Front. Biol. China 2008, 3(2): 170–175
DOI 10.1007/s11515-008-0022-1

RESEARCH ARTICLE

Genetic variation of the genus *Kengyilia* by ISSR markers

Li ZHANG[1], Yonghong ZHOU (✉)[1], Chunbang DING[1], Ruiwu YANG[1], Shigui LIU[2]

1 Triticeae Research Institute, Sichuan Agricultural University, Dujiangyan 611830, China
2 National Laboratory of Grassland Biological Control, Sichuan University, Chengdu 610064, China

© Higher Education Press and Springer-Verlag 2008

Abstract We investigated the genetic variation within 32 accessions distributed to 14 species and one variety by using ISSR (inter-simple sequence repeat) markers. The results showed that genetic variation was relatively higher among the accessions. A total of 593 bands were amplified by 12 ISSR primers, of which 535 bands (90.2%) were polymorphic. Eleven to 80 polymorphic bands were amplified from each prime, with an average of 44.6 bands. The interspecies GS (genetic similarity) value ranged from 0.430 to 0.866, and the average was 0.620. Cluster analysis showed that all accessions could be classified into 4 groups by ISSR markers. The different accessions in a species were clustered together, but they had genetic variation in molecular levels. There was obvious interspecies genetic variation. Species with similar morphological characteristics and from the same areas or neighboring geographical regions were clustered together and had close relationships. ISSR markers are useful in analyzing interspecies variation in *Kengyilia.*

Keywords *Kengyilia*, ISSR markers, genetic variation

1 Introduction

Kengyilia Yen et J. L. Yang was established by Yen and Yang in 1990, with *Kengyilia gobicola* Yen et J. L. Yang as the type species (Yen and Yang, 1990a, 1990b). So far, 26 species and six varieties of the genus have been described worldwide. They are mainly distributed from the Pamir and Qinghai-Tibet plateau to the Karkorum, Altai, Tianshan, and Qilian mountain (altitudes from 1100 m to 5100 m). In China, there are 23 species and 6 varieties that grow from subalpine meadows, montane steppes, and green slopes on forest fringes to semi-desert or even desert-like environments. The species *Kengyilia* can resist cold, drought, and alkali; cereal crops in Triticeae may hybrid with *Kengyilia* to improve resistance.

Morphologically, *Kengyilia* is a genus usually with terminal spikelets. There are one or two spikelets per node and the spikelets are sessile with (5–) 7 or 8 florets. Rachilla is disarticulated above glumes, which are rounded abaxially or keeled only at the apex, and rarely throughout length. Lemma are rounded abaxially, rarely keeled, usually 5-veined, densely pilose or hirsute, and awnless or shortly awned. Palea is apex retuse and obtuse, or 2-lobed. *Kengyilia* is different from *Roegneria* (by having the erect spike with densely placed spikelets, lemmas are densely pilose or hirsute, short-awned) and *Agropyron* (by flat glumes, lemmas with rounded back and not keeled from tip to bottom, and a terminal spikelet frequently presented). The main characters of *Kengyilia* are in agreement with *Roegneria* sect. *paragropyron* Keng et S. L. erected by Keng (1959). Karyotype and genome analysis on *Kengyilia* species showed that they were hexapodies (2n = 6x = 42) and contained the StYP genomes (Jensen, 1990; Zhou, 1994; Zhang et al., 1998, 2003). The StY genomes of *Kengyilia* came from *Roegneria,* while the P genome was derived from *Agropyron* (Yang et al., 1992). Molecular markers have successfully revealed the origin and evolutionary history of polyploids in plants and clarified the nature of different polyploids and hybridization events involved in their formation. The analysis of RAPD and PAMP by Zhou et al. (2000) and Zhang et al. (2003b, c) revealed a high level of genetic diversity in the *Kengyilia* species, which has been investigated at morphological, cytological and molecular levels since their establishment. However, genome constitution, interspecies genetic relationships and interspecies differences are not clear.

