

Fingerprinting and identification of closely related wheat (*Triticum aestivum* L.) cultivars using ISSR and fluorescence-labeled TP-M13-SSR markers

Yanfang Zhu, Jin Hu*, Rui Han, Yang Wang, Shuijin Zhu*

Seed Science Center, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, China

*Corresponding author: jhu@zju.edu.cn; shjzhu@zju.edu.cn**Abstract**

DNA based molecular markers allow precise, objective and rapid cultivar identification. In this study, five ISSR and two sets of fluorescence-labeled TP-M13-SSR markers were applied for discrimination of eight closely related wheat cultivars. Five ISSR primers revealed a total of 43 distinct reproducible bands, 29 (67.44%) of the 43 bands were polymorphic. The number of polymorphic bands detected by each ISSR primer ranged from 3 to 8 with an average of 4.8 per primer. One ISSR primer UBC 849 was able to identify all the eight wheat cultivars. The two sets of fluorescence-labeled TP-M13-SSR markers produced a total of 29 alleles with an average of 14.5 per locus in the eight wheat cultivars. All of the 29 alleles were polymorphic, and the numbers and sizes of alleles revealed were used for cultivar discrimination. Combination of the two sets of fluorescence-labeled TP-M13-SSR primers could distinguish all the eight closely related wheat cultivars. Finally, two fluorescence-labeled TP-M13-SSR markers successfully fingerprinted the eight closely related wheat cultivars. The result suggested that fluorescence-labeled TP-M13-SSR markers could be used as a potential and valuable method for fingerprinting and identification of wheat cultivars, and fluorescence-labeled TP-M13-SSR markers should be given priority in fingerprinting and identification of wheat cultivars compared with ISSR for its high-throughput and high accuracy. DNA fingerprinting of wheat cultivars can be expanded by additional cultivars and markers, and the data obtained can be used for cultivar protection.

Keywords: Cultivar identification; DNA Fingerprinting; ISSR; TP-M13-SSR; Wheat.**Abbreviation:** CTAB-Hexadecyl trimethyl ammonium Bromide; FAM-6-carboxy-fluorescein; HEX- hexachloro-6-carboxy-fluorescein; ISSR-inter-simple sequence repeat; SSR-simple sequence repeat; TET- tetrachloro-6-carboxy-fluorescein; TP-M13-SSR-PCR performed with three primers, a sequence-specific forward primer with universal primer M13 tail at its 5' end, a sequence-specific reverse primer, and the fluorescence-labeled universal primer M13; RFU- relative fluorescence unit; ROX-6-carboxy-X-fluorescein.**Introduction**

Wheat (*Triticum aestivum* L.) is one of the most important crops in China. High-quality and elite cultivars play a crucial role in wheat production. Because of the limited parent germplasm resources in breeding, the number of similar or related cultivars is growing rapidly, which lead to seriously cultivar mix in seed market. With the number of new similar or closely related wheat cultivars increasing yearly, rapid, unambiguous and economic cultivar identification is essential for cultivar registration, certification, and breeder's right protection. Fingerprinting with DNA-based molecular markers, allows precise, objective and rapid cultivar identification compared with field plot growth test and isozyme electrophoresis. Inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) are two popular molecular markers in plant cultivar identification (Crespel et al., 2009; Lu et al., 2009). ISSR markers detect polymorphisms in inter-microsatellite loci using a single primer composed of a simple sequence repeat (SSR) sequence anchored at the 3' or 5' end by 2 – 4 arbitrary nucleotides. ISSR markers have the advantages of relatively low cost, high polymorphism and good reproducibility. What's more, it is not required to know DNA sequence for ISSR primer design. ISSR markers have been used for cultivar identification of wheat (Nagaoka and Ogiyama, 1997), radish (Liu et al., 2008), lotus (Chen et al., 2008), Indian

