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Full Length Research Paper

Genetic diversity analysis of cocksfoot (*Dactylis glomerata* L.) accessions with sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) markers

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Sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR), two advanced molecular markers for genetic research in grass and forage, were used to analyze the genetic diversity among 44 accessions of cocksfoot collected from seven countries and regions. 21 SRAP primer combinations generated 476 bands, of which 401 were polymorphic (84.24%). Using 12 ISSR primers, 100 polymorphic bands out of 115 bands in total were generated (86.96%). The coefficient of genetic similarity from SRAP and ISSR data ranged from 0.6838 to 0.9686 and from 0.6935 to 0.9231, respectively. Based on unweighted pair group method with arithmetic mean (UPGMA) cluster and principal component analysis (PCA) on a series of genetic characteristics, all accessions were divided into three clusters and four clusters using two markers, respectively. Those, accessions collected from the identical continent were classified into the same cluster, suggesting the geographical distribution of genetic diversity of cocksfoot. The genetic diversity of Chinese cocksfoot except for three Chinese cultivars was rather rich and greater than that of other regions. We proposed that both SRAP and ISSR markers were considered as useful tools for evaluating the genetic diversity of cocksfoot. Especially, SRAP detected more variance and gave clearer cluster groups.

Key words: Cocksfoot, genetic diversity, sequence-related amplified polymorphism (SRAP), inter-simple sequence repeat (ISSR), germplasm.

INTRODUCTION

Cocksfoot (*Dactylis glomerata* L.) is a kind of valuable grazing resource in North America (Van Santen and Sleper, 1996; Lawrence et al., 1995), Europe (Casler et al., 2000) and Japan (Mitui, 1981), which is widely distributed in areas with temperate climates. It is indigenous to the Northern Hemisphere, including regions within Eurasia and North Africa (Beddows, 1959). Cocksfoot germplasm resources are abundant in China (Cheng and Jia, 2002) and many natural populations have been collected from more than 10 provinces of China. Particularly, three cultivated cultivars were originally registered in China in 1997, including “BaoXing”, “GuLin”, and “ChuanDong” (Peng and Zhang, 2005). Due to high forage yield and good quality, cocksfoot has been extensively utilized in cultivated pastures (Lumaret and Barrientos, 1990). In addition, it serves an important role in animal husbandry (Lindner et al., 1999). Genetic diversity is a level of biodiversity that refers to the total number of genetic characteristics in the genetic makeup

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Abbreviations: SRAP, Sequence-related amplified polymorphism; **ISSR,** inter-simple sequence repeat; **PCA,** principal component analysis; **UPGMA,** unweighted pair group method with arithmetic mean.

of a species. Therefore, it is important for current cocksfoot breeding to study its genetic diversity.

Over the last 10 years, polymerase chain reaction (PCR) technology has led to the development of two simple and rapid techniques called sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) (Li and Quiros, 2001; Zietkiewicz et al., 1994). SRAP detects nucleotide sequence polymorphisms, using a pair of primers of arbitrary nucleotide sequence (Ferriol et al., 2003), while ISSR, allows the detection of repeat polymorphisms within microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Fang and Roose, 1997). SRAP combining the convenience of random amplification of polymorphic DNA (RAPD) and the reliability of amplified fragment length polymorphisms (AFLP) has been successfully applied to detect genetic diversity in many species such as Brassica, potato, rice, and cotton (Ferriol et al., 2003; Li et al., 2003). However, it has not been incorporated into the grass and forage research except for Buffalo grass (Budak et al., 2004). ISSR markers have been successfully employed for genome fingerprinting in a multitude of species for many years (Bornet and Branchard, 2001), which are suggested to be more repeatable and reliable than RAPD markers, and have a higher cost-benefit ratio than restriction fragment length polymorphism (RFLP). Both SRAP and ISSR are unique PCR methods that do not require prior knowledge of deoxyribonucleic acid (DNA) sequence for primer design (Budak et al., 2004).

As a consequence, the application of SRAP and ISSR as alternative methods, for the analysis of genetic diversity in cocksfoot, could contribute significantly to the understanding of this species. At present, there are still few reports on the genetic variability of cocksfoot with molecular markers, much fewer reports on the comparison of SRAP and ISSR (Kölliker et al., 1999; Reeves et al., 1999). Herein, SRAP and ISSR are used to analyze the genetic diversity among 44 accessions of cocksfoot collected from seven countries and regions in our researches. However, what is more important is we compared their differences and effects on analysis of genetic diversity in cocksfoot. The objectives of our researches were to compare ISSR and SRAP markers for the molecular characterization of cocksfoot, including evaluations on the degree of polymorphisms generated, and to evaluate the degree and patterns of genetic diversity of cocksfoot.

MATERIALS AND METHODS

Plant materials

44 accessions of cocksfoot, including 25 ecotypes and 19 cultivars, were used in this study (Table 1), representing seven geographically and environmentally distinct countries and regions. Plant materials consisted of young leaves sampled from cocksfoot individuals that grew from basic seeds of the aforementioned 44 accessions in an experimental field of Sichuan Agricultural University.

Total deoxyribonucleic acid (DNA) extraction

Total DNA was extracted from fresh young leaves by a cetyl trimethyl ammonium bromide (CTAB) protocol (Sun et al., 1997). The quality and quantity of the DNA were estimated by using a Beckman-Coulter DU800 nucleic acid/protein analyzer (Beckman-Coulter, Fullerton, CA, USA). The total DNA isolated was stored at -20°C until needed.