Inter simple sequence repeats (ISSR) are a new kind of molecular marker involving PCR amplification of DNA by a single primer 16–18 bp in length and composed of a repeated sequence (anchored at the 3′ or 5′ end by 2–4 arbitrary nucleotides). ISSR markers provide a novel fingerprinting approach for taxonomic and phylogenetic

Translated from *Guihaia*, 2006, 26 (4): 375–380 [译自: 广西植物]

E-mail: zhouyh@sicau.edu.cn

comparisons and are used as a mapping tool in a wide range of organisms (Zietkiewicz et al., 1994). Fernandez et al. (2002) found that ISSR markers provide a quick, reliable and highly informative system for DNA fingerprinting, and that it is also a reliable method to evaluate genetic relationships and gene types in barley. The genetic maps of seven varieties of wheat were constructed by Kojima et al. (1998) by using ISSR and RAPD markers, and 9 ISSR sites were mapped in 5 chromosomes. In this study, we adopted ISSR markers to investigate genetic variation within or among populations of *Kengyilia*, and investigate the efficiency of ISSR fingerprinting in detecting genetic diversity in *Kengyilia* accessions.

2 Materials and methods

2.1 Plant materials

Thirty-two accessions of *Kengyilia*, representing 14 species and one variety, were analyzed in the study (Table 1). All seeds were kindly provided by the Trticeace Research Institute, Sichuan Agriculture University

Table 1 The materials used in this study

species	abbreviation	No.	chromosom No.	genome	entry	geographic origin
K. rigidula (Keng) J.L. Yang, Yen & Baum	KRI	1	42	StYP	W622130	Xiahe, Gansu, China
		2	42	StYP	Y2330	Xiahe, Gansu, China
K. stenachyra (Keng) J.L. Yang, Yen & Baum	KST	3	42	–	W622138	Xiahe, Gansu, China
		4	42	–	W622128	Xiahe, Gansu, China
		5	42	–	Y2723	Xiahe, Gansu, China
		6	42	–	Y2305	Xiahe, Gansu, China
K. hirsuta (Keng) J.L. Yang, Yen & Baum	KHI	7	42	StYP	PI531618	Lanzhou, Gansu, China
		8	42	StYP	Y2860	Qilian, Qinghai, China
		9	42	StYP	Y2364	Xiahe, Gansu, China
		10	42	StYP	Y2368	Xiahe, Gansu, China
		11	42	StYP	Y1235	Rikaze, Tibet, China
		12	42	StYP	PI504457	Qinhai lake, Qinghai, China
		13	42	StYP	Y2876	Geerm, Qinghai, China
K. batalinii (Krassn.) J.L. Yang, Yen & Baum	KBA	14	42	StYP	PI531562	Kyrgyzstan
		15	42	StYP	PI565002	Kazakhstan
		16	42	StYP	PI547361	Kyrgyzstan
		17	42	StYP	PI314623	Siberia
K. tahelacana J.L. Yang, Yen & Baum	KTA	18	42	–	Y0582	Wensu, Xinjiang, China
		19	42	–	Y0599	Wensu, Xinjiang, China
K. melanthera var. tahopaica (Keng) S.L. Chen	KMT	20	42	–	Y2885	Xinghai, Qinghai, China
	KME	21	42	StYP	PI504458	Qinhai lake, Qinhai, China
		22	42	StYP	Y2891	Maduo, Qinghai, China
		23	42	StYP	Y2708	Hongyuan, Sichuan, China
K. melanthera (Keng) J.L. Yang, Yen & Baum		24	42	StYP	Y2709a	Hongyuan, Sichuan, China
K. laxiflora (Keng) J.L. Yang, Yen & Baum	KLA	25	42	StYP	PI531631	Shiqu, Sichuan, China
K. zhaosuensis J.L. Yang, Yen & Baum	KZH	26	42	–	Y2633	Zhaosu, Xinjiang, China
K. gobicola Yen & J.L. Yang	KGO	27	42	StYP	Y9503	Tashikuergan, Xinjiang, China
K. grandiglumis (Keng) J.L. Yang, Yen & Baum	KGR	28	42	StYP	Y2857	Haiyan, Qinghai, China
K. alatavica (Drobow) J.L. Yang, Yen & Baum	KAL	29	42	StYP	Y9519	Tianzhu, Gansu, China
K. thoroldiana (Oliver) J.L. Yang, Yen & Baum	KTH	30	42	StYP	Y2878	Geerm, Qinghai, China
K. mutica (Keng) J.L. Yang, Yen & Baum	KMU	31	42	StYP	Y2873	Geerm, Qinghai, China
K. kokonorica (Keng) J.L. Yang, Yen & Baum	KKO	32	42	StYP	Y2880	Gonghe, Qinghai, China

(Dujiangyan, Sichuan, China) and American National Plant Germplasm System (Pullmam, Washington, USA).

