ISSR and AFLP Markers Differ among American Watermelon Cultivars with Limited Genetic Diversity

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ABSTRACT. Wide phenotypic diversity exists among American heirloom cultivars of watermelon (*Citrullus lanatus* var. *lanatus*). However, in published studies, low or no polymorphism was revealed among those heirlooms using isozyme or randomly amplified polymorphic DNA (RAPD) markers. In this study, experiments with inter-simple sequence repeat (ISSR) [also known as simple sequence repeat-(SSR-) anchored primers] and amplified fragment-length polymorphism (AFLP) markers produced high polymorphisms among watermelon heirloom cultivars. ISSR (111) and AFLP (118) markers (229 total) identified 80.2% to 97.8% genetic similarity among heirloom cultivars. The phylogenetic relations based on ISSR and AFLP markers are highly consistent with the parental records available for some of the heirloom cultivars, providing confidence in the dendrogram constructed for heirlooms based on similarity values. As compared with RAPD markers, ISSRs and AFLPs are highly effective in differentiating among watermelon cultivars or elite lines with limited genetic diversity.

Watermelon is grown in 44 states in the United States. The largest production areas are in Florida, California, Texas, Georgia, and Arizona. U.S. watermelon production has increased from 1.2 M tons in 1980 to 3.9 M tons in 2002 with a farm value of \$291 million in 2002 [U.S. Dept. of Agriculture (USDA), 2003]. In recent years, there has been an increased demand by consumers for seedless (triploid) watermelons. Consequently, production of triploid watermelons has increased significantly. During 2002, >82% and 51% of the watermelons produced in California and in the Southeast of the United States (including Virginia, North Carolina, South Carolina, Georgia, and Florida), respectively, were seedless (USDA, 2003). There is a continuous need to develop new seedless watermelon cultivars suitable to consumer needs.

A large number of American heirloom cultivars are kept at the USDA, ARS, National Seed Storage Laboratory, Fort Collins, Colo. (Germplasm Resources Information Network; <http://www.ars-grin.gov/cgi-bin/npgs/html/croplist.pl>). These cultivars vary in fruit quality (fruit shape, size, flesh color and texture, sugar, and carotenoid content) and can be useful for further develop-

ment of elite watermelon cultivars, particularly triploid seedless watermelons. However, there is insufficient information regarding phylogenetic relationships among many of those heirlooms (Elmstrom, 1999). Furthermore, although there is wide phenotypic diversity among watermelon heirloom cultivars, most of them appeared to have a narrow genetic background reflected in a large number of monomorphic and a few polymorphic RAPD markers among cultivars (Levi et al., 2001; Zhang et al., 1994). Thus, it is vital for breeders and researchers to employ in their breeding programs highly polymorphic DNA markers that accurately differentiate among closely related watermelon cultivars and breeding lines.

The objectives of this study were to 1) determine whether inter-simple sequence repeat (ISSR) and amplified fragment-length polymorphism (AFLP) markers detect higher polymorphism among watermelon cultivars as compared to randomly amplified polymorphic DNA (RAPD) markers, 2) construct a dendrogram based on AFLP and ISSR markers that delineate phylogenetic relationships among watermelon heirlooms with limited genetic diversity, 3) determine to what degree the phylogenetic relationships based on ISSR and AFLP markers are consistent with the parental records provided for watermelon (Elmstrom, 1999), and 4) construct a DNA fingerprinting table based on polymorphic ISSR and AFLP markers that can be useful in differentiating among heirloom cultivars or elite watermelon lines.

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Material and Methods

PLANT MATERIAL AND DNA ISOLATION. Seeds of 44 heirloom watermelon cultivars were obtained from various sources (Table 1) and germinated in the greenhouse (26 °C day/20 °C night temperatures). Young leaves were collected from four to five plants (3 weeks old) of each of the cultivars and stored at -80 °C. To avoid co-isolation of polysaccharides, polyphenols, and other secondary compounds that damage DNA, we used an improved procedure for isolation of DNA from young leaves of watermelon (Levi and Thomas, 1999; Levi et al., 2001).

