Genes & Genomics (2012) 34: 51-57
DOI 10.1007/s13258-011-0102-5

RESEARCH ARTICLE

Development and use of novel SSR markers for molecular genetic diversity in Italian millet (*Setaria italica L.*)

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Received: 18 May 2011 / Accepted: 07 September 2011 / Published online: 31 January 2012

Abstract

Italian millet is a commercially important grain crop. Nineteen polymorphic simple sequence repeat (SSR) markers, developed through construction of an SSR-enriched library from genomic DNA of Italian millet (*Setaria italica* L., P. Beauv.), were used for assessment of molecular genetic diversity against 40 accessions of *S. italica*. In total, 85 alleles were detected, with an average of 4.5 alleles per locus. The average gene diversity and polymorphism information content (PIC) values were 0.412 and 0.376, ranging from 0.02 to 0.88 and from 0.02 to 0.87, respectively. Values for observed (H_O) and expected (H_E) heterozygosities ranged from 0 to 0.73 and from 0.03 to 0.89, respectively. Nine loci deviated from Hardy-Weinberg equilibrium. The mean similarity coefficient among accessions was 0.6593. Based on the UPGMA algorithm, six different groups were successfully identified. In this clustering analysis, all Korean accessions grouped in one cluster, indicating that Korean accessions are genetically quite distinct from other introduced accessions. These newly developed microsatellite markers should be very useful tools for several genetic studies, including an assessment of diversity and population structure in Italian millet.

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Keywords Genetic diversity; Heterozygosity; Italian millet; Simple sequence repeat (SSR) markers; *Setaria italica*.

Introduction

The genus *Setaria* belongs to the tribe Paniceae of the subfamily Panicoideae (family Poaceae) and comprises about 150 species widely distributed in warm and temperate parts of the world. *Setaria italica*, commonly known as foxtail or Italian millet, is an important grain crop in Asian countries for use as a staple food and, to a lesser extent, in Europe for animal feed. It is relatively drought-tolerant and well adapted to arid and semiarid growing conditions. The species is thought to have played an important role in early agriculture in the Old World (Sakamoto, 1987) and is therefore an interesting subject for investigations into the development and evolution of grass species (Doust et al., 2005). Genetic traits such as self-pollination, diploidy, and small genome size make it an ideal model plant for genetic and molecular biology research (Wang et al., 1998). However, in-depth research on Italian millet is scarce since it only became a minor cereal crop in the 1980s (Jia et al., 2009). Now, however, with the development of consumers' health consciousness, demand for Italian millet is increasing and the nutrient value and functionality of millet are being reevaluated.

In recent years, several studies based on various DNA markers have been conducted to address the genetic diversity of this crop (Wang et al., 1998; Li et al., 1998; Schontz and Rether, 1999; Le Thierry d'Ennequin et al., 2000; Fukunaga et al., 2002, 2005). Simple sequence repeats (SSRs) have recently emerged as a simple but powerful tool for genetic studies in a diverse group of plant taxa (Li et al., 2003). SSRs are reported to be more variable than RFLP or RAPD, and have been widely utilized in plant genomic studies (He et al., 2003).

However, widespread use of these markers is often limited by the time and cost involved in their development, which

requires DNA library construction, sequencing, identification of SSR-containing clones, and, finally, the design of primer pairs from flanking sequences of SSR motifs. The recent development of library enrichment techniques and automated sequencing has made production of these markers simple, rapid, and cost effective (Zane et al., 2002). There is little information available regarding isolation and development of SSR sequences from the genome of Italian millet or on the development of an SSR-based marker system for this crop (Jia et al., 2007, 2009). However, more SSR markers will be required to develop an estimate of genetic diversity and to analyse population structures in this species. Therefore, given the need for these markers, the present study reports on development and characterization of 19 novel microsatellite markers for analyze of genetic diversity and phylogenic relationships of Italian millet using the enrichment method.