bitter melon (Behera et al., 2008), rose (Crespel et al., 2009) and so on. SSR markers, DNA fragments containing tandem repeats of a short sequence (1–6 nucleotides) and detection of polymorphisms in microsatellite loci. SSR markers have the advantages of abundance, co-dominance, high polymorphism, and ease of assay by polymerase chain reaction (PCR) (Kuleung et al., 2004). SSR markers have been applied for cultivar identification of pear (Kimura et al., 2002), potato (Coombs et al., 2004), rice (Rahman et al., 2009), wheat (Fujita, 2009), rape (Louarn et al., 2007), soybean (Tantasawat et al., 2011) and so on. To analyze the exact length of SSR fragments on a laser detection system, one of the SSR primers has to carry a fluorescent dye label, which may be 6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), hexachloro-6-carboxy-fluorescein (HEX) or 6-carboxy-X-fluorescein (ROX) in conventional fluorescence-labeling method which is high cost. In order to overcome this financial burden, Schuelke (2000) put forward and demonstrated fluorescence-labeled TP-M13-SSR PCR method. In this method, PCR was performed with three primers, a sequence-specific forward primer with universal primer M13 tail at its 5' end, a sequence-specific reverse primer, and the fluorescence-labeled universal primer M13. Fluorescence-labeled TP-M13-SSR markers have the advantages of high-throughput and high accu-

Table 1. Eight wheat cultivars used in the study

No.	Cultivar Name	pedigree
1	Yangmai 158	Yang mai 4/ST1472/506
2	Yangmai 12	Yangmai 158/3/TP114/Yangmai 5//85-853
3	Yangmai 5	Yangjian 2/(ST1472/506)
4	Yangmai 11	Yangmai 158/3/Y.C/Yangjian 2//85-85
5	Yangmai 10	85-85//Y.C/Yangjian 21///Yangmai 158
6	Yangmai 3	Yangmai 158/1-9012
7	Yangmai 15	89-40/Chuanyu 21526
8	Yangmai 17	92F101/Chuanyu 21526

Table 2. Sequences of ISSR primers used in the study

Primer name	Primer Sequence
UBC811	GAGAGAGAGAGAGAGAC
UBC819	GTGTGTGTGTGTGTGTA
UBC846	CACACACACACACACART
UBC849	GTGTGTGTGTGTGTGYA
UBC856	ACACACACACACACACYA
UBC857	ACACACACACACACACYG

Table 3. Sequences of SSR primers used in the study

Primer name	Forward Primer	Reverse Primer
<i>Xgwm174</i>	GGGTCCTATCTGGTAAATCCC	GACACACATGTCCTGCCAC
<i>Xgwm46</i>	GCACGTGAATGGATTGGAC	TGACCCAATAGTGGTGGTCA

racy, which have been successfully used for genotype identification of *Sorghum* and rice (*Oryza sativa* L.) (Li et al., 2005). However, there is no report on fingerprinting and cultivar identification of wheat by fluorescence-labeled TP-M13-SSR markers. The eight wheat cultivars used in this study are a series of closely related wheat cultivars with similar morphological characteristics. The objectives of the study were to: (1) determine whether fluorescence-labeled TP-M13-SSR markers can be used to fingerprint and distinguish closely related wheat cultivars; (2) fingerprint and identify the eight closely related wheat cultivars; (3) compare the two different marker systems in fingerprinting and identification of closely related wheat cultivars.

Results

ISSR analysis and cultivar identification

Six ISSR primers were screened against the eight closely related wheat cultivars, and all of them were polymorphic. The six polymorphic primers generated a total of 43 distinct reproducible bands with an average of 7.2 bands per primer. The sizes of amplified fragments ranged from 300 bp to 2000 bp. 29 (67.44%) of the 43 bands produced were polymorphic. The number of polymorphic bands detected by each ISSR primer ranged from 3 (by primer UBC811) to 8 (by primer UBC819) with an average of 4.8 per primer. Primer UBC849 could produce up to eight types of banding pattern and distinguish all the eight wheat cultivars (Fig. 1). Three sets of primer combination, (UBC819-UBC846), (UBC819-UBC856) and (UBC819-UBC857), were also able to distinguish the eight closely related wheat cultivars, respectively.