DNA AMPLIFICATION CONDITIONS USING ISSR PRIMERS AND GEL ELECTROPHORESIS. ISSR primers (15 to 20 decamer oligonucleotides) were purchased from the Univ. of British Columbia, Vancouver, B.C., Canada (primer 800-899). Polymerase chain reactions (PCR) were performed in 25 µL reaction buffer containing 20 µM NaCl, 50 mM Tris-HCl pH 9, 1% Triton-X-100, 0.01 %

gelatin, 1.6 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP, and dTTP (Sigma), 0.2 mM primer, 7 units Taq DNA Polymerase (Promega, Madison, Wis., supplied in storage buffer A), and 15 ng template DNA (Levi et al., 1993). Amplification reactions were carried out for 45 cycles in a PTC-200 thermocycler (MJ Research, Watertown, Mass.), programmed for 60 s to denature DNA at 92 °C, 70 s for DNA annealing at 48 to 62 °C (Table 2), and 120 s for DNA transcription at 72 °C. The DNA annealing temperature was optimized for each primer based on its melting-temperature (T_m) (Table 2). Amplification products were separated by electrophoresis in 1.4% agarose gels in 0.5× Tris-borate buffer (Sambrook et al., 1989). The gels were stained with 0.5 µg·mL⁻¹ ethidium bromide solution for 30 min and destained for 15 min in distilled water. DNA fragments were visualized under UV light and photographed using a still video system (Gel Doc 2000; Bio-Rad, Hercules, Calif.). The molecular weights of the amplification products were calculated using 1-kb DNA ladder standards (Gibco BRL, Gaithersburg, Md.).

Table 1. Watermelon heirlooms examined for genetic similarity using AFLP and ISSR markers.

