbcL	0.0055±0.0007	0.0052±0.0028
rps11	0.0006±0.0005	0.0007±0.0009
rps16	0.0020±0.0021	0.0100±0.0071

Table4 Wilcoxon rank sum test for intraspecific variation

Gene1	Gene2	Wilcoxon rank sum test	Result
matK	*rbcL*	N1=62,N2=19,W=476,P=0.04212	matK > rbcL
matK	*rps11*	N1=62,N2=3,W=56,P=0.04522	*matK >rps11*
rps16	*matK*	N1=61,N2=62,W=1128,P=5.586e-6	*rps16 > matK*
rbcL	*rps11*	N1=19,N2=3,W=29.5,P=0.5628	*rbcL <= rps11*
rbcL	*trnL-F*	N1=19,N2=34,W=334,P=0.4099	*rbcL <= trnL-F*
rps16	*rbcL*	N1=61,N2=19,W=406,P=0.02011	*rps16 > rbcL*
rps16	*trnL-F*	N1=61,N2=34,W=1311.5,P=0.01161	*rps16 > trnL-F*
rps11	*rps16*	N1=3,N2=61,W=57.5,P=0.8716	*rps11 <= rps16*
rps11	*trnL-F*	N1=3,N2=34,W=57.5,P=0.3475	*rps11 <= trnL-F*
rps11	*atpB-rbcL*	N1=3,N2=2,W=6,P=0.06932	*rps11 <= atpB-rbcL*
atpB-rbcL	*matK*	N1=2,N2=62,W=109,P=0.004033	*atpB-rbcL > matK*
atpB-rbcL	*rbcL*	N1=2,N2=19,W=38,P=0.007844	*atpB-rbcL > rbcL*
atpB-rbcL	*rps16*	N1=2,N2=61,W=101.5,P=0.05251	*atpB-rbcL <= rbcL*
atpB-rbcL	*trnL-F*	N1=2,N2=34,W=59,P=0.02438	*atpB-rbcL > trnL-F*
trnL-F	*matK*	N1=34,N2=62,W=898,P=0.04959	*trnL-F > matK*

Table5 Wilcoxon rank sum test for interspecific variation

Gene1	Gene2	Wilcoxon rank sum test	Result
atpB-rbcL	*matK*	N1=229,N2=373,W=50772,P=2.648e-5	*atpB-rbcL > matK*
atpB-rbcL	*rbcL*	N1=229,N2=359,W=64128,P=2.2e-16	*atpB-rbcL > rbcL*
atpB-rbcL	*rps11*	N1=229,N2=168,W=35888,P=2.2e-16	*atpB-rbcL > rps11*

rps16	*atpB-rbcL*	N1=1067,N2=229,W=74166.5,P=2.2e-16	*rps16 > atpB-rbcL*
atpB-rbcL	*trnL-F*	N1=229,N2=1001,W=126870,P=0.005114	*atpB-rbcL > trnL-F*
matK	*rbcL*	N1=373,N2=359,W=84913,P=1.447e-11	*matK > rbcL*
matK	*rps11*	N1=373,N2=168,W=43337,P=1.718e-15	*matK > rps11*
rps16	*matK*	N1=1067,N2=373,W=87802,P=2.2e-16	*rps16 > matK*
trnL-F	*matK*	N1=1001,N2=373,W=143485,P=5.94e-12	*trnL-F > matK*
rbcL	*rps11*	N1=359,N2=168,W=40836,P=6.625e-13	*rbcL > rps11*
rps16	*rbcL*	N1=1067,N2=359,W=66004.5,P=2.2e-16	*rps16 > rbcL*
trnL-F	*rbcL*	N1=1001,N2=359,W=118899.5,P=2.2e-16	*trnL-F > rbcL*
rps16	*rps11*	N1=1067,N2=168,W=15641.5,P=2.2e-16	*rps16 > rps11*
trnL-F	*rps11*	N1=1001,N2=168,W=40507.5,P=2.2e-16	*trnL-F > rps11*
rps16	*trnL-F*	N1=1067,N2=1001,W=796887.5,P=2.2e-16	*rps16 > trnL-F*

3.3 Assessment of Barcoding gap

In an ideal situation, the minimum value of interspecific genetic distance should be greater than the maximum value of intraspecific genetic distance. Thus, the formation of barcoding gap when the two non-overlapping[24]. As shown in Figure 1, the interspecific variation of *rps16* was the highest and barcoding gap exists in parts of the species. The *matK*、*atpB-rbcL*、*rbcL*、*trnL-F* and *rps11* are no obvious barcoding gap.

