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Novel method to establish molecular identity using inter-simple sequence repeat markers in cotton (*Gossypium hirsutum*) cultivars

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ABSTRACT. Plant molecular identity (ID) is used to describe molecular characteristics of plants, which should contain all of the necessary information. Using inter-simple sequence repeat (ISSR) primers, molecular ID can be described in a way that reflects the polymerase chain reaction (PCR) conditions, annealing temperature, and the bands obtained in PCR amplification. A new complete molecular ID system is described in this study, which can be easily used and expanded to include more information. Using three cotton cultivars, we analyzed the products of PCR with ISSR primers and discussed the strategy for establishing their molecular ID. Using the segmented naming method, we designate the simple names and the full name systems of these three cultivars.

Key words: Cotton; Molecular identity; Simple name; Full name; Segmented naming method

INTRODUCTION

DNA markers have proven to be an efficient tool for molecular characterization in plant breeding, and are widely used in fingerprinting, diversity analyses, and gene mapping (Joshi et al., 1999, Reddy et al., 2002). Microsatellites or simple sequence repeats (SSRs) are ubiquitous in eukaryotic genomes. Single-locus SSR markers have been developed for a number of species. However, there is a major bottleneck in developing SSR markers, whereby flanking sequences must be known in order to design 5'-anchors for polymerase chain reaction (PCR) primers. The inter simple sequence repeat (ISSR) technique is another PCR-based method, which involves amplification of the DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions. The technique uses microsatellites, usually 16-25-bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to mainly amplify ISSR sequences of different sizes. ISSR-PCR is a simple, quick, and efficient technique with high reproducibility. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Moreno et al., 1998; Reddy et al., 2002).

In 2009, a molecular identity (ID) dataset, based on numeralized data from polyacrylamide gel electrophoresis (PAGE) bands, was established with ID Analysis software for 83 soybean cultivars in Heilongjiang, China (Gao et al., 2009). Subsequently, more molecular IDs of more plants were established in China, including those of sweet sorghum (Wang et al., 2011), kenaf (Zheng et al., 2010), flax (Hao et al., 2011), hybrid rice (Yan et al., 2011), peach (Chen et al., 2011), sugarcane (Liu et al., 2010), peanut (Zhao et al., 2010), and *Spiraea* (Liu et al., 2009). These molecular IDs have proven very helpful in spice classification, identification, and germplasm protection. However, molecular IDs can vary a lot depending on the methods for naming the IDs as well as the construction system of the molecular IDs. ISSRs have high reproducibility, which is possibly due to the use of longer primers (16-25 mers); only the faintest bands are not reproducible. Approximately 92-95% of scored fragments could be repeated across DNA samples of the same cultivar and across separate PCR runs when detected using PAGE (Fang and Roose, 1997; Moreno et al., 1998). It would be possible to establish a plant ISSR-based ID system if a standard naming system were developed that contains enough information to reflect PCR conditions, such as primers used in the experiment for a certain plant, annealing temperatures, and PCR products. In general, the same crop should have the same molecular ID, and different plants should be distinguished with ISSR markers. Using three cotton cultivars, a complete ISSR-based molecular ID system is described in this study, which can be easily used and expanded with much more information.

MATERIAL AND METHODS

Three commonly used major cotton (*Gossypium hirsutum*) cultivars (Baimian No 1, Xinyan 9648, and Zhongzi No 6) developed in Henan Province, China, were used in the present study.

Genomic DNA was isolated by a modified cetyl-trimethylammonium bromide (CTAB) protocol (Bornet and Branchard, 2001). PCR was performed with 37 anchored or non-anchored primers. Sequences of each primer (Invitrogen, China) are shown in Table 1. The PCR mixture contained: approximately 10 ng template DNA, 10 µL 2X Taq MasterMix, 1 µL 10 µM primer, and topped with distilled water to a total volume of 20 µL. The PCR program was as follows: 5 min at 94°C for pre-denaturation; 36 cycles each at 94°C for 1 min,

step down annealing temperature (Tm -8°C, Tm +2°C) for 55 s, and 72°C for 1 min; with a final extension at 72°C for 10 min. Each reaction was repeated at least twice and three plants of each accession were analyzed as replicates of the cultivars tested.

Table 1. Serial numbers and sequences of the ISSR primers.