Cultivar	Source of seeds	Year Released	Breeding Parentage	Closest Cultivars on Dendrogram (Figure 2) and their similarity value (%) with heirloom
Allsweet	Sunseeds ^Z	1972	[Miles x Peacock] x Charleston Gray	Calhoun Gray (96.4%)
AU-Golden Producer	Hollar ^Y	1993	A selection from AU-Producer	AU-Producer (93.0%)
AU-Jubilant	Hollar	1985	Jubilee x PI 271778	Black Diamond (93.4%)
AU-Producer	Hollar	1985	Crimson Sweet x PI 189225	AU-Golden Producer (93.0%)
Black Diamond	Sunseeds	---	---	Blackstone (94.4%)
Blackstone	Hollar	---	Black Diamond, Fairfax	Black Diamond (94.4%)
Calhoun Gray	Sunseeds	---	---	Allsweet (96.4%), CalSweet (93.9%)
CalSweet	Sunseeds	---	---	Calhoun Gray (93.9%), Family Fun (93.4%)
Charleston Gray	NSL-5267 ^X	1954	Africa 8, Iowa Belle, Garrison, Hawkesbury, Leesburg	Dixielee (90.6%)
Coles Early	Sunseeds	1892	---	Dixie-Queen (83.7%), Fairfax (80.2%)
Congo	Syngenta ^W	1949	(African x Iowa Belle) x Garrison	Blackstone (90.8%)
Crimson Sweet	Hollar	1963	(Miles x Peacock) x Charleston Gray	AU-Producer (89.9%), Garrison (88.4%)
Dixielee	Hollar	1979	Texas W5, Wilt resistant Peacock, Fairfax, Summit	Family Fun (91.5%), Charleston Gray (90.6%)
Dixie Queen	Sunseeds	1890	F1 Hybrid ---	Jubilee (92.5%)
Dunbarton	NSL-6637	1953	(African x Iowa Belle) x Garrison	Klondike-Strip (89.1%)
Fairfax	Sunseeds	1952	Garrison, African, Iowa Belle, Leesburg, Hawkesbury	Garrisonian (85.3%), Coles Early (83.7%)
Family Fun	Syngenta	1973	F1 Hybrid ---	CalSweet (93.4%), Dixielee (91.5%)
Garrison	NSL-2053	---	---	Garrisonian (92.5%), Crimson Sweet (88.4%)
Garrisonian	Willhite ^V	1957	Africa 8, Iowa Belle, Garrison, Hawkesbury, Leesburg	Garrison (92.5%)
Georgia Rattlesnake	Seed Savers ^U	---	---	Leesburg (90.6%)
Golden Honey	Hollar	1954	Mixed Strain from Japan Seed, Co.	Hawkesbury (89.3%)
Golden Midget	NSL-5288	1959	New Hampshire Midget x Pumpkin Rind	Mickylee (92.3%)
Hawkesbury	Syngenta	1936	---	Golden Honey (89.3%)
Iopride	Syngenta	---	---	Kleckley's Sweet (89.4%)
Ironsides	NSL-7369	1950	[Leesburg x Hawkesbury] x Garrison	New Hampshire Midget (85.6%)
Jubilee	Hollar	1963	Africa 8, Iowa Belle, Garrison, Hawkesbury, Leesburg	Dixie Queen (92.5%), Georgia Rattlesnake (90.1%)
King and Queen	Hollar	---	---	Leesburg (88.6%), Iopride (87.4%)
Kleckley's Sweet	Seed Savers	---	---	Miles (91.8%)
Klondike	Syngenta	1959	---	Dunbarton (89.1%)
Leesburg	NSL-7368	1936	Selection from Kleckley Sweet	Georgia Rattlesnake (90.6%)
Mickylee	Hollar	1986	Texas W5, Fairfax, Summit, Graybelle	Minilee (94.6%), Golden Midget (92.3%)
Miles	NSL-6688	1948	Dixie Queen x Klondike R-7	Parker (96.2%)
Minilee	Hollar	1986	Texas W5, Fairfax, Summit, Graybelle	Mickylee (94.6%)
New Hampshire Midget	Syngenta	1951	Favorite Honey x Dakota Sweet	Minilee (90.8%),
Northern Sweet	Syngenta	1932	---	Summit (92.9%), Picnic (92.6%)
Parker	Willhite	---	F1 Hybrid ---	Miles (96.2%), Summit (92.9%)
Peacock	Hollar	1939	---	Sweet Princes (92.4%), Prince Charles (90.6%)
Picnic	Syngenta	1972	Peacock type	Prince Charles (97.7%), Northern Sweet (92.6%)
Prince Charles	Hollar	1978	F1 Hybrid ---	Picnic (97.7%)
Sugar Baby	Sunseeds	1955	Tough Sweets selection	Klondike-Strip (88.7%)
Stone Mountain	Hollar	1924	---	Stone Mountain #5 (89.8%), Sweet Princes (86.9%)
Stone Mountain #5	Syngenta	1936	Stone mountain x Iowa Belle	Stone Mountain (89.8%)

^ZSunseeds Co. (Acampo, California). ^YHollar Seeds, Inc. (Rocky Ford, Colorado). ^XAccession number provided for each of the heirloom cultivars kept at the USDA National Seed Storage Laboratory (Fort Collins, Colorado). ^WSyngenta Seeds, Inc. (Napels, Florida). ^VWillhite Seed, Inc. (Poolville, Texas). ^USeed Savers Exchange (Decorah, Iowa).

^TInformation unavailable.

Table 2. The nucleotide sequence of ISSR primers, the optimal annealing temperature in PCR reactions, and the number of markers produced by each primer (number of polymorphic markers in parentheses).