Sequence-related amplified polymorphism (SRAP) polymerase chain reaction (PCR) analysis

The PCR amplification was carried out on a Thermo Hybaid Cycler (Bio-Rad, Hercules, CA, USA). On the basis of primer sequences successfully screened out from kindred plant, primer screening was done on high quality DNA extracted from two varieties of cocksfoot with great morphological difference. 21 oligo-primer combinations (Table 2) used for the SRAP marker evaluations were produced by Beijing SBS Genetic Company (SBS Genetech, Beijing, China). The SRAP protocol for the analysis was based on Li and Quiros (2001). Each 25 µL PCR reaction mixture consisted of 60 ng genomic DNA, 200 µM deoxyribonucleotide triphosphates (DNTPs) (TaKaRa Biotechnology (Dalian) Company, Dalian, China), 1.6 mM $MgCl_2$, 0.3 µM primer, 2.5 µL 10×Taq buffer, and 1 unit Taq polymerase (TaKaRa Biotechnology (Dalian) Company, Dalian, China). Samples were subjected to the following thermalcycler program: the first five cycles were run at 94°C, 1 min, 35°C, 1 min, and 72°C, 1 min, for denaturing, annealing and extension, respectively. Then the annealing temperature was raised to 50°C for another 35 cycles, followed by another extension step of 10 min at 72°C, and then followed by a 4°C holding temperature. The DL2000 molecular weight marker (TaKaRa Biotechnology (Dalian) Company, Dalian, China) was mixed with 12 µL loading buffer to identify amplification product size. All PCR products were run on a 6% denatured polyacrylamide gel [acrylamide-bisacrylamide (19:1), 0.5×Tris-borate-EDTA (TBE)]. The gel was pre-run in 0.5×TBE buffer at 200 V constant voltage prior to loading the samples. Electrophoresis was carried out in a DYY-6C vertical electrophoresis chamber (Beijing LiuYi Instrument Factory, Beijing, China). Following the pre-run, samples were loaded and run at 400 V constant voltage for 1.5 to 2 h. Gel temperature was maintained at 50°C to avoid breaking the gel-support glass. After electrophoresis, the gel was stained with $AgNO_3$ solution (Wang, 2003) then photographed by a Gel Doc™ XR System (Bio-Rad, Hercules, CA, USA).

Inter-simple sequence repeat (ISSR) polymerase chain reaction (PCR) analysis

12 randomly selected oligos (Table 3) were used in the ISSR evaluations and were produced by the Shanghai ShengGong Genetic Research Company (Shanghai, China). The protocol for the ISSR analysis was that of Wang et al. (2003). The final ISSR reaction mixture consisted of a total volume of 20 µL systems containing 1.6 mM Mg^{2+}, 250 µM dNTP, 1.0 U Taq polymerase, 0.25 µM primer, 100 ng DNA. Both DNTP and Taq polymerase were the same one utilized in the SRAP approach. Amplification reactions were conducted for one initial step of 5 min denaturation at 94°C, followed by 45 cycles of 45 s denaturation at 94°C, 1 min annealing at 52°C, 1.5 min extension at 72°C, followed by another extension step of 5 min at 72°C, and then followed by a 4°C holding

Table 1. Cultivars name and origin of cocksfoot used in this study.

Code	Accession number	Origin	Cultivar name
1	YA02-107	Baoxing County, Sichuan Province, China	-
2	YA02-108	Baoxing County, Sichuan Province, China	-
3	YA02-109	Baoxing County, Sichuan Province, China	-
4	YA90-70	Kangdin County, Sichuan Province, China	-
5	YA02-111	Zhongdian County, Yunnan Province, China	-
6	YA02-106	Baoxing County, Sichuan Province, China	-
7	YA90-130	Maoxian County, Sichuan Province, China	-
8	YA02-114	Qujing County, Yunnan Province, China	-
9	YA02-116	Kunming County, Yunnan Province, China	-
10	YA02-105	Dazhou County, Sichuan Province, China	-
11	YA05-262	Dazhou County, Sichuan Province, China	“ChuanDong”
12	YA01-101	Bijie County, Guizhou Province, China	-
13	YA91-103	Yuexi County, Sichuan Province, China	-
14	YA91-7	Hanyuan County, Sichuan Province, China	-
15	YA02-101	Guizhou Province, China	-
16	YA00850	Xinjiang Province, China	-
17	YA79-9	Lushan Mountain, Jiangxi Province, China	-
18	YA02-117	Denmark	“Amba”
19	02685	Gulin County, Sichuan Province, China	“GuLin”
20	YA91-2	Sichuan Agricultural University, China	“BaoXing”
21	01071	England	“Justus”
22	YA79-118	The Netherlands	-
23	01822	Denmark	-
24	02123	Sweden	“Porto”
25	YA01-104	New York, USA	-
26	00737	The Netherlands	-
27	02683	Sweden	-
28	YA79-14	Denmark	-
29	YA96011	Germany	-
30	01996	Sweden	“Datuoce”
31	01824	Sweden	“PG693”
32	01993	Denmark	“Daketa”
33	02684	Denmark	“Sparta”
34	01823	Denmark	“2060”
35	01995	Sweden	“Datus 2”
36	01076	England	“81-Justus”
37	01767	Sweden	“Trode”
38	02682	Sweden	“Tamus”
39	02122	Germany	“Wana”
40	YA79-15	Denmark	-
41	02681	Denmark	-
42	YA98-101	USA	“Potomac”
43	YA98-102	USA	“Tekapo”
44	00937	Canada	“Juno”

temperature. PCR products were mixed with 4 µL loading buffer and were separated on 1.5% agarose (TaKaRa Biotechnology (Dalian) Company, Dalian, China) gel in 1×TBE buffer. Gels were run at 80 V for 3.5 h by using the DYY-6C electrophoresis apparatus and stained with ethidium bromide. Gels were photographed using the Gel Doc™ XR system.