Materials and Methods

Plant materials and DNA isolation

A world collection comprising 40 accessions of *Setaria italica* L. was used. This collection includes 13 accessions from South Korea, while the rest were collected from 6 different countries including China, India, Nepal, Russia, Thailand, and Uzbekistan (Table 1). All accessions were obtained from the Rural Development Administration (RDA) (http://genebank.rda.go.kr), Republic of Korea. The seeds of each accession were grown in a glass greenhouse, and DNA was extracted from the fresh leaves of 15-day-old seedlings using a QIAGEN DNA extraction kit (QIAGEN, Germany).

The relative purity and concentration of extracted DNA were estimated using NanoDrop ND-1000 (NanoDrop Technologies Inc., USA). The final concentration of each DNA sample was adjusted to 20 ng/μℓ.

Construction of an SSR-enriched library

A microsatellite-enriched library was constructed using a modified biotin-streptavidin capture method based on Dixit et al. (2005). Briefly, total genomic DNA of Italian millet was digested with seven restriction enzymes (*Eco*RV, *Dra*I, *Sma*I, *Pvu*I, *Alu*I, *Hae*III, and *Rsa*I) in separate reactions. After pooling, the digested sample was size-fractionated on a 1.4% agarose gel. Fragments ranging from 300 to 1500 bp were eluted from the gel followed by purification using a gel extraction kit (QIAGEN, Germany). DNA fragments (1 μg) were ligated with 1 μg of double-stranded adaptor molecules (AP11- 5'-CTCTTGCTTAGATCTGGACTA-3' and AP12- 5'-TAGTCCAGATCTAAGCAAGAGCACA-3'). The adaptor-ligated DNA was hybridized with a mixture of biotin-labeled SSR probes [$(GA)_{20}$, $(AC)_{20}$, $(AGC)_{15}$, $(GGC)_{15}$, $(AAG)_{15}$, $(AAC)_{15}$, $(AGG)_{15}$]. The hybridized DNA fragments were captured with streptavidin-coated magnetic beads (Promega, USA). After stringent washing, the 5 captured DNA fragments were eluted in 50 ml of distilled water. Final eluates were amplified with the AP11 primer by PCR (94ºC for 7 min; 94ºC for 30 sec, 53ºC for 30 sec, 72ºC for 1 min, 30 cycles; 72ºC for 10 min) and cloned into the pGEM-T Easy vector (Promega, USA).

Characteristics of the enriched library, primer design, and marker development

In total, 504 recombinant clones were randomly picked from primary transformation plates containing ampicillin, 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (XGal), and iso-

Table 1. Information on the 40 Italian millet accessions [*Setaria italica* (L.) P. Beauv.] used in this study.

No.	Code	No. in genebank	Origin
1	1_CHN	153461	China
2	2_CHN	153464	China
3	3_CHN	153466	China
4	4_CHN	153468	China
5	5_CHN	153471	China
6	6_IND	123875	India
7	7_IND	123878	India
8	8_IND	123881	India
9	9_IND	123885	India
10	10_IND	123888	India
11	11_KOR	K011497	Goseong city, Republic of Korea
12	12_KOR	185907	Goseong city, Republic of Korea
13	13_KOR	105772	Myeongju city, Republic of Korea
14	14_KOR	185899	Myeongju city, Republic of Korea
15	15_KOR	K011787	Samcheok city, Republic of Korea
16	16_KOR	123801	Samcheok city, Republic of Korea
17	17_KOR	185929	Samcheok city, Republic of Korea
18	18_KOR	K011718	Yanggu city, Republic of Korea
19	19_KOR	K011719	Yanggu city, Republic of Korea
20	20_KOR	185903	Yangyang city, Republic of Korea
21	21_KOR	185946	Yangyang city, Republic of Korea
22	22_KOR	900073	Yeongwol city, Republic of Korea
23	23_KOR	185896	Wonju city, Republic of Korea
24	24_NPL	109442	Nepal
25	25_NPL	109443	Nepal
26	26_NPL	109445	Nepal
27	27_NPL	109446	Nepal
28	28_NPL	109449	Nepal
29	29_RUS	153487	Russia
30	30_RUS	153488	Russia
31	31_RUS	153491	Russia
32	32_RUS	153493	Russia
33	33_RUS	153496	Russia
34	34_THA	137683	Thailand
35	35_THA	137690	Thailand
36	36_THA	137693	Thailand
37	37_THA	137695	Thailand
38	38_THA	137699	Thailand
39	39_UZB	K015667	Uzbekistan
40	40_UZB	214844	Uzbekistan