Primer	Sequence	Annealing temp (°C)	No. of markers
808	AGAGAGAGAGAGAGAGC	59	5 (4)
809	GAGGAGAGAGAGAGAGG	59	4 (3)
810	GAGAGAGAGAGAGAGAT	53	5 (3)
812	GAGAGAGAGAGAGAGAA	53	2 (1)
813	CTCTCTCTCTCTCTT	53	1 (1)
815	CTCTCTCTCTCTCTG	48	1 (1)
816	CACACACACACACAT	54	2 (1)
817	CACACACACACACAA	54	2 (1)
818	CACACACACACACAG	54	4 (3)
820	GTGTGTGTGTGTGTC	59	4 (2)
821	GTGTGTGTGTGTGTT	59	2 (0)
822	TCTCTCTCTCTCTCA	54	2 (0)
823	TCTCTCTCTCTCTCC	54	6 (2)
824	TCTCTCTCTCTCTCG	54	2 (0)
825	ACACACACACACACT	54	6 (2)
826	ACACACACACACACC	62	4 (4)
828	TGTGTGTGTGTGTGA	54	3 (0)
829	TGTGTGTGTGTGTGC	62	1 (0)
834	AGAGAGAGAGAGAGYT	59	4 (4)
835	AGAGAGAGAGAGAGYC	59	5 (2)
836	AGAGAGAGAGAGAGYA	54	1 (1)
840	GAGAGAGAGAGAGAYT	54	4 (4)
841	GAGAGAGAGAGAGAYC	54	3 (1)
842	GAGAGAGAGAGAGAYG	59	2 (2)
843	CTCTCTCTCTCTCTRA	54	3 (3)
844	CTCTCTCTCTCTCTRC	54	3 (3)
845	CTCTCTCTCTCTCTRG	54	3 (0)
847	CACACACACACACAAGC	62	2 (2)
849	GTGTGTGTGTGTGTCTA	54	3 (2)
851	GTGTGTGTGTGTGTCTG	62	1(0)
852	TCTCTCTCTCTCTCAGA	54	3 (3)
853	TCTCTCTCTCTCTCAGT	54	2 (1)
854	TCTCTCTCTCTCTCAGG	54	1 (1)
856	ACACACACACACACCTA	62	2 (1)
864	ATGATGATGATGATG	54	5 (5)
880	GGAGAGGAGAGGAGA	54	4 (4)
889	AGTCGTAGTACACACACAC	62	5 (3)
891	ACTACGACTTGTGTGTGTGTG	62	2 (2)

AFLP PROCEDURE. The AFLP procedure developed by Vos et al. (1995) was modified using a commercially available kit (Plant Mapping Kit—Regular Plant Genome; Applied Biosystems, Foster City, Calif.). According to the manufacturer's protocol, a high-quality genomic DNA (500 ng; intact and with a 260/280 O.D. ratio of 1.8) of each cultivar was added to a restriction–ligation mixture containing *EcoRI* (5 U), *MseI* (1 U), T4 ligase (1 U), 1× T4 ligase buffer (New England BioLabs, Beverly, Mass.), 50 ng·μL⁻¹ BSA, and 50 mM NaCl. Additionally, *MseI* and *EcoRI* adapters (20 and 2 nm·μL⁻¹, respectively), and sterile distilled water were added to bring the mixture to a final volume of 11 μL (New England BioLabs). Following 2 h incubation at 37 °C, the restriction–ligation reaction mix was diluted 18-fold by adding 189 μL purified water.

Each preselective PCR amplification contained 4 μL of the diluted restriction–ligation reaction, 1 μL of *EcoRI* / *MseI* primer solution, and 15 μL AFLP Core Mix (Plant Mapping Kit—Regular Plant Genome, Applied Biosystems). All PCRs were performed

in a PTC 200 thermocycler (MJ Research, Watertown, Mass.). Preselective PCR amplifications were carried through 20 cycles, each consisting of 20 s to denature DNA at 94 °C, 30 s for DNA annealing at 56 °C, and 120 s for DNA transcription at 72 °C. Then, the final cycle included 30 min incubation at 60 °C to complete any DNA annealing and transcription, and cooling at 4 °C. Each of the 20 μL of preselective amplification reactions were diluted 10-fold by adding 180 μL sterile distilled water.