2.2 Methods

2.2.1 Total DNA isolation

Total DNA was extracted from 3 g fresh leaves of 6–10 individuals based on the method of Sharp et al. (1988).

2.2.2 PCR amplification

Thirty-five primers from Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. were tested for PCR. Amplification of genomic DNA was made on a PTC-200 TM thermocycler. The 25 μL PCR mixture contains 1 × PCR buffer (10 mmol/L Tris-HCl pH8.3, 50 mmol/L KCl, 0.001% gelatin), 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.2 μmol/L primers, 20–50 ng of DNA template and 1 U Taq polymerase (Promega, Shanghai). DNA amplification was performed for 2 min at 94°C for initial denaturation, followed by 45 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C, with a final extension reaction of 10 min at 72°C.

2.2.3 Examination of PCR products (sliver staining)

The PCR products were denatured at 95°C for 5 min and immediately put on ice, and the denatured products were mixed with 5 μL buffer (10% DMF, 10 mol/L EDTA pH8.0, 0.025% Bromophenol Blue, 0.025% Xylene Cyanole). Samples were separated by denatured polyacrylamide gel electrophoresis (4% w/v) at 2500 V. The gel was put into 10% acetic acid and swayed until the indicator colour faded. The faded gel was washed with distilled water for 5 min 3 times. The gel was equilibrated in 2 L $AgNO_3$ solution (1 g/L) for 30 min and rocked several times to incorporate the stain. The gel (stained by $AgNO_3$) was fixed in 10% acetic acid for 5 min, then washed with distilled water and dried in the air and photographed.

2.2.4 ISSR data scoring and analysis

ISSR bands were scored as presence (1) or absence (0) of a DNA fragment which was treated as an independent character without consideration of the quantitative aspects of the results, i.e., band intensity. The data matrix was then used to calculate genetic similarity (GS) coefficients, $GS = 2N_{ij}/(N_i + N_j)$, where N_{ij} is the number of ISSR bands shared by accessions *i* and *j*, and N_i and N_j are the total number of ISSR bands observed for accessions *i* and *j* respectively (Nei and Li, 1979). Based on the GS matrix, a dendrogram showing the genetic similarity between accessions was constructed by using the unweighted pair group method with arithmetic average (UPGMA) through the software NTSYS-pc (Rohlf, 1993).

3 Results

3.1 ISSR polymorphisms

Thirty five primers were used for ISSR analysis, and the sequences of these primers had been shown by Fang (1997) and Nagaoka (1997). The 35 primers were tested to amplify any two plants of accessions. Twelve primes were selected (Table 2). Figure 1 showed the amplification of 32 *Kengyilia* samples from primer 5′-$(CA)_8$G-3′. A total of 593 fragments (average of 49.4 for per primer) were amplified by the 12 primers, of which 535 bands (90.2%) were polymorphic. Eleven to 80 polymorphic bands could be amplified for each primer with an average of 44.6 bands (Table 2).

3.2 Genetic similarities

All the 593 bands generated from 12 ISSR primers were used to calculate GS coefficients among 14 species and one variety of *Kengyilia* (Table 3). The results showed that the GS ranged from 0.430 to 0.866, with a mean of 0.620. The highest GS value was found between *K. thoroldiana* and *K. mutica* (0.866), which also showed the nearest genetic distance (GD = 1–GS), while the lowest GS value was observed between *K. laxiflora* and *K. rigidula* (0.430). The mean GS of accessions among the 6 species of *Kengyilia*, namely, *K. rigidula*, *K. stenachyra*, *K. hirsuta*, *K. batalinii*, *K. tahelacana* and *K. melanthera*, was 0.849. The highest GS value was found among the

Table 2 ISSR primer sequences and the results of PCR amplification

primer	sequence	DNA bands amplified		primer	sequence	DNA bands amplified	
		total	polymorphic bands			total	polymorphic bands
1	5′-HVH$(TG)_7$T-3′	66	58	7	5′-$(CT)_8$G-3′	33	28
2	5′-VHVG(TG)-3′	54	49	9	5′-$(AC)_8$C-3′	16	11
3	5′-HVH$(TCC)_5$-3′	60	55	10	5′-$(AG)_8$YT-3′	85	80
4	5′-$(AG)_8$YC-3′	33	29	11	5′-$(GA)_8$YC-3′	85	79
5	5′-$(AC)_8$YG-3′	43	38	12	5′-$(AC)_8$YT-3′	23	20
6	5′-$(GA)_8$C-3′	43	40	total		593	535

Note: Y=G/C, H=A/T/C, V=A/G/C. The primers 1–5 and 6–12 had been shown by Fang (1997) and Nagaoka (1997) respectively.