propyl-beta-D-thiogalactopyranoside (IPTG). Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and sequenced using an ABI 3100 DNA sequencer (Applied Biosystems, USA). SSR identification within cloned sequences and primer design were carried out using the SSR MANAGER program (Kim, 2004). Of 504 sequenced clones, 23 (4.6%) were redundant. Of the remaining 481 unique clones, 163 (33.89%) were found to harbor microsatellite sequences. A total of 145 primer pairs were designed and evaluated for polymorphism in a panel of ten accessions using the procedure described by Dixit et al. (2005). Nineteen primer pairs gave reproducible polymorphic bands and were further characterized using a diverse set of 20 accessions. The M13-tailPCR method of Schuelke (2000) was used to measure the size of the PCR products. The method involves three primers; the forward SSR-specific primer with the M13 (18 bp) tail at the 5' end, the reverse SSR-specific primer, and a fluorescent dye-labeled (FAM, HEX, or NED) M13 (18 bp) universal primer. The amount of forward primer was adjusted to less than half of the reverse primer. Microsatellite alleles were resolved on an ABI PRISM 3100 DNA sequencer (Applied Biosystems,USA) using GENESCAN 3.7 software and sized precisely using GeneScan 500 ROX (6-carbon-X-rhodamine) molecular size standards with GENOTYPER 3.7 software (Applied Biosystems, USA).

Statistical analysis

The variability at each locus was measured in terms of number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E), genetic distance between each pair of accessions, and Hardy-Weinberg equilibrium (HWE) using the genetic analysis package POPGENE Version 1.31 (Yeh et al., 1999). Major allele frequency (MAF), gene diversity (GD), and polymorphic information content (PIC) were measured by calculating the shared allele frequencies using PowerMarker V3.23 (Liu and Muse, 2005). The UPGMA algorithm was used to construct an unrooted phylogram from a distance matrix using MEGA4 software (Tamura et al., 2007).

Results

SSR development

A library enriched for several di- and tri-nucleotide SSRs was constructed from the genomic DNA of Italian millet. Developmental steps for the construction of the enriched library and its characteristic features are summarized in Table 2. A total of 504 putative recombinant clones from the enriched library were sequenced and analyzed for the presence of SSRs. Twenty-three clones (4.6%) were redundant. Of the remaining 481 unique clones (95.4%), 163 clones (33.9%) were found to harbor SSR sequences.

Sequence analysis of all SSR-containing clones revealed a predominance of trinucleotide SSRs (69.2%) over di-nucleotide SSRs (20%). Additionally, a few (10.8%) tetra/ penta/ hexa nucleotide SSRs were identified in the library (Table 3). Among the di-nucleotide SSRs, the AG/GA class of repeat motif was the most frequently identified (69.2% of the total di-nucleotide SSRs), followed by the AC/CA class (23%). Among the tri-nucleotide SSRs, the AGC/GCA/CAG class of repeat motifs was predominant (28.2%), followed by AGG/GGA/GAG (22.2%) and CCG/CGC/GCC (21.5%; Table 3).

In total, 143 primer pairs could be designed from flanking sequences of different repeat motifs and screened for polymorphism. Newly designed SSR markers were tested for polymorphisms in a panel of four Italian millet accessions using the procedure described by Dixit et al. (2005). As a result, only 19 primers produced very distinct and reproducible polymorphic bands in the germplasm analyzed, and were further characterized using a set of 40 accessions from seven different countries. The 19 newly developed SSRs, including sequences of the primer sets, repeat motifs, annealing temperatures, and

Table 2. Summary of SSR-enriched library screening of Italian millet [*Setaria italica* (L.) P. Beauv.].