Primer No.	Sequence	Primer No.	Sequence
001	$(GTG)_3GC$	020	$(GAG\ AGA\ )_2GAG\ AA$
002	$(CAA)_5$	021	$(CT)_8T$
003	$(AGC)_5$	022	$(CTC\ TCT)_2CTC\ TG$
004	$(CAC\ )_4GC$	023	$(ACA\ CAC)_2ACA\ CT$
005	$(GACA\ )_4$	024	$(CT)_8RA$
006	$(GATA\ )_2(\ GACA)_2$	025	$(CA)_8RG$
007	$(ACTG)_2\ ACCG\ ACTG$	026	$(ACC)_6$
008	$(AGA\ GAG\ )_2\ AGA\ GC$	027	$(ATG)_6$
009	$(AGA\ GAG\ )_2\ AGA\ GG$	028	$TCT(TA)_7$
010	$(GAG\ AGA)_2GAG\ AC$	029	$(AGA\ GAG)_2AGA\ GYC$
011	$(CTC\ TCT\ )_2CTC\ TG$	030	$(AGA\ GAG)_2AGA\ GYA$
012	$(TCT\ CTC\ )_2\ TCT\ CG$	031	$(GAG\ AGA)_2GAG\ AA$
013	$(ACA\ CAC\ )_2ACACC$	032	$(CAC\ ACA)_2CAC\ ARC$
014	$(GAGAGA)_2GAGACT$	033	$(ACA\ CAC)_2ACA\ CYT$
015	$(GAGAGA)_2GAG\ ATC$	034	$(ACA\ CAC\ )_2ACA\ CYA$
016	$(GAGAGA)_2GAG\ ATG$	035	$(ACA\ CAC)_2ACA\ CYG$
017	$CAC\ (\ TGT\ GTG)_2TG$	036	$HBH\ (AGA\ GAG)_2AG$
018	$(GAC\ A)_4$	037	$DBD\ (ACA\ CAC\ )_2AC$
019	$(AGA\ GAG)_2AGAGT$		

PCR products were electrophoresed on 2% agarose gels. DNA fragment size (bp) was calculated with Gel imaging analysis system software (Shanghai Shanfu) by comparing the DNA bands to a D2000 DNA reference marker (Kangweishiji, China).

RESULTS

Proper ISSR primers were selected based on clearly detectable PCR bands under optimized PCR conditions. The reaction products were electrophoresed on a 2% agarose gel. Clearly detectable amplified ISSR bands were obtained ranging in size from 122 to 2108 bp for the three cotton cultivars. Among the 37 primers, 24 were suitable for Baimian No 1, 14 were suitable for Xinyan 9648, and 17 were suitable for Zhongzi No 6. The primers and suitable annealing temperature for each primer are listed in Table 2. Eleven of the 37 primers (001-005, 013, 020, 026, 027, 031, and 034) could be used for all three cultivars, and there were no PCR products observed for seven primers (011, 012, 015, 025, 030, 032, and 035).

The ISSR fingerprinting profile for Baimian No 1, Xinyan 9648, and Zhongzi No 6, based on the selected primers and annealing temperatures, are shown in Figure 1, 2, and 3.

A complete molecular ID was used to describe the ISSR fingerprinting features for a certain plant. The ID should contain the molecular ID naming part and the related explanatory part. The explanatory part should contain information related to the suitable primer sequences, optimized annealing temperature for the ISSR-based PCR reaction, the PCR reaction system, and the electrophoresis image. Considering the informative characteristics and ease of use, we named the molecular ID with two forms: a simple name and a full-informative name. For the simple name, we designated the molecular ID with numbers reflecting the bands obtained from PCR. The complete name contains detailed information of the ISSR fingerprint profile.

Table 2. Suitable annealing temperature for each selected primer of three cultivars.

Primer No.	Baimian No1 Tm (°C)	Xinyan 9648 Tm (°C)	Zhongzi No6 Tm (°C)
001	48.0	52.0	52.0
002	48.0	48.8	48.8
003	58.8	60.8	62.0
004	51.0	50.3	46.0
005	51.2	53.0	56.4
006	45.0		
007	54.2		
008	54.0		
009		55.0	54.3
010		57.0	56.3
013	55.2	57.0	56.0
014	54.3		
016			
017	55.2		56.0
018	57.0		
019	51.0		53.3
020	51.0	53.3	53.0
021	52.2		
022			
023	54.6		
024	52.0		
026	62.2	67.4	60.2
027	49.2	53.2	50.3
028			55.3
029	55.0		
031	51.0	54.0	54.0
033	55.4		
034	54.2	55.3	55.3
036	52.0		
037		56.0	57.0

The simple name was designed with six digital serial numbers (Figure 4). The 1st three digits represent the primer number (e.g., 001), and the 4th represents band numbers smaller than and including 500 bp, the 5th represents band numbers between 500 and 1000 bp, and the 6th represents band numbers larger than 1000 bp.