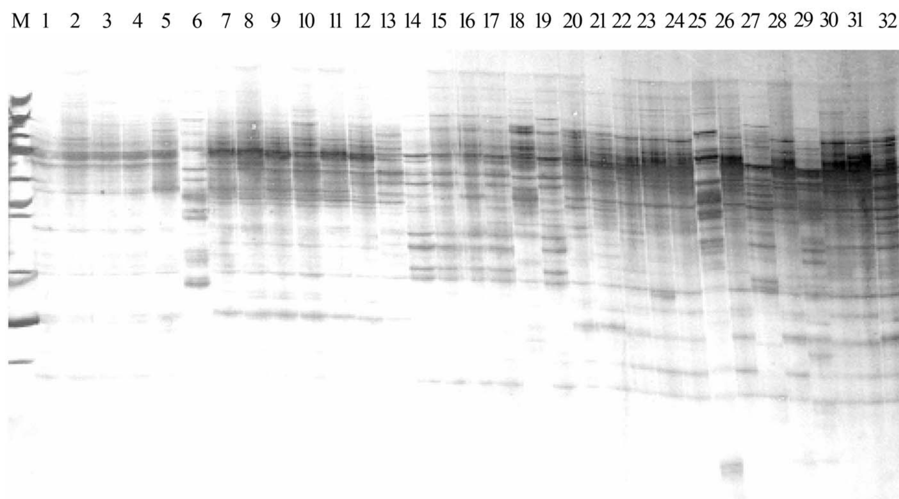

Fig. 1 The ISSR amplification of primer 8. Note: Lanes 1 to 32 correspond to the material numbers in Table 1; M stands for GeneRuler™ 50 bp DNA Ladder Plus (MBI)

accessions of the *K. rigidula* (0.965), while the lowest GS value was observed in *K. tahelacana* (0.775).

3.3 Dendrogram analysis

The genetic relationships among 32 *Kengyilia* accessions were analyzed using the UPGMA method (Figure 2). Group I included only one species, *Kengyilia laxiflora,* while Group II contained *K. batalinii* and *K. tahelacana*. Group III included two subgroups (IIIa, IIIb), of which IIIa included three accessions and IIIb included 16 accessions. In IIIa there were three taxa: *K. zhaosuensis*, *K. gobicola* and *K. alatavica*. In IIIb there were six species and one variety: *K. hirsuta*, *K. melanthera*, *K. melanthera* var. *tahopaica*, *K. grandiglumis*, *K. thoroldiana*, *K. mutica* and *K. kokonorica*. In group IV there were six accessions of *K. rigidula* and *K. stenachyra*.

4 Discussion

ISSR markers were based on the size-polymorphism of 200–2500 bp long inter-microsatellite spacers, which can be amplified by a single-primer PCR. During the long period of evolution, the genetic variations of simple sequence had been accumulated. As a consequence, ISSR amplification revealed a much larger number of polymorphic fragments (Jing et al., 2000). Besides, among the accessions with very close relationships, the polymorphic fragments could be distinguished (Li et al., 2002). Qian et al. (2000) used ISSR to detect the genetic variation of *Oryza granulata*, and they found that the percentage of polymorphic *Oryza granulata* was 72.95%. Du et al. (2002) studied the genetic diversity of wheat, and the polymorphic bands that were amplified by each primer ranged from 11 to 38, with an average of 18.8. The