Screening steps	Number (Percentage)
Sequenced clones	504
Redundant clones	23 (4.6%)
Unique clones	481 (95.4%)
SSR clones	163 (33.9%)
Polymorphic markers	19

Table 3. Characteristics of the SSR sequences identified from the enriched library of Italian millet [*Setaria italica* (L.) P. Beauv.].

Type of SSR	Repeat class	Number	(%)
Di-nucleotide			
	AC/CA	9	23.1
	AG/GA	27	69.2
	AT/TA	1	2.6
	GC/CG	2	5.1
Total		39 (20.0%)	
Tri-nucleotide			
	AAC/ACA/CAA	9	6.7
	AAG/AGA/GAA	3	2.2
	AAT/ATA/TAA	0	0.00
	ACC/CCA/CAC	12	8.9
	ACG/CGA/GAC	11	8.2
	ACT/CTA/TAC	0	0.0
	AGC/GCA/CAG	38	28.2
	AGG/GGA/GAG	30	22.2
	ATC/TCA/CAT	3	2.2
	CCG/CGC/GCC	29	21.5
Total		135 (69.2%)	
Others (Tetra/Penta/Hexa)		21 (10.8%)	
Total Repeat Motifs		195	

Table 4. Characteristics of 19 microsatellite loci developed from an enriched library of Italian millet [*Setaria italica* (L.) P. Beauv.].

Marker	GenBank accession nos.	Primer sequence		Repeat motif	T_A (°C)	Size range (bp)	M_{AF}	N_A	H_O	H_E	GD	PIC
		Forward	Reverse									
GB-SIM-002	EF117793	CCCATGGACAGAGTTGC	GACCAGAGCTTGCTGCAC	$(CTC)_7$	55	273-276	0.95	2	0.00	0.10	0.10	0.09
GB-SIM-012*	EF117794	CGCTAGCCATGTAGGCAG	AATCCTCGCGTCATCTCA	$(CT)_{20}$	55	216-254	0.23	12	0.18	0.89	0.88	0.87
GB-SIM-018	EF117795	TTGCCCGGCATCATC	CAGCAGCCCTCGAACTTA	$(GGC)_3$-(GGA)-$(GGC)_2$	58	218-236	0.75	4	0.50	0.41	0.41	0.38
GB-SIM-030*	EF117796	AGCAAAACCCAGTCAGCA	GCAACGGCAACAAGGTTA	$(AG)_6$, $(GCCA)_4$, $(GA)_{18}$	55	293-353	0.51	10	0.03	0.71	0.70	0.68
GB-SIM-039*	EF117797	GCTGCGTGCCTAGGAAAT	GCATTGCCAGCGTAAAAG	$(CCG)_7$	55	179-218	0.56	5	0.03	0.59	0.58	0.52
GB-SIM-043	EF117798	TGCTTGCAGAGGAGAAGC	CATGCTCGAGACACCCTC	$(TGG)_5$	55	249-261	0.61	2	0.73	0.48	0.47	0.36
GB-SIM-044	EF117799	TTTGGTTCTGCTGCTGCT	GCTCTACCACCCTGTCCC	$(GA)_7$	55	237-241	0.99	2	0.03	0.03	0.02	0.02
GB-SIM-046*	EF117800	CTACGATCGGATTGCGAG	CGCCACTTTATTCTCCCC	$(CTG)_3$-(G)-$(CTG)_4$	55	160-175	0.78	3	0.05	0.36	0.35	0.30
GB-SIM-051	EF117801	TCCTCTACTTCGACGGCA	AGGCGAGTGAGGGGTCTA	$(AGC)_5$	55	215-227	0.53	4	0.35	0.63	0.62	0.56
GB-SIM-054	EF117802	AGGCTGGGAGACAGGAAG	GCAGGTGAGATGTTTGGC	$(GCT)_6$, $(CTG)_4$, $(CTG)_5$	55	301-334	0.78	4	0.03	0.37	0.36	0.32
GB-SIM-066*	EF117803	AGAATAGCCTTGAGCCCG	TTACCAAATGGGTCGTGG	$(TC)_2$-(TT)-$(TC)_4$	55	118-176	0.86	2	0.03	0.24	0.24	0.21
GB-SIM-068	EF117804	TACGAGACACCCCCTCCT	GTCCATCTCTCGCGTCTG	$(GGGA)_3$	55	192-252	0.99	2	0.03	0.03	0.02	0.02
GB-SIM-069	EF117805	GTGGGCTACCGGACTTTC	CCACCCCATACTTAGGACG	$(ACC)_3$-(AAC)-$(ACC)_2$	55	266-284	0.63	4	0.70	0.52	0.52	0.44
GB-SIM-077*	EF117806	TCACGGGATTACCACTCG	TACCTTGGAGGTGTTGCG	$(GA)_5$-$(AGC)_5$-$(AGCA)_4$	55	91-272	0.56	5	0.53	0.63	0.63	0.59
GB-SIM-105	EF117807	TCCACTGCACCCATCATT	GTACCCCGAAAAAGGACG	$(TGG)_4$-TGT-$(TGG)_4$	55	146-176	0.73	3	0.55	0.41	0.41	0.33
GB-SIM-113*	EF117808	TCACCCCAAATTCAGCAC	ACTGCTGTGACGGACACC	$(TG)_{16}$	55	264-302	0.44	8	0.05	0.74	0.73	0.70
GB-SIM-117*	EF117809	CCCAATGCAAAAGATCCA	TAGGCTGTTAGGTCGCCA	$(CTC)_4$-CG-$(CT)_6$-CA-$(CT)_{11}$	55	261-323	0.66	7	0.60	0.53	0.53	0.50
GB-SIM-130*	EF117810	GCGTACGAAAAATGTCGC	CTTTCGTCGGATCTGCTG	$(TG)_8$-$(GTGG)_5$	55	210-248	0.89	4	0.10	0.21	0.21	0.20
GB-SIM-135	EF117811	AGCTCCGGCTGGAGTAAC	CGGAAGGATGGAACAGTG	$(GCA)_4$, $(CCT)_4$	55	233-296	0.98	2	0.05	0.05	0.05	0.05
Mean							0.705	4.5	0.238	0.417	0.412	0.376