The full-informative name should contain all of the details of the ISSR fingerprinting profile. It is segregated into four parts, which include primer (A), annealing temperature (B), PCR bands number (C), I, II, III, etc., to indicate the PCR fragment size (bp), and a final part, @hist, which provides information about the institute (Figure 5).

Based on the naming system described above and the ISSR fingerprinting profiles shown in Figure 1 to Figure 3, all simple and full-informative names of the three cotton cultivars were designated as follows:

Simple name of Baimian No 1:

001130-002031-003140-004120-005100-006021-007010-008010-013111-014111-017020-018100-019001-020220-021100-023221-024001-026101-027201-029210-031120-033130-034120-036220.

Simple name of Xinyan 9648:

001120-002112-003140-004211-005022-009320-010001-013011-020211-026101-027200-031100-034120-037300.

Simple name of Zhongzi No 6:

001120-002122-003211-004131-005330-009211-010320-013220-017210-019300-020120-026201-027020-028210-031120-034021-037101.

Full name of Baimian No 1:
A001B48.0C04I400II536III655IV842@hist
A002B48.0C04I676II802III971II1073@hist
A003B58.8C05I289II536III584IV729V956@hist
A004B51.0C03I384II773III987@hist
A005B51.0C01I378@hist
A006B45.0C03I584II985III1111@hist
A007B54.2C01I829@hist
A008B54.0C01I574@hist
A013B55.2C03I414II574III1111@hist
A014B54.3C03I305II884III1811@hist
A017B55.2C02I775II956@hist
A018B57.0C01I407@hist
A019B51.0C01I1092@hist
A020B51.0C04I250II475III884IV985@hist
A021B52.2C01I475@hist
A023B54.6C05I394II451III593IV971V1237@hist
A024B52.0C01I1282@hist
A026B62.2C02I356II1215@hist
A027B49.2C03I332II421III1305@hist
A029B55.0C03I279II338III518@hist
A031B51.0C03I434II879III965@hist
A033B51.0C04I483II512III771IV959@hist
A034B54.2C03I266II600III896@hist
A036B52.0C03I409II490III543IV862@hist
Full name of Xinyan 9648:
A001B52.0C03I399II533III672@hist
A002B48.8C04I318II619III1018IV1098@hist
A003B60.8C05I282II559III614IV761V1000@hist
A004B50.3C04351II419III797IV1018@hist
A005B53.0C04I614II785III1037IV1626@hist
A009B55.0C05I224II278III440IV542V935@hist
A010B57.7C01I1018@hist
A013 B57.0C02I605II1187@hist
A020B53.3C04I246II461III909IV1037@hist
A026B67.4C02I334II1286@hist
A027B53.2C02I146II292@hist
A031B54.0C01I454@hist
A034B55.3C03I283II633III974@hist
A037B56.0C03I250II362III500@hist
Full name of Zhongzi No 6:
A001B52.0C03I386II761III940@hist
A002B48.8C05I329II521III699IV1019V1427@hist
A003B62.0C04I278II352III725IV1123@hist
A004B46.0C05I409II552III682IV826V1751@hist

A005B56.4C06I233II340III478IV699V813VI1000@hist
A009B54.3C04I241II444III925IV2108@hist
A010B56.3C05I226II268III432IV521V853@hist
A013B56.0C04I122II363III511IV911@hist
A017B56.0C03I233II432III868@hist
A019B53.3C03I226II319III489@hist
A020B53.0C03I233II582III840@hist
A026B60.2C03I218II329III1066@hist
A027B50.3C02I716II896@hist
A028B55.3C03I278II397III868@hist
A031B54.0C03I421II562III985@hist
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A037B57.0C02I329II1038@hist.