Table 3 Genetic similarity (GS) values

	KRI	ST	KHI	KBA	KTA	KMT	KME	KLA	KZH	KGO	KGR	KAL	KTH	KMU
KRI	0.965													
KST	0.810	0.838												
KHI	0.645	0.664	0.813											
KBA	0.558	0.580	0.581	0.883										
KTA	0.509	0.561	0.566	0.645	0.775									
KMT	0.566	0.599	0.686	0.586	0.598									
KME	0.586	0.615	0.689	0.571	0.571	0.807	0.817							
KLA	0.430	0.473	0.508	0.512	0.518	0.503	0.519							
KZH	0.588	0.625	0.648	0.581	0.572	0.681	0.726	0.512						
KGO	0.543	0.582	0.567	0.614	0.550	0.590	0.613	0.502	0.769					
KGR	0.610	0.644	0.664	0.586	0.565	0.699	0.727	0.496	0.736	0.630				
KAL	0.578	0.637	0.599	0.617	0.586	0.632	0.652	0.501	0.734	0.749	0.690			
KTH	0.596	0.611	0.625	0.590	0.553	0.695	0.746	0.503	0.722	0.627	0.749	0.670		
KMU	0.580	0.608	0.623	0.574	0.558	0.680	0.732	0.498	0.710	0.622	0.729	0.646	0.866	
KKO	0.591	0.618	0.631	0.563	0.589	0.719	0.685	0.533	0.659	0.591	0.662	0.642	0.741	0.757