Data analysis

The same analysis method was used in two markers. SRAP and ISSR amplified fragments were scored for band presence (1) or absence (0) and two binary qualitative data matrices were constructed. Data analyses were performed using the NTSYS-pc

Table 2. Primer sequences used in SRAP analysis of cocksfoot.

Primer	Primer sequence (5'-3')	Primer	Primer sequence (5'-3')
me1	TGAGTCCAAACCGGATA	em6	GACTGCGTACGAATTGCA
me2	TGAGTCCAAACCGGAGC	em9	GACTGCGTACGAATTCGA
me4	TGAGTCCAAACCGGACC	em10	GACTGCGTACGAATTCAG
me8	TGAGTCCAAACCGGTGC	em12	GACTGCGTACGAATTATG
me10	TGAGTCCAAACCGGTTG	em13	GACTGCGTACGAATTAGC
me11	TGAGTCCAAACCGGTGT	em14	GACTGCGTACGAATTACG
em2	GACTGCGTACGAATTTGC	em15	GACTGCGTACGAATTTAG
em3	GACTGCGTACGAATTGAC	em16	GACTGCGTACGAATTTCG
em4	GACTGCGTACGAATTTGA		

Table 3. The amplification results of SRAP primers.

Primer combination	Total band	Polymorphic band	The percentage of polymorphic band (%)
me10+em2	25	21	84
me11+em14	19	17	89.47
me11+em15	21	18	85.71
me10+em10	22	18	81.82
me10+em9	26	22	84.62
me10+em4	26	22	84.62
me10+em13	19	16	84.21
me1+em4	36	30	83.33
me2+em4	22	18	81.82
me10+em15	21	18	85.71
me11+em4	25	21	84
me11+em9	21	17	80.95
me2+em6	24	20	83.33
me8+em12	20	17	85
me1+em2	23	19	82.61
me2+em16	19	17	89.47
me10+em14	15	12	80
me2+em2	34	29	85.29
me2+em14	27	23	85.19
me1+em3	18	15	83.33
me4+em4	13	11	84.62
Mean	22.67	19.10	84.24
Total	476	401	

(Numerical Taxonomy and Multivariate Analysis System; Rohlf, 2001) version 2.1 computer program package. The Dice similarity coefficient matrix (Nei and Li, 1979), generated from binary qualitative data matrices using the qualitative data module from NTSYS, was used to construct the UPGMA dendrograms (Sneath and Sokal, 1973) for clustering analysis (SAHN module from NTSYS). In order to highlight the resolving power of the ordination, the principal coordinate analysis (PCA) was finally performed using the EIGEN module from NTSYS (Seman et al., 2003). In addition, the percentages of polymorphic bands (PPB) and the total number of bands (TNB) were surveyed based on binary qualitative data matrices. Then, the genetic similarity coefficient (GS) was estimated according to the Dice similarity coefficient matrix. All of them were used for the comparison of two markers.

RESULTS

Genetic polymorphism analysis of PCR products

21 oligo-primer pairs, combinations of 16 primers, were used to evaluate the genetic diversity of the 44 accessions In SRAP analysis (Table 2). Primer combinations, total bands, polymorphic bands, the

Table 4. Sequences and amplification results of ISSR primers.

Primer	Sequence (5'-3')	Total band	Polymorphic band	The percentage of polymorphic band (%)
SG13	$(GACA)_4$	10	9	90
SG17	TAG ATC TGA TAT CTG AAT TCC C	10	9	90
SG18	AGA GTT GGT ACG TCTTGA TC	9	8	88.89
SG19	ACT ACG ACT$(TG)_7$	10	9	90
SG20	ACT TCC CCA CAG GTT AAC ACA	8	7	87.5
SG23	$(CT)_8$AGA	8	7	87.5
SG39	$(AG)_8$TC	10	8	80
SG40	$(AC)_8$GCT	10	9	90
SG41	$(GA)_8$GCC	9	7	77.78
SG62	ACT CGT ACT $(AG)_7$	11	10	90.91
SG65	CGT AGT CGT $(CA)_7$	9	8	88.89
SG66	AGT CGT AGT $(AC)_7$	11	9	81.82
Mean		9.58	8.33	86.96
Total		115	100	