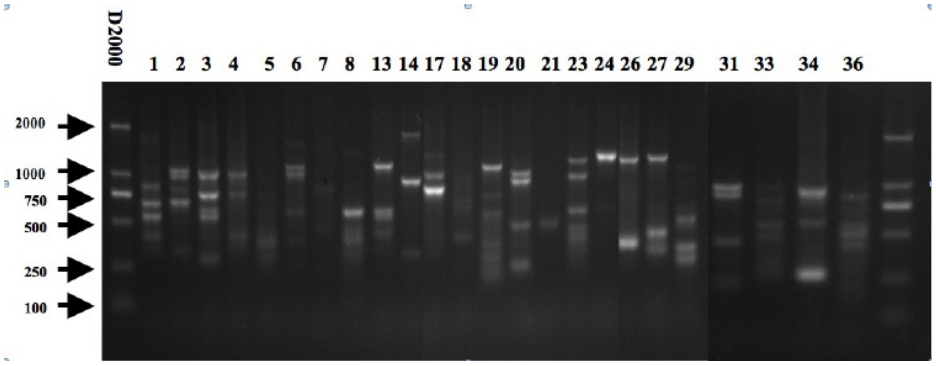

Figure 1. ISSR fingerprinting profile of Baimian No.1 with different primers listed on the top of the gel. D2000 = DNA marker; *lanes 1* to *36* = primers used in the experiment.

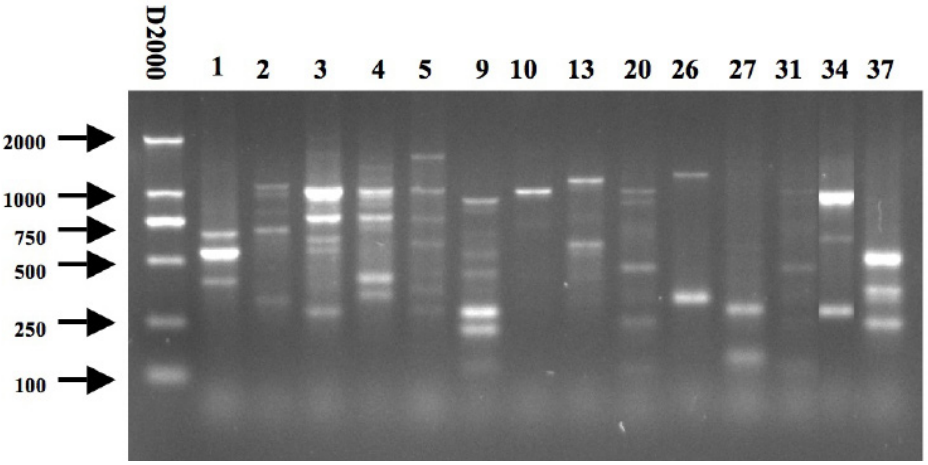

Figure 2. ISSR fingerprinting profile of Xinyan-9648 with different primers listed on the top of the gel. D2000 = DNA marker; *lanes 1* to *37* = primers used in the experiment.

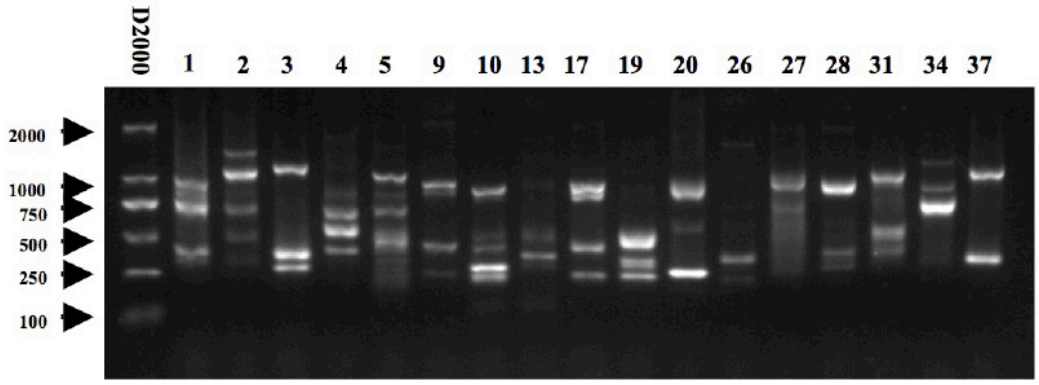

Figure 3. ISSR fingerprinting profile of Zhongzi-No. 6 with different primers listed on the top of the gel. D2000 = DNA marker; *lanes 1* to *37* = primers used in the experiment.