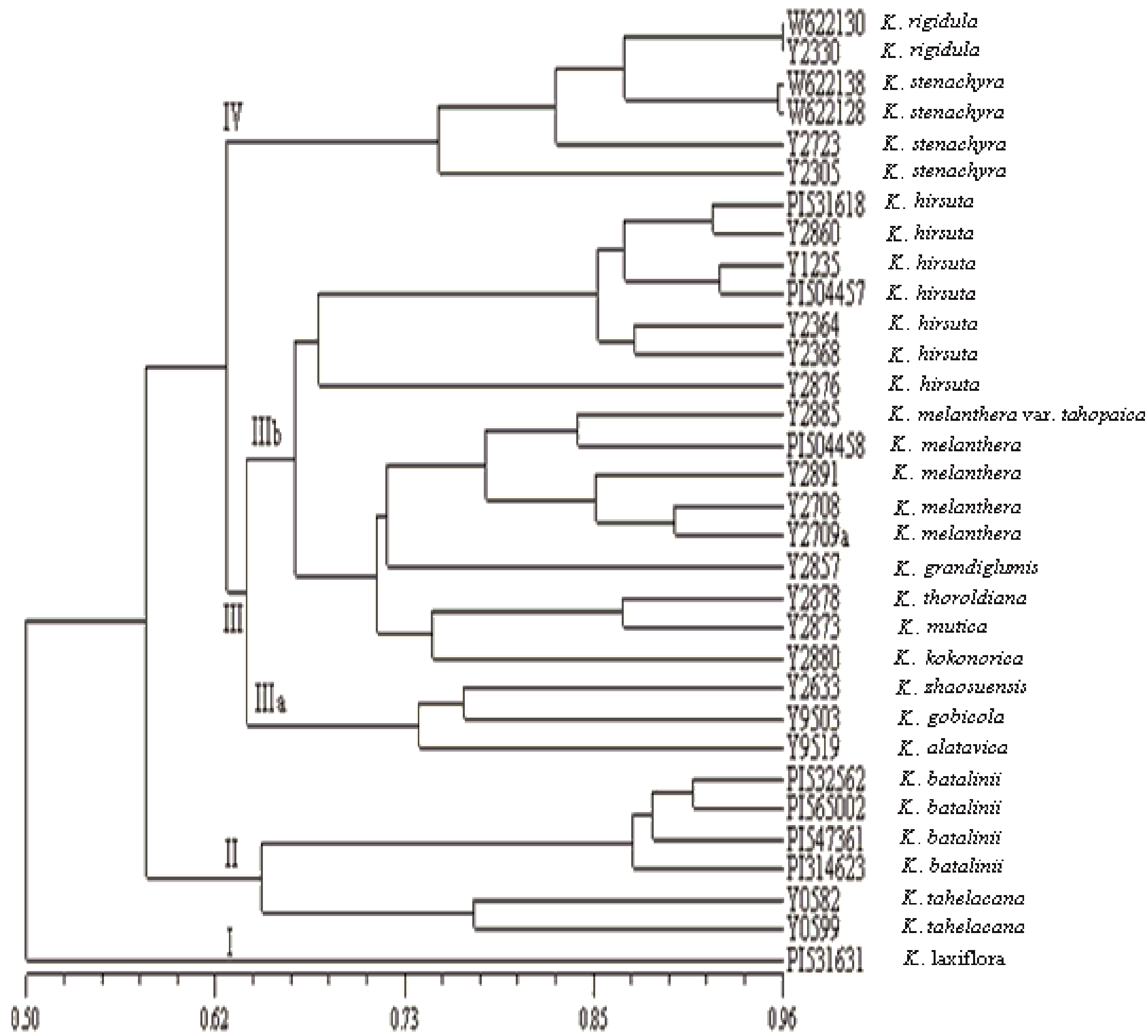

Fig. 2 Dendrograms of 32 *Kengyilia* accessions constructed from a matrix of similarity based on ISSR data

percentage of polymorphic bands was 87.4%. In this study, the polymorphisms among 32 *Kengyilia* samples were detected with all the 12 primers. These primers generated a total of 593 fragments with a mean of 49.4, ranging from 16–85. Polymorphism marked 535 out of 593 bands (90.2%). Eleven to 80 polymorphic bands could be amplified per primer, with 44.6 polymorphic bands on average. This study suggested that there was a high level of variation in the DNA sequence of *Kengyilia* accessions, and the polymorphisms revealed by ISSR markers were high. Polymorphism by ISSR markers was higher than RAPD (Zhou et al., 2000; Zhang et al., 2003b) and RAMP markers (Zhang et al., 2003 c), and the results supported those of Gilbert et al (1999), Yang et al. (1996), and Jonsson et al.(1996).

The dendrogram based on ISSR data indicated that the different accessions of a species were generally clustered together, which suggested that they had large genetic similarities and close relationships. The GS in the accessions of *K. rigidula* was the highest with 0.965, which indicated that the accessions of *K. rigidula* had large genetic similarities, and all accessions came from Xiahe, Qinghai, China. The accessions of *K. tahelacana* had the lowest GS (0.775), and were distributed in Wensu, Xinjiang, China. The results suggested that differentiations not only existed in different populations of a species, but also in different regions of a species.

The results of cluster analysis based on ISSR data indicated that all 32 accessions were divided into four groups. Group I included only one species, *K. laxiflora*, and that had a comparatively distant relationship with other species. *K. laxiflora* was mainly distributed in the Sichuan Province of China with lax spikes. *K. laxiflora* was treated as Roegneria Sect. Clinelymus (Guo et al., 1987). Cytological study indicated that *K. laxiflora* contained StYP genomes (Zhang et al., 1998). Therefore, *K. laxiflora* was a valid taxon. In group II, there were two species: *K. batalinii* and *K. tahelacana*. They belonged to Sect. *Kegyilia* and Sect. *stenachyra*, respectively, according to their morphology (Cai et al.,

1999). In subgroup IIIb, *K. thoroldiana* and *K. mutica* were clustered together and had large morphological similarities and close relationships. *K. melanthera* and *K. melanthera* var. *tahopaic*a were clustered together, which had the closest relationships. Cai (1999) treated morphologically *K. melanthera* and *K. melanthera* var. *tahopaica* as *K. thoroldian*a var. *melanthera* and *K. hirsuta* var. *tahopaica*, respectively. In this study, the treatment of *K. melanthera* and *K. melanthera* var. *tahopaica* was reasonable, which was consistent with the results of Zhou et al. (2000) and Zhang et al. (2003 b, 2003 c). In group IV, *K. rigidula* and *K. stenachyra* were clustered together. They were distributed in the Gansu Province of China. Morphologically, *K. rigidula* differed from *K. stenachyra* only by length, width of glume vein and length of lemma. Therefore, it is concluded that the ISSR technique is an effective and reliable additional method to analyze genetic diversity and similarity among *Kengyilia* species.