Fluorescence-labeled TP-M13-SSR analysis and cultivar identification

In the analysis, all the eight wheat cultivars were successfully amplified with the two sets of fluorescence-labeled TP-M13-SSR primers (TP-M13-*Xgwm*-

m46 and TP-M13-*Xgwm174*). TP-M13-*Xgwm 46* and TP-M13-*Xgwm174* were both polymorphic, and produced a total of 29 alleles with an average of 14.5 per locus in the eight wheat cultivars. TP-M13-*Xgwm46* produced 18 alleles, and the sizes of the alleles ranged from 165 bp to 201 bp (Fig. 2). TP-M13-*Xgwm174* revealed 11 alleles, and the sizes of the alleles ranged from 197 bp to 240 bp (Fig. 3). The numbers and sizes of the alleles revealed by TP-M13-*Xgwm46* and TP-M13-*Xgwm174* were used for identification of the eight wheat cultivars. Combination of the two sets of fluorescence-labeled TP-M13-SSR primers could distinguish all the eight wheat cultivars. The eight wheat cultivars were fingerprinted by TP-M13-*Xgwm46* (Table 4) and TP-M13-*Xgwm174* (Table 5).

Discussion

DNA fingerprinting and cultivar identification by fluorescence-labeled TP-M13-SSR markers

In order to utilize DNA-based molecular markers for cultivar identification of wheat in practice, it is essential to find rapid, cost-effective and high accurate marker systems. This investigation was a successful attempt to use fluorescence-labeled TP-M13-SSR markers for fingerprinting and identification of closely related wheat cultivars. It was reported that fluorescence-labeled TP-M13-SSR markers might not be suitable for genotype identification of varieties with large genome in previous study (Li et al., 2005). However, the result of this study suggested that fluorescence-labeled TP-M13-SSR markers could be applied for fingerprinting and cultivar identification of wheat with a large genome. It was indicated that fluorescence-labeled TP-M13-SSR marker system might be a potential and valuable method for fingerprinting and identification of closely related or low genetic diversity germplasm in further study because of its high-throughput, high sensitiveness and accuracy. DNA fingerprinting of wheat cultivars studied

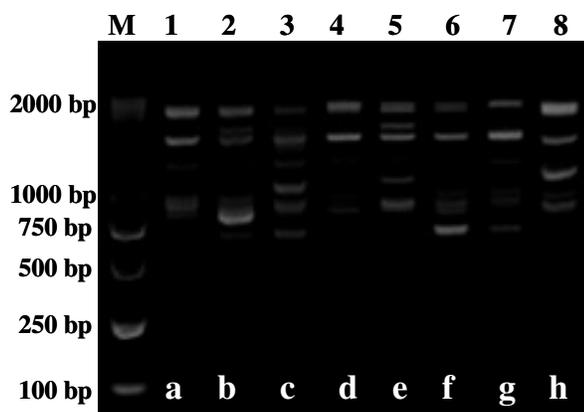


Fig 1. Amplification profile of ISSR primer UBC 849 from 8 wheat cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 8 correspond to the wheat cultivars listed in Table 1. The letters a ~ h represent the different types of banding pattern.