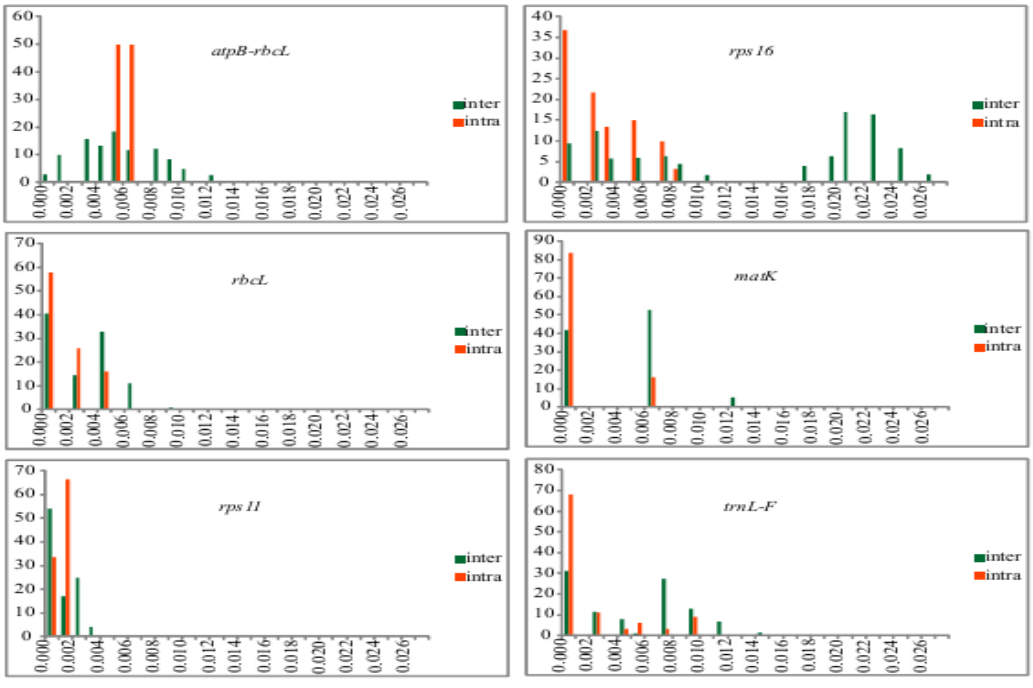

Figure 1. Barcoding gap of six chloroplast regions.
X-axis: K2P genetic distances ; Y-axis: Distribution for intra-specific and inter-specific genetic distances（%）

3.4 Identification efficiency for single region and combination of regions

In our study, Identification efficiency for single region and combination of regions in *Leymus* observed

by UPGMA methods. In the single region level, identification success rate *rps16* is 58%, *atpB-rbcL* identification success rate is 50%, second only to *rps16* and *rps11* identification success rate of only 25% (Figure 2). The results shown that the distinguishable ability of single region is outlined below, from best to worst: *rps16*, *atpB-rbcL*, *trnL-F*, *matK*, *rbcL*, *rps11*. After comprehensive comparison, the *rps16* and *atpB-rbcL* are relatively the best. We thus conducted analyses using different combinations of the datasets. *rps16+atpB-rbcL+trnL-F* identification success rate of 62.1% and *rps16+atpB-rbcL+trnL-F+matK* identification success rate of 63.3%, other combinations are less than or equal to 60%.