For selective amplification, 1.5 μL of diluted reaction was mixed with one of the following selective primer sets: pair 1) 5'GAATTC(*EcoRI*)-AAG and 5'TTAA(*MseI*)-CAT, pair 2) 5'GAATTC(*EcoRI*)-AAG and 5'TTAA(*MseI*)-CTC, pair 3) 5'GAATTC(*EcoRI*)-ACC and 5'TTAA(*MseI*)-CAT, and pair 4) 5'GAATTC(*EcoRI*)-ACC and 5'TTAA(*MseI*)-CAA according to manufacturer's instructions (Plant Mapping Kit—Regular Plant Genome; Applied Biosystems). The first part of the selective amplification reaction consisted of a cycle of 140 s at 94 °C to denature DNA, 30 s for DNA annealing at 65 °C, and 120 s

Fig. 1. PCR-ISSR patterns (on 1.4% agarose-gel) of watermelon cultivars produced by ISSR primers UBC-844, and UBC-849. Lanes are: M) 100 bp DNA ladder markers (Life Technologies, Rockville, Maryland), 1) 'Allsweet', 2) 'Picnic', 3) 'AU-Golden Producer', 4) 'AU-Jubilant', 5) 'AU-Producer', 6) 'Blackstone', 8) 'Calhoun Gray', 9) 'Calsweet', 10) 'Charleston Gray' (NSSL), 11) 'Charleston Gray' (NK Lawn & Garden Co), 12) 'Coles Early', 13) 'Congo', 14) 'Crimson Sweet', 15) 'Dixielee', 16) 'Dixie Queen', 17) 'Dunbarton', 18) 'Fairfax', 19) 'Family Fun', 20) 'Garrison', 21) 'Garrisonian', 22) 'Georgia Rattlesnake', 23) 'Golden Honey', and 24) 'Golden Midget'.

Table 3. ISSR and AFLP markers polymorphic among American heirloom cultivars: Allsweet (AS), AU-Jubille (AJ), AU-Golden Producer (AG), AU- Producer (AP), Black Diamond (BD), Blackstone (BS), CalSweet (CS), Calhoun Gray (CG), Charleston Gray (CY), Coles Early (CE), Congo (CO), Crimson Sweet (CT), Dixielee (DL), Dixie Queen (DQ), Dunbarton (DB), Fairfax (FX), Family Fun (FF), Garrison (GR), Garrisonian (GN), Georgia-Rattlesnake (GS), Golden Honey (GH), Golden Midget (GM), Hawkesbury (HU), Iopride (IP), Ironsides (IS), Jubilee (JB), King & Queen (KQ), Kleckely's Sweet (KS), Klondike (KD), Leesburg (LB), Mickylee (MK), Miles (MS), Minilee (MN), New Hampshire Midget (NM), Northern Sweet (NS), Parker (PK), Peacock (PO), Picnic (PI), Prince Charles (PC), Stone Mountain (SM), Stone mountain #5 (S5), Sugar Baby (SB), Summit (SU), and Sweet Princes (SP).

ISSR																																																
Markers	AS	AJ	AG	AP	BD	BS	CS	CG	CY	CE	CO	CT	DL	DQ	DB	FX	FF	GR	GN	GS	GH	GM	HU	IP	IS	JB	KQ	KS	KD	LB	MK	MS	MN	NM	NS	PK	PO	PI	PC	SM	S5	SB	SU	SP				
808-700	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
808-675	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
808-525	1	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
808-375	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
809-2000	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0		
809-875	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0		
809-450	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0		
810-1350	0	1	1	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
810-600	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	
815-800	1	1	1	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
818-625	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
820-660	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
820-650	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
822-750	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1	0	1	1	1	1	0	0	1	0	0	1	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
834-575	1	1	0	0	1	1	1	1	0	0	1	0	0	0	1	0	1	1	0	0	1	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
834-375	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
834-325	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
840-1550	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	
840-1400	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	
840-675	0	0	0	1	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
841-725	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
843-725	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
843-375	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	0	0	1	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
844-1000	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	
844-950	0	0	0	1	1	1	0	1	0	0	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
AFLP																																																
Markers	AS	AJ	AG	AP	BD	BS	CS	CG	CY	CE	CO	CT	DL	DQ	DB	FX	FF	GR	GN	GS	GH	GM	HU	IP	IS	JB	KQ	KS	KD	LB	MK	MS	MN	NM	NS	PK	PO	PI	PC	SM	S5	SB	SU	SP				
AAG-CAT-79	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAG-CAT-86	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAG-CAT-110	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAG-CAT-118	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAG-CAT-119	0	0	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AAG-CAT-163	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0														