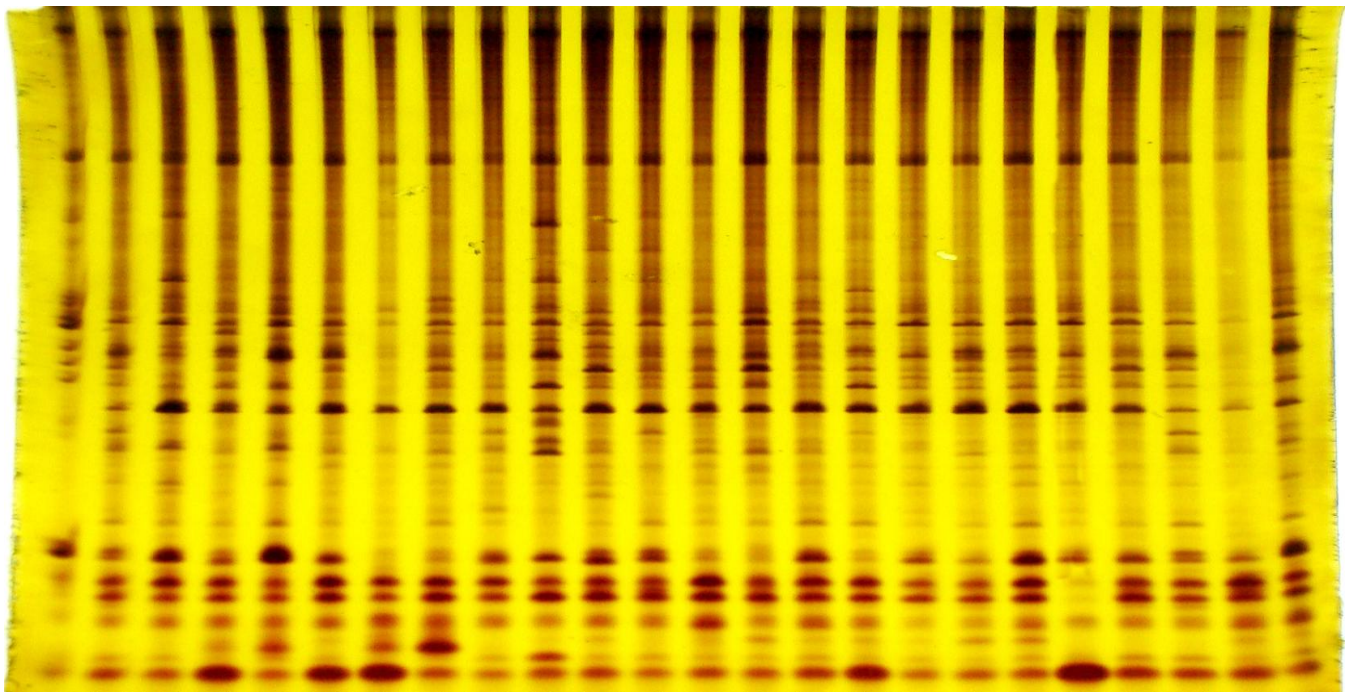

Figure 1. SRAP fingerprinting patterns amplified by primer combination M2+E14.

percentage of polymorphic bands (%) of SRAP primers were presented in Table 3. A total of 476 bands were generated from 21 SRAP primer combinations, of which 401 were polymorphic, accounting for 84.24% of the total number. On average, each primer combination could amplify 19.53 polymorphic bands.

Table 4 shows the sequences and amplification results of ISSR primers. 12 ISSR primers produced 100 polymorphic bands out of 115 bands in total (86.96%). The number of total bands produced by each primer ranged from eight to 11, with an average of 9.58 bands each primer, whereas the number of polymorphic bands ranged from seven to 10, with an average of 8.33 polymorphic bands per primer. In addition, two examples of SRAP fingerprinting patterns amplified by primer combination M2 + E14 and ISSR patterns amplified by ISSR primer SG13 are shown in Figures 1 and 2, respectively. Judging from Figures 1 and 2, both SRAP