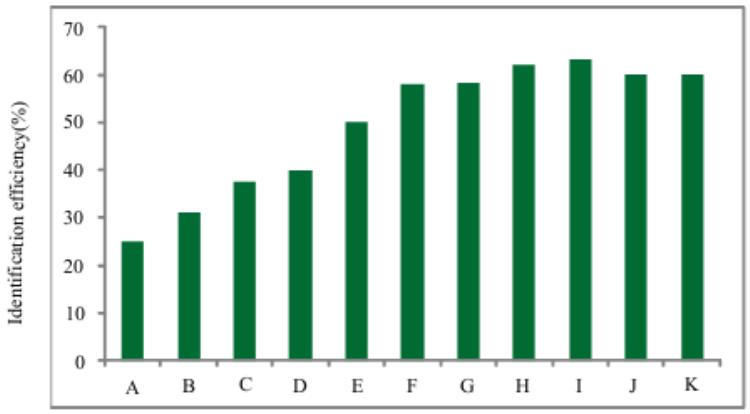

Figure 2 Identification efficiency for single region and combination of regions observed by UPGMA methods. A: *rps11*, B: *rbcL*, C: *matK*, D: *trnL-F*, E: *atpB-rbcL*, F:*rps16*, G: *rps16+atpB-rbcL*, H:*rps16+atpB-rbcL+trnL-F*, I:*rps16+atpB-rbcL+trnL-F+matK*, J:*rps16+atpB-rbcL+trnL-F+matK+rbcL*, K:*rps16+atpB-rbcL+trnL-F+matK+rbcL+rps11*

IV. CONCLUSION

The ideal DNA barcode should have some potential benefits including universal, the appropriate sequence length and good ability to distinguish between species[25-26]. There was insignificant genetic variation and in interspecific of the regions in which the *matK*, *rbcL*, *trnL-F*, *rps11*,and *atpB-rbcL*. At the same time, these regions had no clear barcoding gap. The *rbcL* and *rps11* had low identification reliability. Especially *rps11*, intraspecific and interspecific genetic distance were less than other regions. Thus it can be seen that *rbcL* has high identification reliability in high classification[27], but it has great limitation in low classification[28-39]. *matK* had high identification reliability in Cyperaceae and Orchidaceae[30]. Due to the difference is only found in a handful of Leymus species that *matK* had low identification reliability while it has the most variable and parsimony informative sites. Thus,the number of variable site is not reliable standard by which identification efficiency of region should be judged. In contrast, *rps16* has a relatively high resolution, while it has clear barcoding gap, moderate sequence length.

Intraspecific and interspecific variation, barcoding gapa and identification efficiency with UPGMA method was used to evaluate these chloroplast regions. The result showed that using of these six chloroplast regions, i.e. *matK*, *atpB-rbcL*, *rbcL*, *trnL-F*, *rps11* and *rps16* solely, are not suitable for the candidate barcoding of *Leymus*. This is true of many other studies[31-32]. Low resolution of single region is mainly because of less variation sites. But combinations should deal with this problem. For example, the combination *ndhF+matK+trnH-psbA+rps8-rpl36* can be correctly identified by the rate of 92% in Crocus[33]. In our study, the combination *rps16+atpB-rbcL+trnL-F+matK* can be correctly identified by the rate of 63.3% in *Leymus*. Addition of *rbcL* and *rps11* did not give better results.

Our results indicate that the *rps16* and *atpB-rbcL* showed a good ability to distinguish the species of *Leymus*. Meanwhile, we propose *matK* and *trnL-F* as additional barcode. Although *rps16+atpB-rbcL+trnL-F+matK* has the highest identification rate, but there is a difficult way for accurate identification of *Leymus* species. Hence, some aspects need to be looked at in future studies: The more species and more samples of each species should be considered. We should explore new regions of the genome, even consider to search for highly variable regions from chloroplast genome of closely related species. We expected to find a suitable specific DNA barcode of *Leymus*. Developing highly variable regions is not only demands of *Leymus* species identification but also the basis and prerequisite for phylogenic analysis and species objective evaluation.

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Appendix 1 The sequences of all species belonging to Leymus downloaded from GenBank.