for DNA transcription at 72 °C, followed by a cycle of 120 s at 94 °C to denature DNA, 30 s for DNA annealing at 64 °C, and 120 s for DNA transcription at 72 °C. The second cycle was followed by seven cycles of same conditions, but with only 20 s at 94 °C to denature DNA, and the annealing temperature was gradually reduced by 1 °C in each sequential cycle from 63 °C at the first cycle to 57 °C at the seventh cycle. The second part of the selective amplification consisted of 25 cycles of 20 s at 94 °C to denature DNA, 30 s for DNA annealing at 56 °C, and 120 s at 72 °C for DNA transcription, followed by 30 min at 60 °C for any additional annealing and DNA transcription, and an extended cooling period at 4 °C.

Reactions were prepared for electrophoresis analysis by mixing 1 µL of the final selective amplification reaction with 1.2 µL of formamide : blue dextran (30 mg·L⁻¹) (mixed in a ratio of 5:1), and 0.3 µL of ROX 500 Genescan standard (Applied Biosystems). Samples (2.2 µL) were loaded on a 6% polyacrylamide gel, and electrophoresed at 3000 V using a Perkin Elmer ABI-373 or ABI-377 Sequencer (according to manufacturer's protocol). Fragment sizes were determined using Genescan and Genotyper software (Applied Biosystems).

Fig. 2. Dendrogram of watermelon cultivars produced by UPGMA cluster analysis of similarity matrix based on 229 ISSR and AFLP markers.

DATA ANALYSIS. A pairwise similarity matrix was generated using the Nei-Li similarity index (Nei and Li, 1979) according to the equation: $\text{Similarity} = 2N_{ab}/(N_a + N_b)$, Where N_{ab} is the number of fragments (ISSRs and AFLPs) shared by two genotypes (a and b) and N_a and N_b are the total number of fragments analyzed in each genotype. A dendrogram was constructed based on the similarity matrix data by applying the unweighted pair-group method with arithmetic average (UPGMA) cluster analysis using the Numerical Taxonomic and Multi-Variant Analysis System for PC (NTSYS-PC version 2) (Rohlf, 1993).

Results and Discussion

ISSR MARKERS. The ISSR markers identified significant polymorphisms and were consistent among experiments due to optimization of the annealing temperature in PCR for each of the ISSR primers (Table 2). Thirty-one (81.6%) of the 38 ISSR primers produced polymorphic markers. Seventy-two (64.5%) of 111 ISSR markers were polymorphic among cultivars (Table 2, Fig. 1). Previously Levi et al. (2001) observed only 85 (29.5%) of 288 RAPD markers that were polymorphic among the same cultivars. ISSRs exhibited more polymorphisms than RAPD markers in various plant species, as shown in experiments with rice varieties (Davierwala et al., 2000) and with Finger millet (*Eleusine coracana*) accessions (Salimath et al., 1995). Simple sequence repeats are present throughout the genome and are likely to be clustered near unique gene sequences (Temnykh et al., 2001; Wang et al., 2002). ISSR markers have been used successfully to detect polymorphisms in crop plants as shown for cauliflower (Bornet et al., 2002), fruit plants including strawberry, apple and Ribes species (Korbin et al., 2002) and in *Cucurbita pepo* (Paris et al., 2003). Bornet et al. (2002) showed that ISSR primers were useful in detecting simple sequence repeat regions across the *Brassica oleracea* genome, and that many ISSR-internal regions had homologies with known gene sequences (mainly with genes coding for proteins implicated in DNA interaction or gene expression).