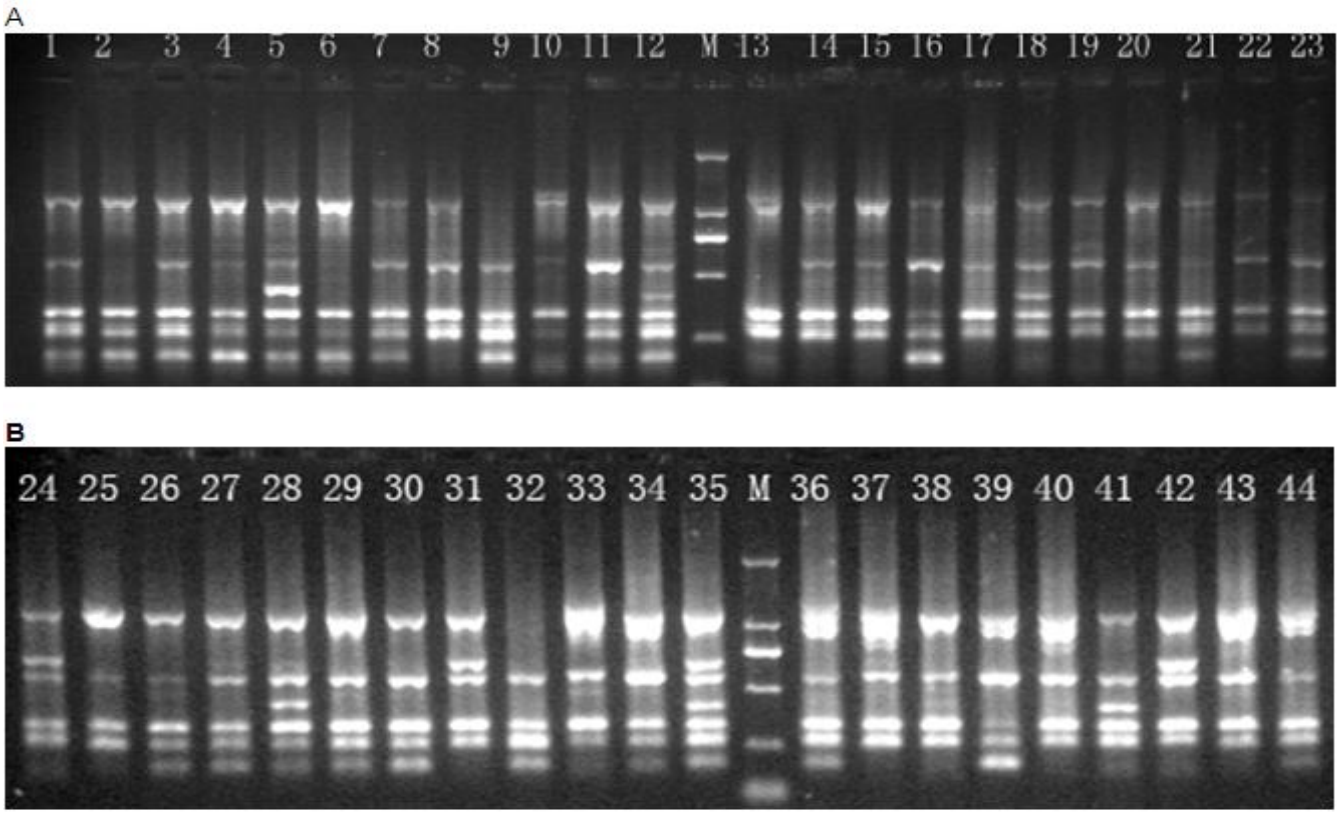

Figure 2. ISSR patterns amplified by primer SG13 in 1 to 23 DNA (A) and 24 to 44 DNA (B) of *D. glomerata*.

and ISSR are efficient measures for detecting the degree of polymorphism within cocksfoot. However, SRAP markers are considered inferior to ISSR markers in genetic polymorphism analysis of cocksfoot to a certain degree, since they can detect a large number of informative bands.

Genetic similarity analysis

According to the Dice similarity matrix, the SRAP-based genetic similarity coefficient (GS) among 44 accessions of cocksfoot ranged from 0.6838 to 0.9686 with an average of 0.8013. Of these, the maximum value of GS (0.9686) occurred between accessions YA02-105 and YA91-2, obtained from Sichuan Province of China, which suggested that the two accessions had higher genetic similarity with little genetic distance (GD). The GS between accessions YA02-107 and YA02-109, obtained from Sichuan Province, was the second largest (GS=0.9538). On the other hand, the GS was the smallest between accessions YA02-101 and YA02-117 (GS=0.6838), obtained from Guizhou Province of China and Denmark respectively, indicating low genetic similarity and a high degree of genetic diversity.

As for the identical accessions, the range of ISSR-based GS was from 0.6935 to 0.9231, with the average being 0.8044. The Dice similarity matrix revealed that the largest GS (0.9231) existed between accessions YA02-107 and YA02-109, and the smallest GS (0.6935) occurred between accessions YA02-101 and YA02-117. These results are similar to those observed in the SRAP analysis.

Our analysis also encompassed the evaluation of 16 Chinese ecotypes and three registered Chinese cocksfoot cultivars. The genetic similarity coefficient of 16 Chinese ecotypes ranged from 0.7269 to 0.9686 in SRAP analysis and from 0.6880 to 0.9231 in ISSR analysis, whereas that of three Chinese cocksfoot cultivars ranged from 0.8799 to 0.8977 and from 0.8636 to 0.8640, respectively. A separate analysis of European accessions indicated GS ranged from 0.7482 to 0.8498 with SRAP and from 0.7752 to 0.8855 with ISSR which were more limited than those of Chinese ecotypes. The wide GS ranges showed that the investigated Chinese ecotypes in this study possessed a high level of genetic diversity. Nevertheless, the narrow GS ranges implied that the genetic relationship of three Chinese cocksfoot cultivars was large, and their genetic basis was rather narrow.

Both SRAP and ISSR matrix analysis indicated a relatively high level of genetic diversity across the evaluated cocksfoot accessions. SRAP and ISSR