Species	***atpB-rbcL***	***matK***	***rbcL***	***trnL-F***	***rps16***	***rps11***
	Genbank No.	Genbank No.	Genbank No.	Genbank No.	Genbank No.	Genbank No.
Leymus ambiguus	JN382031.1			EF581905.1	EF485906.1	
					EF485907.1	
					EF485908.1	
Leymus angustus	JN382055.1	AF164404	EU636660.1	EF581909.1	EF485912.1	GU140035.1
			GU140024.1	EF581910.1	EF485913.1	EU623072.1
Leymus akmolinensis			GU140021.1	EF581897.1	EF485904.1	GU140032.1
				EU366396.1	EF485905.1	
Leymus arenarius	JN382053.1	JN894789	GU140017.1	EF581906.1	EF485914.1	GU140028.1
	JN382058.1	KF277154	JN891800.1	EU366397.1	EF485915.1	
		KF277155	JN893061.1	GQ245074.1		
			JN893062.1			
Leymus chinensis	JN382062.1		Z49843.1	EF581896.1	EF485916.1	EU623073.1
			EU636661.1	EF581898.1	EF485917.1	
				JQ627774.1		
				JQ627775.1		
Leymus cinereus	JN382033.1		GU140019.1	EF581899.1	EF485927.1	GU140030.1
				EU366402.1	EF485931.1	
					EF485932.1	
					EF485933.1	
Leymus crassiusculus	JN382061.1					
Leymus condensatus					EF486177.1	
					EF486178.1	
Leymus erianthus			GU140015.1	EU366398.1		GU140026.1
Leymus flexus	JN382052.1					
Leymus flavescens					EF486179.1	
					EF486180.1	
Leymus innovatus	JN382032.1	JN966347	JN965627.1	EF581901.1	EF486181.1	GU140025.1
			JN965628.1	EU366403.1	EF486182.1	
			GU140014.1	GQ245075.1		
Leymus interior				GQ245076.1		
				GQ245077.1		
Leymus karelinii			EU636664.1	EF581907.1		GU140033.1
			GU140022.1			EU623076.1
Leymus leptostachyus	JN382056.1					
Leymus mollis	JN382048.1	JN966348	EU636666.1	EF581902.1	EF486188.1	EU623078.1
		JN966349	JN965629.1	GQ245078.1	EF486189.1	
		JN966350	JN965630.1			

		KC474953	JN965631.1			
		KC474955				
		KC474950				
		KC474954				
		KC474949				
		KC474951				
		KC474952				
Leymus multicaulis	JN382050.1				EF486190.1	
					EF486191.1	
					EF486215.1	
					EF486216.1	
Leymus ovatus	JN382068.1					
Leymus paboanus	JN382063.1		EU636662.1			GU140034.1
			GU140023.1			EU623074.1
Leymus pseudoracemosus	JN382059.1		GU140020.1	EU366399.1		GU140031.1
Leymus pubescens				JQ627786.1		
				JQ627787.1		
				JQ627788.1		
Leymus racemosus	JN382054.1		EU636663.1	EF581903.1	EF486196.1	EU623075.1
				EU366400.1	EF486197.1	
					EF486198.1	
Leymus ramosus	JN382051.1				EF486199.1	
					EF486200.1	
Leymus sabulosus			EU636665.1		EF486203.1	EU623077.1
Leymus salinus	JN382035.1		GU140018.1	EF581908.1	EF486187.1	GU140029.1
				EU366401.1	EF486204.1	
					EF486205.1	
					EF486206.1	
					EF486207.1	
					EF486208.1	
					EF486209.1	
Leymus secalinus	JN382049.1		EU636667.1	EF581904.1	EF486210.1	EU623079.1
	JN382057.1				EF486211.1	
					EF486212.1	
					EF486213.1	
					EF486214.1	
Leymus shanxiensis	JN382060.1					
Leymus triticoides	JN382034.1		GU140016.1	EU366404.1	EF486194.1	GU140027.1
					EF486195.1	
					EF486201.1	

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