*Loci deviated from the Hardy-Weinberg equilibrium (HWE); T_A, annealing temperature; M_{AF}, major allele frequency; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; GD, gene diversity; PIC, polymorphic information content.

fluorescent dyes are given in Table 4.

SSR polymorphism

The variability at each SSR locus was measured in terms of numbers of alleles and expected heterozygosity. A total of 85 alleles, ranging from two (GB-SIM-002, GBSIM-043, GB-SIM-044, GB-SIM-066, GB-SIM-068, and GB-SIM-135) to 12 (GBSIM-012), were observed among 40 Italian millet accessions at 19 microsatellite loci with an average of 4.5 alleles per locus. The database of allelic frequencies showed that rare alleles (with a frequency < 0.05) comprised 32.9% of all alleles, while intermediate (0.05 < frequency < 0.50) and abundant alleles (frequency > 0.50) comprised 47.1% and 20.0% of all detected alleles, respectively. Major allele frequency varied from 0.23 (GBSIM-012) to 0.99 (GB-SIM-068) (Table 4). The average gene diversity and PIC values were 0.412 and 0.376, ranging from 0.02 (GB-SIM-044 and GB-SIM-068) to 0.88 (GBSIM-012) and from 0.02 (GB-SIM-044 and GB-SIM-068) to 0.87 (GB-SIM-012), respectively. The values for H_O ranged from 0.00 at GB-SIM-002 to 0.73 at GB-SIM-043 with an average of 0.238, whereas those of H_E ranged from 0.03 at GB-SIM-044 and GB-SIM-068 to 0.89 at GB-SIM-012 with an average of 0.417 for all loci studied (Table 4). We tested for HWE at a significance level of $P < 0.05$ after applying a Bonferroni correction/adjustment for multiple comparisons. Nine loci deviated from HWE ($P < 0.05$) (Table 4).