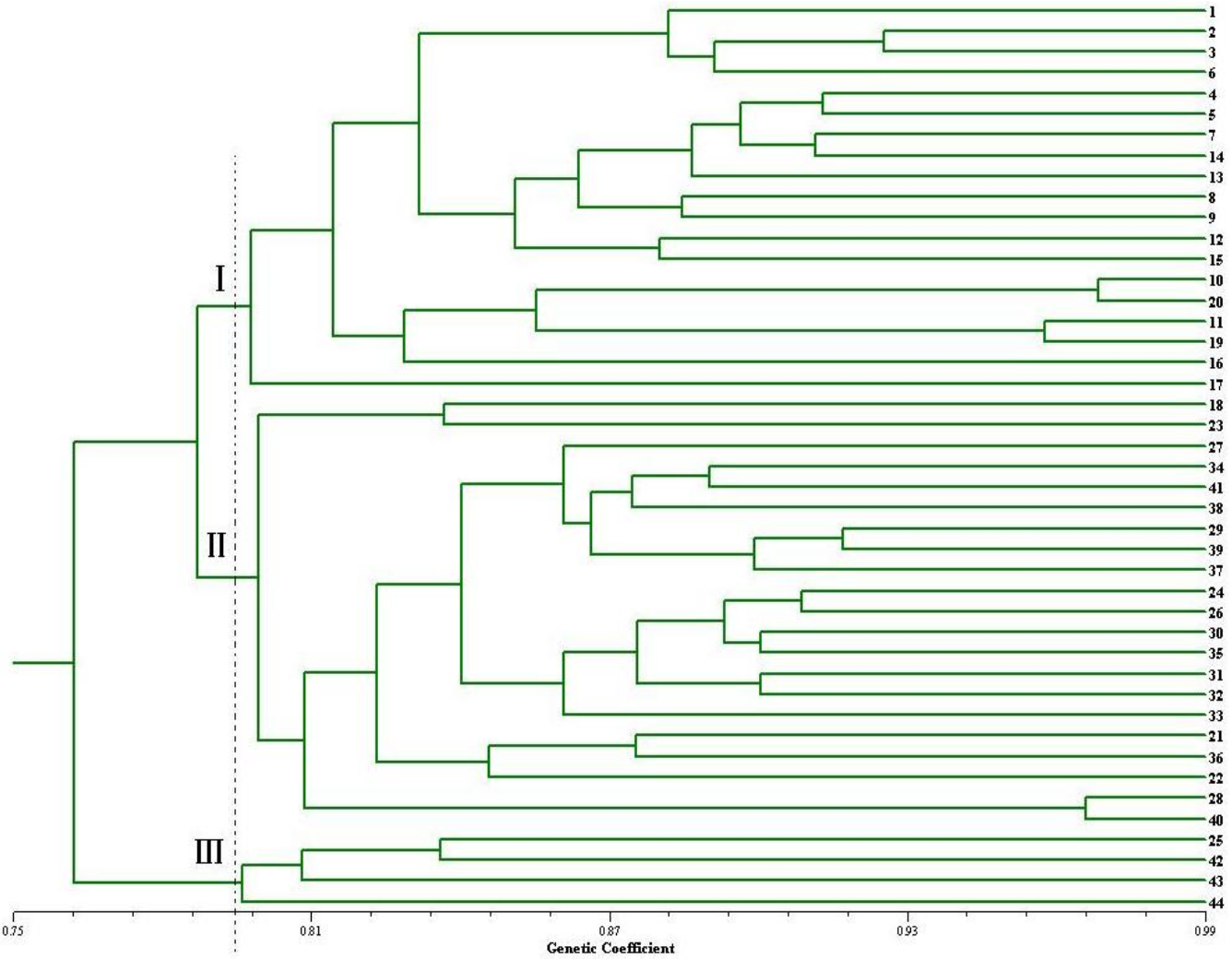

Figure 3. UPGMA dendrogram for SRAP of cocksfoot based on Dice genetic coefficient.

analysis provided similar results across identical accessions. However, a higher level of genetic variance was revealed by SRAP analysis.

Dendrogram

Two dendrograms for cocksfoot were constructed using SRAP and ISSR data from UPGMA cluster analysis based on the Dice genetic similarity coefficient which were basically consistent with each other. Using SRAP marker, 44 accessions of cocksfoot were grouped into three major clusters with an average GS value of 0.8013: cluster I of Chinese accessions, cluster II of all European accessions and cluster III of all the USA accessions (Figure 3). The SRAP dendrogram was in line with the geographical source of the cocksfoot accessions. From the ISSR analysis, four major clusters were identified with an average GS value of 0.8044, including cluster I of all Chinese accessions, cluster II of all European accessions with the exception of cultivar Amba, cluster III of the USA accessions and cluster IV of Amba cultivar (YA02-117) from Denmark (Figure 4). Since the SRAP analysis can provide clearer cluster groupings, it is suggested that SRAP methodology may be superior to ISSR in characterizing geographical origins of cocksfoot accessions.

Principal component analysis

To better visualize the genetic relationship of cocksfoot among 44 accessions, the PCA of GS matrixes generated by SRAP and ISSR dataset was performed using the NTSYS 2.1 software package (Figures 5 and 6). The adjacent accessions had close genetic relationships so that they were grouped into the same cluster. On the contrary, those with more distant relationships did not