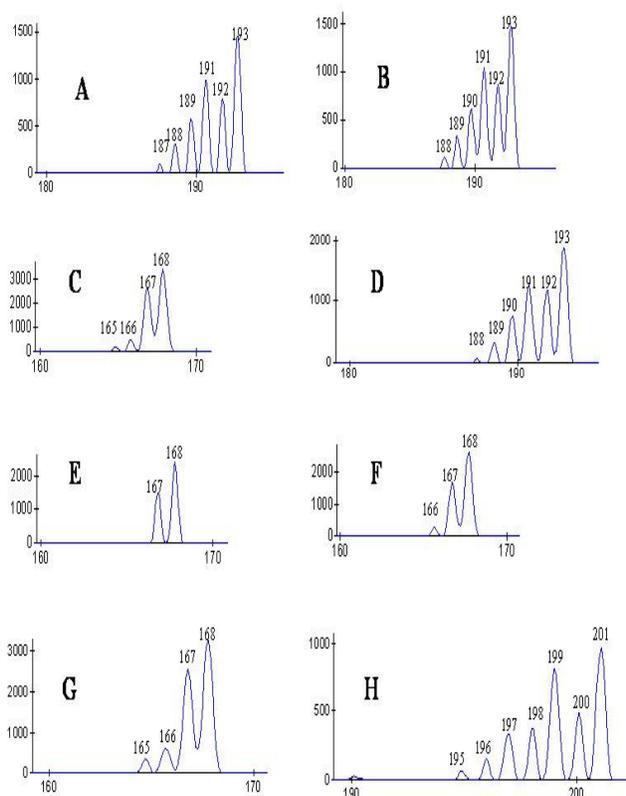


Fig 2. Amplification profile of TP-M13-Xgwm46 from 8 wheat cultivars detected using Mega BACE 1000 DNA Sequencer (Pharmacia, USA). X axis represents DNA molecular mass marker in base pairs (bp), Y axis represents RFU (relative fluorescence unit). The capital letters A-H correspond to the wheat cultivars 1~8 listed in Table 1. The peaks correspond to loci detected from 8 wheat cultivars using Mega BACE 1000 DNA Sequencer. The number marked on the top of each peak is the nucleotide size of corresponding PCR fragment.

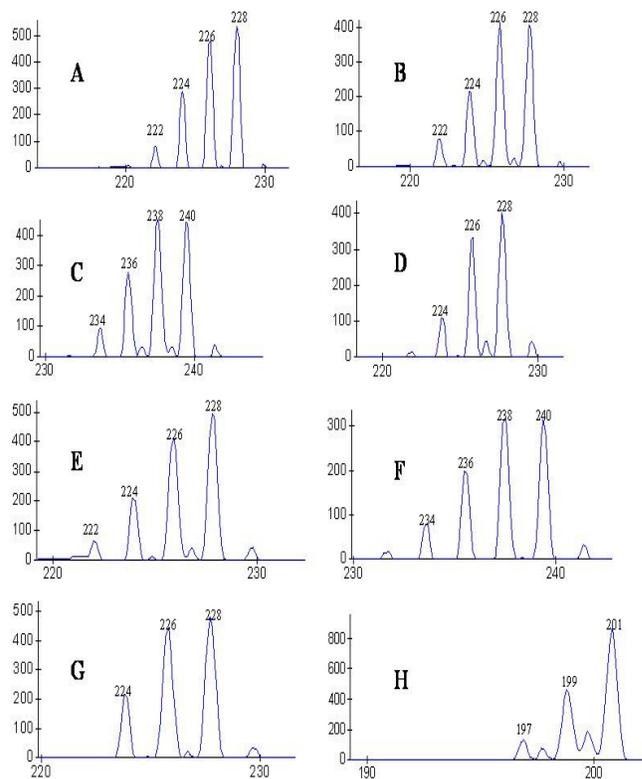


Fig 3. Amplification profile of TP-M13-Xgwm174 from 8 wheat cultivars detected using Mega BACE 1000 DNA Sequencer (Pharmacia, USA). X axis represents DNA molecular mass marker in base pairs (bp), Y axis represents RFU (relative fluorescence unit). The capital letters A-H correspond to the wheat cultivars 1~8 listed in Table 1. The peaks correspond to loci detected from 8 wheat cultivars using Mega BACE 1000 DNA Sequencer. The number marked on the top of each peak is the nucleotide size of corresponding PCR fragment.

in this research can be expanded as the number of additional cultivars and markers increasing, and the data obtained can be used for germplasm identification and cultivar protection in practice.