AFLP MARKERS. All AFLP fragments produced by the four *EcoRI* / *MseI* primer pairs in this study were polymorphic among cultivars. AFLP primer pairs 1, 2, 3, and 4 produced 35, 33, 26, and 24 polymorphic markers, respectively (representative markers are shown in Table 3). Overall, the AFLP markers in this study appeared to be highly polymorphic among watermelon cultivars compared with ISSR and RAPD markers. Ke-peng et al. (2003) examined genetic diversity among watermelon accessions developed in China using eight selective AFLP primer pairs different from those used in this study. In that study, the introduction of *EcoRI* selective primer with two nucleotides at the 3' end (*EcoRI*-AT and *MseI*-CTA, *EcoRI*-AT and *MseI*-CAT, *EcoRI*-AA and *MseI*-CAA, *EcoRI*-AC and *MseI*-CTT, and *EcoRI*-AG and *MseI*-CAA) resulted in producing large numbers of AFLP fragments (94, 120 102, 92, 76, and 87 fragments, respectively). However, in contrast with the results in this study, not all AFLP fragments were polymorphic among the Chinese accessions. These differences might be due to the higher resolution using a DNA sequencer (ABI 373, Applied Biosystems) in this study, versus visual reading of AFLP gels as was performed for the Chinese accessions (Ke-peng et al., 2003). AFLPs are efficient in detecting polymorphisms in crop plants with limited genetic diversity as shown with cotton (Abdalla et al., 2001), cowpea landraces (Tosti and Negri, 2002), and cashew accessions (Archak et al., 2003). Bonnema et al. (2002) indicated that AFLP markers may cover wider genomic regions of tomato

that are not readily covered by RFLP markers.

EFFECTIVE USE OF MOLECULAR MARKERS IN WATERMELON BREEDING PROGRAMS. A commonly held view is that cultivated watermelon has a narrow genetic base due to recurrent selection and continuous inbreeding during 5000 years of cultivation (Navot and Zamir, 1987). The narrow genetic base is notably reflected in no or low isozyme polymorphisms among watermelon cultivars and accessions (Navot and Zamir, 1987). In a previous study (Levi et al., 2001), 288 RAPD markers produced low polymorphisms (92.5% to 98.9% genetic similarity) and could hardly differentiate among some of the heirloom cultivars. In this study, a comparable number of ISSR and AFLP markers (altogether 229) produced higher polymorphisms (80.2% to 97.8% genetic similarity) among all heirlooms. Also, the genetic similarity values among cultivars (Table 1) and their phylogenetic relations (Fig. 2) are consistent with the parental records collected by Elmstrom (1999) (Table 1), e.g., 'Garrison' and 'Garrisonian', 'AU-Producer' and 'AU-Golden Producer', 'Mickylee' and 'Minilee', 'Black-Diamond' and 'Black Stone', and 'Stone Mountain' and 'Stone Mountain #5'. Based on parental information (Elmstrom, 1999) the cultivars in each pair are closely related as shown in the dendrogram (Fig. 2). ISSRs and AFLPs efficiently differentiate among watermelon heirloom cultivars with limited genetic diversity. It is apparent that AFLP technology is more effective than ISSRs and RAPDs in identifying closely related breeding lines of watermelon, and in identifying triploid (seedless) watermelons derived from closely related genotypes. The large number of polymorphic ISSR and AFLP markers also provide better resolution with respect to relatedness among watermelon cultivars as compared with RAPD markers. Conversion of specific AFLP or ISSR fragments to sequenced characterized amplified region (SCAR) markers could enhance the value of these markers for watermelon cultivar identification. Furthermore, AFLP markers appeared to be clustered and cover linkage regions different from ISSR and RAPD markers, as recently shown in a linkage map constructed for melon (*Cucumis melo*) (Perin et al., 2002) and in our mapping experiments for watermelon (A. Levi, unpublished data). Thus, AFLP, ISSR and RAPD markers should complement each other in genetic identification and in covering different regions of watermelon genome.

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