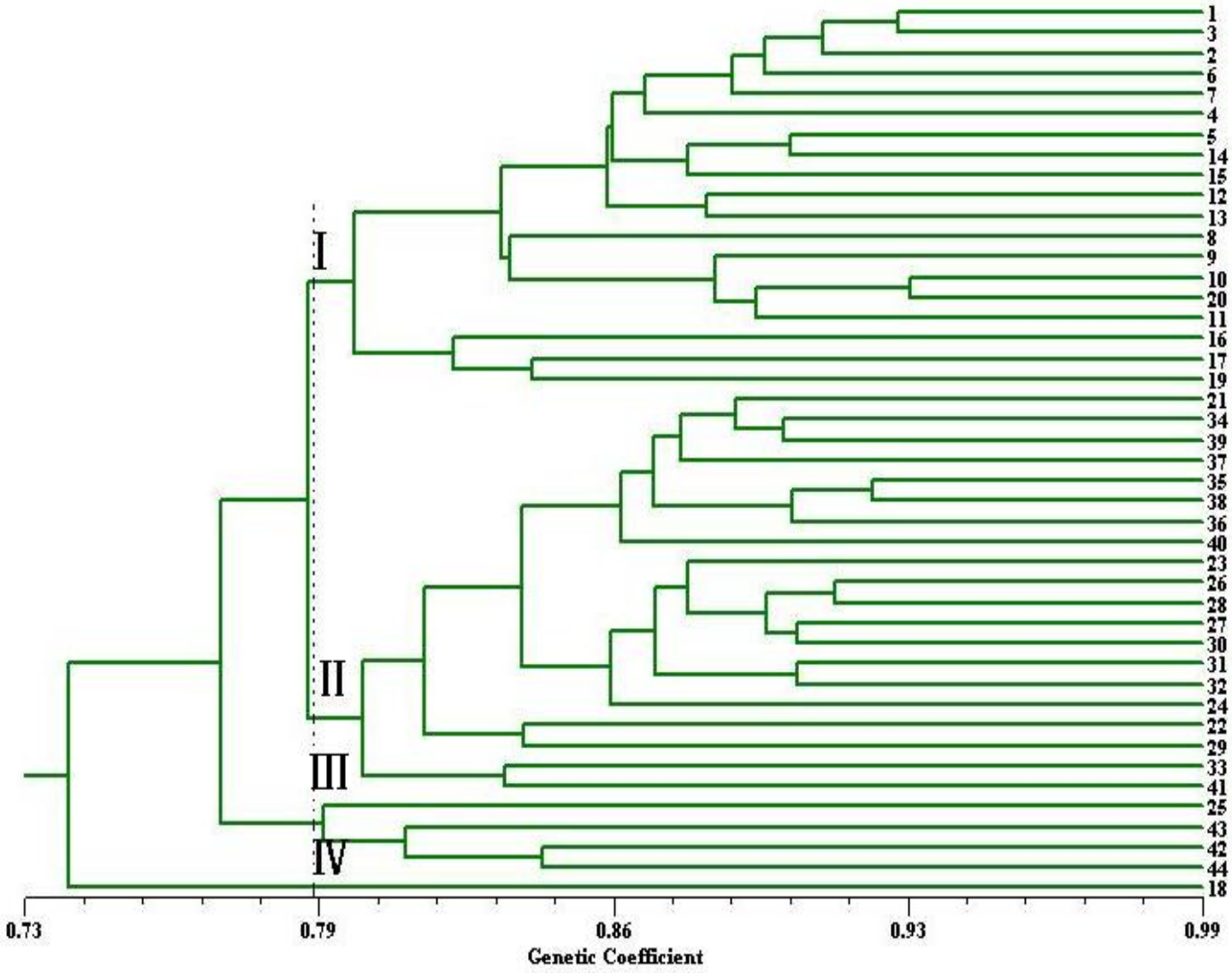

Figure 4. UPGMA dendrogram for ISSR of cocksfoot based on Dice genetic coefficient.

cluster together. The results are identical with the aforementioned dendrograms (Figures 3 and 4), which demonstrated that PCA was superior in identifying the genetic similarity of cocksfoot among the 44 accessions.

DISCUSSION

The use of biochemical and molecular markers could greatly enhance the understanding of the genetic relatedness of complex polyploidy species, including cocksfoot (Dawson et al., 1993). This study first evaluated the degree and patterns of genetic diversity among 44 accessions of cocksfoot collected from seven geographically and environmentally distinct countries and regions using ISSR and SRAP markers.

44 accessions of cocksfoot including 19 cultivars are evaluated by SRAP and ISSR methods to identify which one is more informative in assigning GS coefficients. The results prove that SRAP with higher resolution is superior to ISSR in clustering accessions. This conclusion is coherent with earlier result of Budak et al. (2004), who compared the four marker systems in buffalo grass and figured out the values of average discriminating power as: SRAP>SSR>ISSR>RAPD. A possible explanation is that SRAP marker is based on two-primer amplification of PCR, and its primer combination is more efficient than arbitrary primer of ISSR (Li and Quiros, 2001).

All accessions used in this study exhibit an extensive degree of genetic diversity, especially in 16 Chinese ecotypes. Kölliker et al. (1999) also reported that the genetic diversity of cocksfoot was rich by RAPD marker, and showed that proportion of variability was higher in cocksfoot (85.1%) than that in perennial ryegrass (82.4%)