Comparison of ISSR and fluorescence-labeled TP-M13-SSR markers

In this study, ISSR and fluorescence-labeled TP-M13-SSR markers were both able to successfully distinguish the eight closely related wheat cultivars. With the advantages of rapid detection and high polymorphism, the two marker systems might be both fit for cultivar identification of wheat in practice. Compared with ISSR, fluorescence-labeled TP-M13-SSR marker system can detect more polymorphic loci and identify smaller difference with 1~2 bp in length among the fragments. The high detection ability to identify small difference is of great importance for accurate cultivar identification. However, the analysis technique of fluorescence-labeled TP-M13-SSR marker is much more expensive. Considering balancing, advantages and disadvantages of the two marker systems, it is suggested that fluorescence-labeled TP-M13-SSR marker system can be chosen when there are a large number of samples, otherwise

Table 4. Analysis of loci produced from primer pair *Xgwm46*

No	Cultivars	Bands positions due to primer TP-M13- <i>Xgwm46</i> (bp)																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Yangmai 158					187	188	189		191	192	193							
2	Yangmai 12						188	189	190	191	192	193							
3	Yangmai 5	165	166	167	168														
4	Yangmai 11						188	189	190	191	192	193							
5	Yangmai 10			167	168														
6	Yangmai 3		166	167	168														
7	Yangmai 15	165	166	167	168														
8	Yangmai 17												195	196	197	198	199	200	201

Table 5. Analysis of loci produced from primer pair *Xgwm174*

No	Cultivars	Bands positions due to primer TP-M13- <i>Xgwm174</i> (bp)										
		1	2	3	4	5	6	7	8	9	10	11
1	Yangmai 158				222	224	226	228				
2	Yangmai 12				222	224	226	228				
3	Yangmai 5								234	236	238	240
4	Yangmai 11					224	226	228				
5	Yangmai 10				222	224	226	228				
6	Yangmai 3								234	236	238	240
7	Yangmai 15					224	226	228				
8	Yangmai 17	197	199	201								

ISSR marker system can be given priority to identify wheat cultivars in practice. The fluorescence-labeled TP-M13-SSR marker system may be a better choice for fingerprinting for its high accuracy. In order to make fluorescence-labeled TP-M13-SSR marker system more available in cultivar identification and fingerprinting in practice, it is essential to reduce the cost of the analysis method. Finding new and inexpensive alternative fluorescent dye labels may be the best and critical way to overcome the financial burden in further study.

Materials and methods

Materials

Seeds of eight closely related wheat cultivars (Table 1) were provided by Nanjing Agricultural University, China.

DNA extraction

Genomic DNA was extracted from young leaves using the modified CTAB method (Zhu et al., 2010). DNA was dissolved and diluted to a final concentration of 20 ng/μL with 1×TE buffer (10 mM/L Tris-HCl, pH 8.0; 1 mM/L EDTA) and stored at -20 °C for further use.

ISSR analysis

Six ISSR primers (Table 2) were used in the study. ISSR polymerase chain reaction (PCR) amplification was conducted in a 20 μL volume containing 50 ng of genomic DNA, 1 U *Taq* DNA polymerase, 1.5 mM Mg²⁺, 0.25 mM dNTPs, and 0.2 μM primer. The PCR protocol consisted of an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 45 s, annealing at 52°C for 45 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. All PCR reactions were carried out in a thermal cycler C1000 (Bio-Rad, USA). PCR products were separated on 1.5% agarose gels, stained with GelRed™ (Biotium, USA) and photographed under UV light using Image Lab™ software Version 2.0.1 (Bio-Rad, USA).

Fluorescence-labeled TP-M13-SSR analysis

Two previously developed SSR primer pairs (Table 3) were applied in this analysis. Primer labeling method was following Li et al. (2005), and the universal primer M13 was labeled with fluorescence dye 6-carboxyfluorescein (FAM) at its 5' head. TP-M13-SSR polymerase chain reaction (PCR) amplification was conducted in a 20 μL volume containing 60 ng of genomic DNA, 1 U *Taq* DNA polymerase, 1.5 mM Mg²⁺, 0.2 mM dNTPs, and 0.5 μM of each primer. The PCR protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, annealing at 56 °C for 45 s, 72 °C for 1 min, and a final extension step of 72°C for 10 min. All PCR reactions were carried out in a thermal cycler C1000 (Bio-Rad, USA). PCR products were detected using Mega BACE 1000 DNA Sequencer (Pharmacia, USA).