Genetic diversity and cluster analysis

To evaluate the usefulness of these newly developed SSRs for the study of genetic variations and phylogenetic relationships among Italian millet accessions, a genetic distance-based analysis was performed by calculating the shared allele frequencies among the 40 accessions, and an unrooted phylogram (Fig. 1) was computed using MEGA 4 (Tamura et al., 2007). The similarity coefficient among accessions ranged from 0.3908 to 1.0000, with an average value of 0.6593. The wide variation in genetic similarity among the different Italian millet accessions revealed by SSRs reflected a high level of polymorphism at the DNA level. In the phylogram, all Italian millet accessions except for three (5 CHN, 27 NPL, and 39 UZB) clustered into six groups.

Discussion

SSRs have become one of the most widely used molecular markers for diversity studies, linkage map construction, and marker-assisted selection. Traditionally, SSR loci have been isolated by screening several thousand clones using colony hybridization with repeat-containing probes. Although relatively simple, this approach can be rather laborious and inefficient for plant species with low SSR frequencies (Zane et al., 2002), while the enrichment procedure is faster and less costly than library screening. The work described in this paper involved

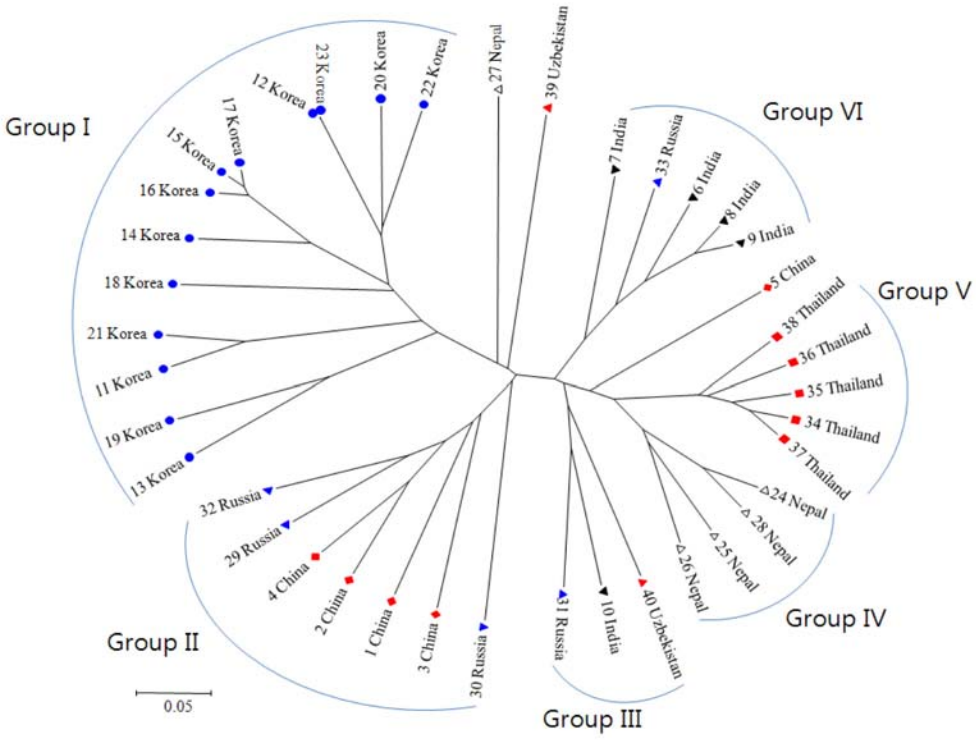

Figure 1. Unrooted UPGMA tree of 40 Italian millet accessions using shared allele distances among 19 microsatellite loci.

the development of 19 polymorphic SSR markers from an enriched library of genomic DNA of Italian millet. Compared to other plants with enrichment efficiencies between 50 and 90% (Butcher et al., 2000), the efficacy of development of SSRs in Italian millet was lower (33.9%), but similar to some studies of other species, e.g., zoysia grasses, that used the same method (Ma et al., 2007). Jia et al. (2009) established an SSR enrichment procedure using the anchored PCR program for foxtail millet, where enrichment levels reached 87.0 and 80.5%. However, only 28.9% and 33.8% of the microsatellite-containing clones from these libraries were suitable for primer design and the others were mainly redundant sequences. The differences may be attributed to genome differences, the size range of the insert, the restriction enzyme used for genomic DNA library construction, and the approach used for SSR enrichment (Gupta and Varshney, 2000).