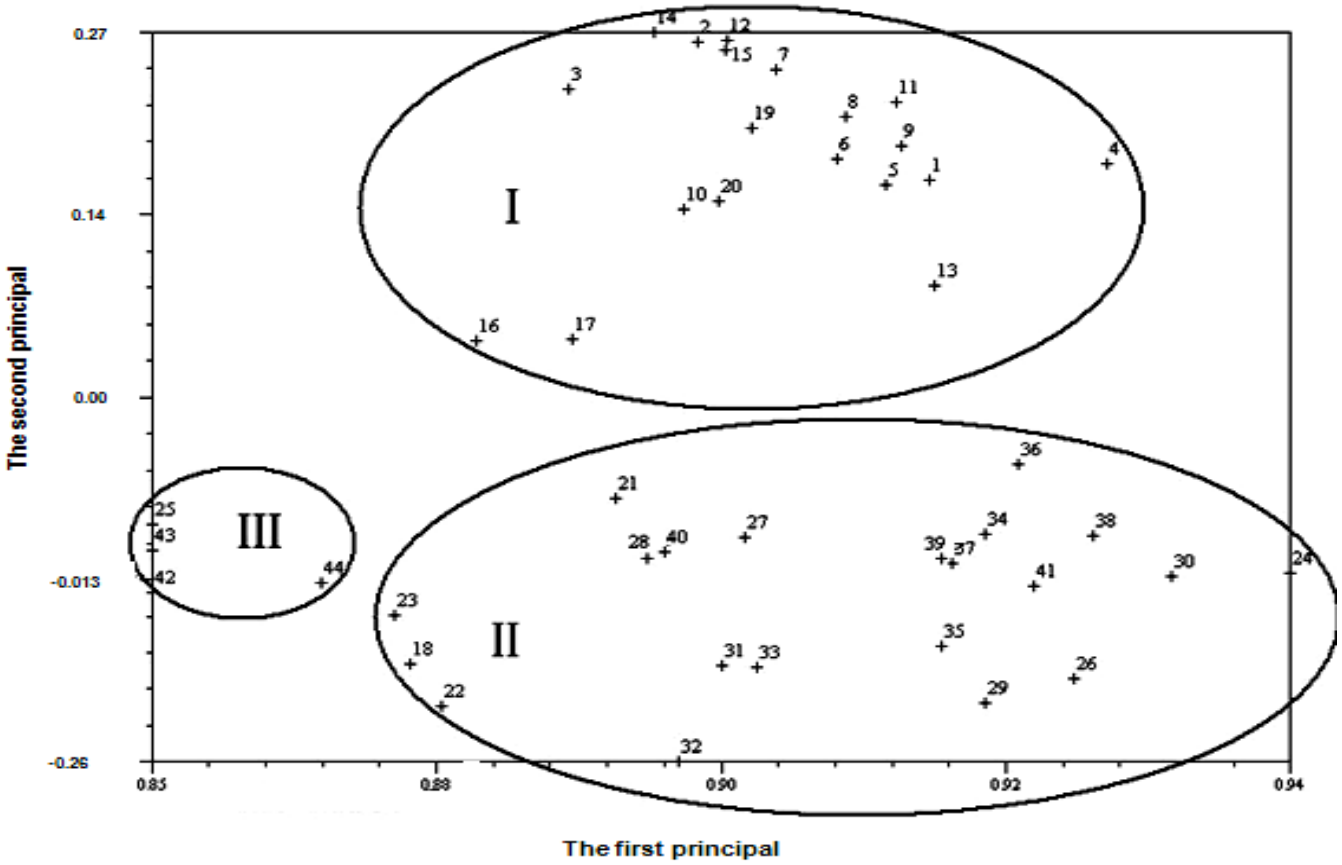

Figure 5. Principal component analysis based on SRAP patterns in cocksfoot.

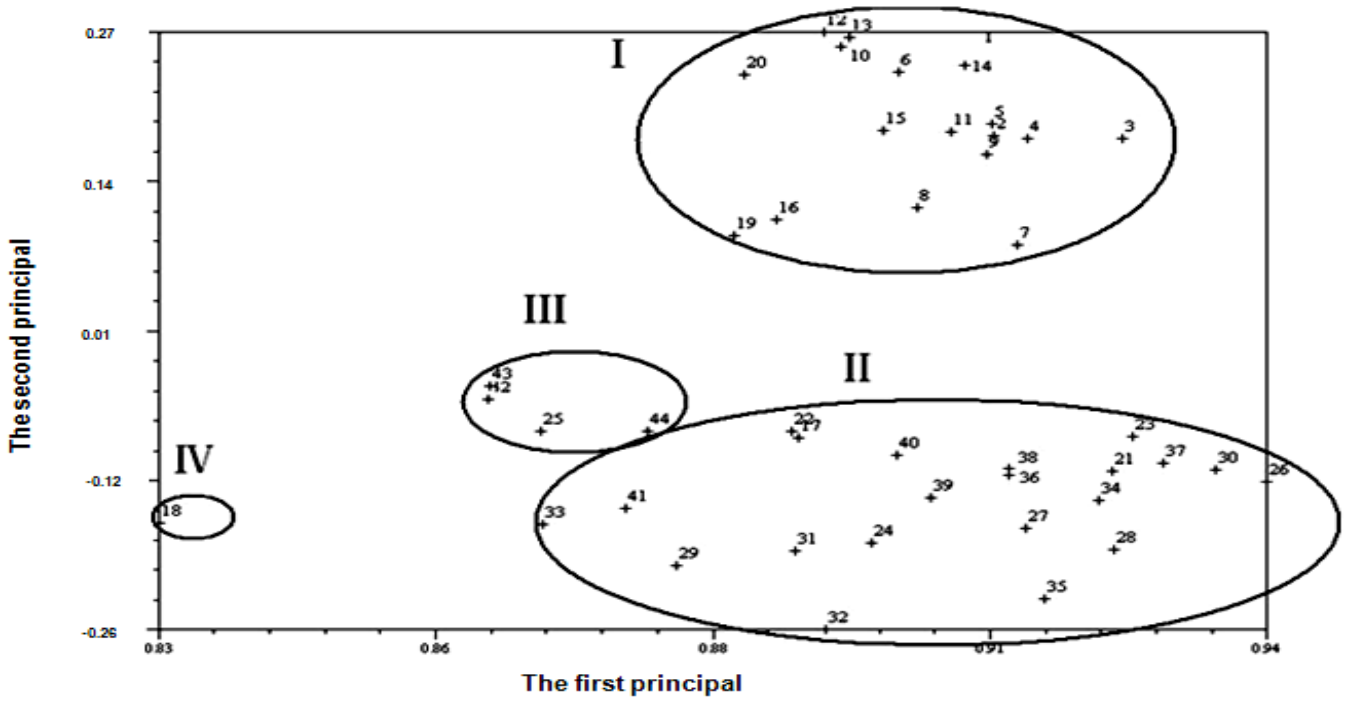

Figure 6. Principal component analysis based on ISSR patterns in cocksfoot.

and meadow fescue (64.6%). Moreover, the AFLP analysis conducted by Peng et al. (2006) suggested the rich genetic diversity of Chinese cocksfoot. High genetic diversity observed in this study using SRAP and ISSR markers is in agreement with the polymorphic nature and wide distribution of cocksfoot in temperate climates. This is confirmed by genetic diversity analysis of AFLP (Peng et al., 2006) too.

In general, the genetic variation analysis benefits the formation of core collection, since we provide genetically based evidence for each accession. Besides, the data are helpful to the establishment of breeding strategies for this germplasm. One of the major results from this study is that the genetic basis of three Chinese cultivars is rather narrow, based on the limited GS range. A reasonable explanation for this finding is that all the three registered cultivars are merely bred in Sichuan Province of China by domestication of wild material, coupled with very limited breeding methods. Additionally, the result suggests that the breeding status of cocksfoot could be developed in China. The rich genetic germplasm resources from different ecotypes origin and all kinds of breeding methods will be used efficiently.

Clustering analysis and PCA indicate that the clusters of the tested cocksfoot accessions are correlated with their geographical origin. This means that SRAP and ISSR analyses can be utilized to separate genotypes according to their clusters. Furthermore, SRAP and ISSR show the similar potential of discriminating the genetic diversity of cocksfoot.

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