References

Cai L B, Zhi L (1999). A taxonomical study on the genus *Kengyilia* Yen et J. L. Yang.Acta Phytotaxonomica Sinica, 37(5): 451–467 (in Chinese)

Du J K, Yao Y Y, Ni Z F, Feng H R, Sun Q X (2002). Genetic diversity revealed by ISSR molecular marker in common wheat spelt compactum and progeny of recurrent selection. Acta Genetica Sinica, 29(5): 445–452 (in Chinese)

Fang D Q, Roose M L (1997). Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor Appl Genet, 95: 408–417

Fernandez M E, Figueiras A M, Benito C (2002). The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. Theor Appl Genet, 104: 845–851

Gilbert J E, Lewis R V, Wilkinson M J, Caligari P D S (1999). Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor Appl Genet, 98: 1125–1131

Guo B Z (1987). Flora of China. Beijing: Science Press, 9(3): 51–104 (in Chinese)

Jensen K B (1990). Cytology and taxonomy of *Elymus kengii*, *E. grandiglumis*, *E. alatavicus*, and *E. batalini* (Poaceae; Triticeae). Genome, 33: 668–673

Jing R C, He Y Q, Huang Q Y, Zhu Y G (2000). Analysis of the fertility restorer gene in the wild-abortive (WA) type cytoplasmic male sterility (CMS) system with the ISSR and SSLP markers. Scientia Agricultura Sinica, 33(2): 1–9 (in Chinese)

Jonsson B O, Jonsdottir I S, Cronberg N (1996). Clonal diversity and allozyme variation in population of the arctic sedge *Carex bigelowii* (Cyperacea). J Ecology, 84: 449–459

Keng Y L (1959). Flora Illustalis Plantarum Primarum Sinicarum (Gramineae). Beijing: Science Press, 342–409

Kojima T, Nagaoka T, Noda K, Ogihara Y (1998). Genetic linkage map of ISSR and RAPD markers in Eikorn wheat in relation to that of RFLP markers. Theor Appl Genet, 96: 37–45

Li J B, Mou T M, Fang X J (2002). Identification and genetic analysis for 12 elite PGMS and TGMS rices based on ISSR markers. Chinese Agricultural Science Bulletin, 18(1): 6–9 (in Chinese)

Nagaoka T, Ogihara Y (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use DNA as markers in comparison to RFLP and RAPD markers. Theor Appl Genet, 94: 597–602

Nei M, Li W H (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA, 76: 5269–5273

Qian W, Ge S, Hong DY (2000). Assessment of genetic variation of *Oryza granulata* detected by RAPDs and ISSRs. Acta Botanica Sinica, 42(7): 741–750 (in Chinese)

Rohlf F J (1993). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Version 1.80 [M/CD]. New York: Exeter Software, Setauket.

Sharp P J, Kresis M, Shewry P R, Gale M D (1988). Location of β–amylase sequences in wheat and its relatives. Theor Appl Genet, 75: 286–290

Yang J L, Yen C, Baum B R (1992). *Kengyilia*: synopsis and key to species. Hereditas, 116: 25–28

Yang W P, Oliveira A C, Godwin I, Scheritz K, Bennetzen J L (1996). Comparison of DNA marker technologies in characterizing plant genome diversity: variability in Chinese sorghums. Crop Sci, 36: 1669–1676

Yen C, Yang J L (1990). *Kengyilia gobicola*, a new taxon from west China. Can J Bot, 68: 1894–1897

Zhang L, Zhou Y H, Zheng Y L, Zhang Y (2003a). Study on karyotypes and evolution of 6 species in *Kengyilia*. Journal of Sichuan University (Natural Science Edition), 40(2): 361–366 (in Chinese)

Zhang L, Zheng Y L, Wei Y M, Liu S G, Zhou Y H (2003b). Genetic variation in *Kengyilia* populations based on randomly amplified polymorphic DNA (RAPD) analysis. Journal of Beijing Forestry University, 25(3): 11–16 (in Chinese)

Zhang L, Zhou Y H, Wei Y M, Zheng Y L, Liu S G (2003c). Relationships among *Kengyilia* species based on random amplified microsatellite polymorphism (RAMP). High Technology Letters, 13(4): 28–33 (in Chinese)

Zhang X Q, Yen C, Yang J L, Yen Y (1998). Cytogenetic analyses of *Kengyilia laxiflora*. Plant Syst Evol, 212: 79–86

Zhou Y H (1994). Study on karyotypes of 5 species of *Kengyilia*. Guihaia, 14(2): 163–169 (in Chinese)

Zhou Y H, Zheng Y L, Yang J L, Yen C, Jia J Z (2000). Relationship among *Kengyilia* species assessed by RAPD marker. Acta Phytotaxonomica Sinica, 38(6): 515–521 (in Chinese)

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. Genomics, 20: 176–183