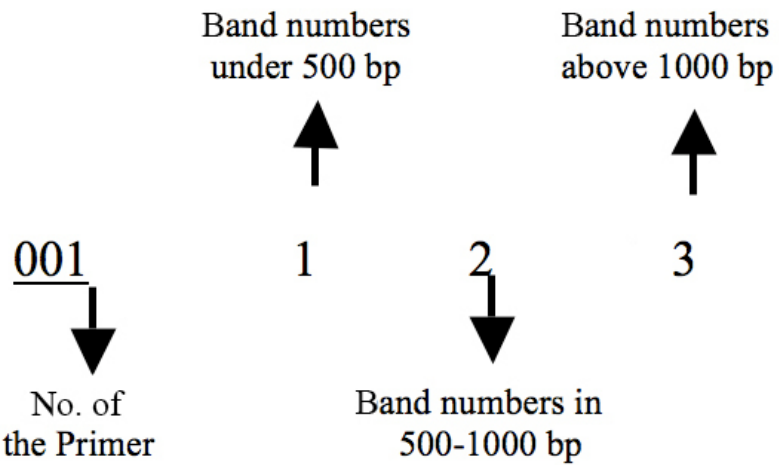

Figure 4. Demonstration of the simple name.

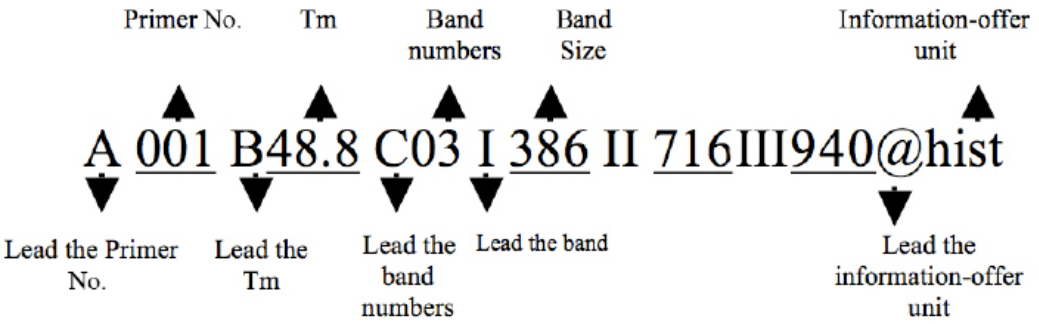

Figure 5. Demonstration of the full name.

DISCUSSION

ISSR marker analysis is an easy, fast, inexpensive, accurate, and reliable technique for genetic analyses of various plant species (Reddy et al., 2002). A complete molecular ID should contain all of the detailed information of target plants. To establish the representative molecular ID, it is critical to collect and reflect all of the information from the amplified ISSR profile. The molecular IDs currently used are relatively quite simple and incomplete. Most of the time, the PCR band is scored as 1 when it is present and as 0 when it absent, and serial numbers are assigned to molecular IDs based on PCR results (Liu et al 2009; Zhao et al., 2010; Wang 2011; Yan et al., 2011). Such molecular IDs are difficult to be widely used across plants due to their limitations and low information content. In the present study, we established two naming systems, which fully reflect the plant characteristics of molecular ID. The names contain information related to primers, annealing temperature, the PCR reaction system, bands obtained, and the information provider. The system is also easy to use. For example, primer No 1 can be used to distinguish Baimioan No 1 and Xinyan 9648 with their simple names 001130 and 001120. Although Zhongzi No 6 has the same simple name as Xinyan 9648, 001120, it is easy to find the full name difference between Xinyan 9648 (A001B52.0C03I399II533III672@hist) and Zhongzi No 6 (A001B52.0C03I386II761III940@hist). Both simple and full names can be easily used or combined according to specific needs. As the 1st three digits represent the primer number, the molecular ID database can also be easily expanded when more primers are introduced into the experiment. For this system, more than 999 primers can be chosen for one plant.

In conclusion, we designated a complete naming system that includes descriptive information and molecular ID information. The descriptive part should contain the detailed information of ISSR amplification conditions, and the molecular ID part should contain the simple name and full name system using the segmented naming method. Therefore, a new complete molecular ID system was established, which can be easily used and expanded as more information becomes available. Hopefully, this system will provide an improved solution for the characterization of ISSR markers.

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