Conclusion

Both ISSR and fluorescence-labeled TP-M13-SSR marker systems were fit for fingerprinting and identification of wheat cultivars in practice. Fluorescence-labeled TP-M13-SSR markers may be a better choice for fingerprinting of closely related wheat cultivars compared with ISSR marker system.

Acknowledgements

The research was supported by Major Science and Technology Special Project (priority subject) of Zhejiang Province (No. 2008C12005-1), Major Special Project of Ministry of Agriculture (No. 2008ZX08005-005) and key project of Natural Science Foundation of Zhejiang Province (No. Z3100150), P. R. China.

References

Behera TK, Singh AK, Staub JE (2008) Comparative analysis of genetic diversity in Indian bitter melon (*Momordica charantia* L.) using RAPD and ISSR markers for developing crop improvement strategies. *Sci. Hortic.* 115: 209–217.

- Chen YY, Zhou RC, Lin XD, Wu KQ, Qian X, Huang SZ (2008) ISSR analysis of genetic diversity in sacred lotus cultivars. *Aquat. Bot.* 89: 311–316.
- Coombs JJ, Frank LM, Douches DS (2004) An applied fingerprinting system for cultivated potato using simple sequence repeats. *Amer J of Potato Res* 81: 243–250.
- Crespel L, Pernet A, Bris ML, Gudin S, Oyant LHS (2009) Application of ISSRs for cultivar identification and assessment of genetic relationships in rose. *Plant Breed.* 128: 501–506.
- Fujita Y, Fukuoka H, Yano H (2009) Identification of wheat cultivars using EST-SSR markers. *Breed. Sci.* 59: 159–167.
- Kimura T, Shi YZ, Shoda M, Kotobuki K, Matsuta N, Hayashi T, Ban Y, Yamamoto T (2002) Identification of Asian pear varieties by SSR analysis. *Breed. Sci.* 52: 115–121.
- Kuleung C, Baenziger PS, Dweikat I (2004.) Transferability of SSR markers among wheat, rye, and triticale. *Theor Appl Genet* 108: 1147–1150.
- Li H, Wang T, Li Y, Shi Y, Song Y, Lu, P (2005) Application of the TP-M13 automated fluorescence-labeled system of SSR genotyping in sorghum. *J Plant Genet Resour* 6: 68–70.
- Liu LW, Zhao LP, Gong YQ, Wang MX, Chen LM, Yang JL, Wang Y, Yu FM, Wang LZ (2008) DNA fingerprinting and genetic diversity analysis of late-bolting radish cultivars with RAPD, ISSR and SRAP markers. *Sci. Hortic.* 116: 240–247
- Louarn S, Torp AM, Holme IB, Andersen SB, Jensen BD (2007) Database derived microsatellite markers (SSRs) for cultivar differentiation in *Brassica oleracea*. *Genet Resour Crop Evol* 54: 1717–1725
- Lu XJ, Liu LW, Gong YQ, Zhao LP, Song XY, Zhu XW (2009) Cultivar identification and genetic diversity analysis of broccoli and its related species with RAPD and ISSR markers. *Sci. Hortic.* 122: 645–648
- Nagaoka T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theor Appl Genet* 94: 597–602
- Rahman MS, Molla MR, Alam MS, Rahman L (2009) DNA fingerprinting of rice (*Oryza sativa* L.) cultivars using microsatellite markers. *Aust J Crop Sci* 3:122–128.
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nat Biotechnol* 18: 233–234
- Tantasawat P, Trongchuen J, Prajongjai T, Jenweerawat S, Chaowiset W (2011) SSR analysis of soybean (*Glycine max* (L.) Merr.) genetic relationship and variety identification in Thailand. *Aust J Crop Sci* 5: 283–290
- Zhu YF, Hu J, Zhang Y, Guan YJ, Huang XX (2010) Transferability of SSR markers derived from cowpea (*Vigna unguiculata* L. Walp) in variety identification. *Seed Sci. Technol.* 38: 730–740.