The different enrichment ratios probably reflect the microsatellite repeat type representation in plant genomes. In plants, the most frequently occurring dinucleotide repeat is TA. However, this type of repeat is not suitable for hybridization because of its autocomplementary feature. In this study, it was found that the predominance of tri-nucleotide SSRs (69.23%) over di-nucleotide SSRs (20%) in the enriched library of Italian millet is a common feature of plant genomes as reported in several recent surveys on the distribution patterns of SSRs in the genomic sequences of various plant species including *Arabidopsis*, *Medicago*, *Lotus*, and *Glycine* (Morgante et al., 2002; Mun et al., 2006). Among the dinucleotiderepeats, both GA and CA repeats appear to be highly abundant, but the frequency of AG/GA repeats was higher than that of the AC/CA repeat type in the Italian millet genome, which is not consistent with a previous report (Jia et al., 2009). The occurrence of the AG/GA class of repeat motif among the di-nucleotide repeats was however consistent with reports from other plant species (Powell et al., 1996). This may be due to the different enrichment methods; the method used in this paper may be more effective for screening of AG/GA than AC/CA repeat types. Among the tri-nucleotide repeats, the AGC/GCA/CAG class was the most common, followed by the AGG/GGA/GAG and CCG/CGC/GCC classes in the Italian millet library. This finding is in contrast to the reported predominance of the CCG/CGC/GCC class as a characteristic feature of cereal genomes (La Rota et al., 2005).

All of the newly developed SSR loci were polymorphic and an average of 4.5 alleles were detected per locus, which is lower than the number reported in Jia et al. (6.2 alleles per locus) (2009), but higher than the number of SSR markers derived from EST sequences (2.5 alleles per locus) (Jia et al., 2007). Some markers showed a large difference between observed and expected heterozygosity. The result might be related to the prevalence of self pollination in Italian millet. In addition, the degree of variation in terms of genetic diversity indices was correlated to some extent with the number of repeats in the SSR loci, as well as the number of alleles.

Correlation analysis revealed that there were very significant positive correlations between PIC and number of repeat units and between PIC and number of alleles per locus, with correlation coefficients of 0.609 and 0.866 ($P < 0.01$), respectively. Moreover, a significant correlation between number of repeat units and number of alleles was also found, with a correlation coefficient of 0.460 ($P < 0.05$). The same results were obtained by Jia et al. (2009).

The SSRs revealed considerable genetic diversity in the 40 Italian millet accessions of diverse origin, with similarity coefficients ranging from 0.3908 to 1.0000, and an average value of 0.6593. The high level of genetic variation observed in this study is consistent with results from previous studies on Italian millet using different molecular markers (Wang et al., 1998; Jia et al., 2009; Li et al., 1998; Schontz and Rether, 1999; Le Thierry d'Ennequin et al., 2000; Fukunaga et al., 2002, 2005), thereby confirming the great diversity in Italian millet accessions. A UPGMA dendrogram inferred from shared allele frequencies among Italian millet accessions clustered 40 accessions into six groups. All Korean Italian millet accessions segregated independently of all other introduced accessions and formed group I, having a remote relationship with other accessions. Group II comprised accessions from Russia and China. Group III consisted of accessions from Russia, India, and Uzbekistan. All accessions from Nepal and Thailand formed groups IV and V, respectively. Group VI included accessions from Russia and India. Three independent accessions (5 CHN, 27 NPL, and 39 UZB) were identified as ungrouped as they shared a genetic background with all six different groups. Cluster analysis revealed that major clusters were moderately related to geographic location.

This workachieved the primary isolation and characterization of Italian millet SSRs. The 19 newly developed SSRs, rich in polymorphisms, should be useful for identification of Italian millet germplasm, core set construction, crop improvement, and assessment of genetic diversity in the future.

Acknowledgments This study was supported by the BioGreen 21 project (#20080401034058) of the Rural Development Administration (RDA), Republic of Korea.

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