Development and Characterization of PCR Markers in Cucumber

Gennaro Fazio,[1] Jack E. Staub,[2] and Sang Min Chung
U.S. Department of Agriculture, Agricultural Research Service, Vegetable Crops Unit, Department of Horticulture, 1575 Linden Drive, University of Wisconsin, Madison, WI 53706

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ABSTRACT. Highly polymorphic microsatellites or simple sequence repeat (SSR), along with sequence characterized amplified region (SCAR) and single nucleotide polymorphisms (SNP), markers are reliable, cost-effective, and amenable for large scale analyses. Molecular polymorhisms are relatively rare in cucumber (*Cucumis sativus* L.) (3% to 8%). Therefore, experiments were designed to develop SSR, SCAR and SNP markers, and optimize reaction conditions for PCR. A set of 110 SSR markers was constructed using a unique, strategically applied methodology that included the GeneTrapper (Life Technologies, Gaithersburg, Md.) kit to select plasmids harboring microsatellites. Of these markers, 58 (52%) contained dinucleotide repeats (CT, CA, TA), 21 (19%) possessed trinucleotide repeats (CTT, ATT, ACC, GCA), 3 (2.7%) contained tetranucleotide repeats (TGCG, TTAA, TAAA), 4 (3.6%) enclosed pentanucleotide repeat (ATTTT, GTTTT, GGGTC, AGCCC), 3 (2.7%) contained hexanucleotide repeats (CCCAAA, TAAAAA, GCTGGC) and 21 possessed composite repeats. Four SCARs (L18-3 SCAR, AT1-2 SCAR, N6-A SCAR, and N6-B SCAR) and two PCR markers based on SNPs (L18-2H19 A and B) that are tightly linked to multiple lateral branching (i.e., a yield component) were also developed. The SNP markers were developed from otherwise monomorphic SCAR markers, producing genetically variable amplicons. The markers L18-3 SCAR and AT1-2 SCAR were codominant. A three-primer strategy was devised to develop a codominant SCAR from a sequence containing a transposable element, and a new codominant SCAR product was detected by annealing temperature gradient (ATG) PCR. The use of a marker among laboratories can be enhanced by methodological optimization of the PCR. The utility of the primers developed was optimized by ATG-PCR to increase reliability and facilitate technology transfer. This array of markers substantially increases the pool of genetic markers available for genetic investigation in *Cucumis*.

Since the advent of the polymerase chain reaction (PCR) (Saiki et al., 1988), many techniques have been developed to detect DNA polymorphisms. Such polymorphisms have utility in plant genetics and breeding (Gupta and Varshney, 2000; Knapp 1998; Lee, 1995; Staub et al., 1996c). DNA fragment and sequence analysis has been used for genetic mapping experiments (Bradeen et al., 2001; Tanksley and Nelson, 1996), diversity analysis (Horejsi and Staub, 1999), and more recently marker-assisted selection in many crop species (Quarrie, 1996; Romagosa et al., 1999; Shen et al., 2001; Yousef and Juvik, 2001). For instance, isozymes, random amplified polymorphic DNA (RAPD) and restriction fragment polymorphisms (RFLP) have been used in cucumber (*Cucumis sativus*) for genetic mapping and diversity analysis (Dijkhuizen et al., 1996; Horejsi et al., 2000; Kennard et al., 1994; Meglic and Staub, 1996a; Meglic and Staub, 1996b; Serquen et al., 1997). The use of these markers has been limited due to the narrow genetic base (3% to 8% on a per band basis) of cucumber (Horejsi and Staub, 1999; Knerr et al., 1989). A higher level of polymorphism has been documented in *C. sativus*, and in *Cucurbita* and *Citrullus* species of the Cucurbitaceae using simple sequence repeats (SSR) (Katzir et al., 1996). Four of the seven SSRs used detected polymorphisms among the 11 cucumber genotypes examined which allowed for the calculation of gene diversity values ranging between 0.18 and 0.64 (Katzir et al.,

1996). More recently, 20 polymorphic SSRs were developed and tested in cucumber to more critically determine their potential value for genetic analysis. Polymorphisms were detected at 12 of the 20 SSR loci (60%) having two to five alleles at each SSR locus examined (Danin Poleg et al., 2000; Danin Poleg et al., 2001).

A moderately large molecular marker database now exists for cucumber that includes isozymes (Meglic and Staub, 1996b), RFLP (Dijkhuizen et al., 1996), RAPD (Horejsi and Staub, 1999), amplified fragment length polymorphisms (AFLP) (Bradeen et al., 2001), SSR (Danin Poleg et al., 2000), and sequence characterized amplified regions (SCAR) (Horejsi et al., 1999) markers. The application of these markers for diversity assessment has allowed for the partitioning of commercial germplasm into market classes, and the grouping of accessions in the U.S. National Plant Germplasm System into geographic proximity and country of origin. Their limitations for use in legal applications stems from their paucity, degree of consistency, and cost (Staub, 1999; Staub et al., 1996b; Staub and Meglic, 1993).

The characterization of additional polymorphic SSR, single nucleotide polymorphisms (SNP), and SCAR markers would allow increased precision in genetic similarity estimation among cucumber cultivars as well as in the mapping of qualitative and quantitative traits. Despite their high development costs, microsatellites have played an important role in genetic studies of higher eukaryotes, such as sweetpotato (*Ipomoea batatas* L. Lam.) (Buteler et al., 1999), cassava (*Manihot esculenta* L.) (Chavarriagaaguirre et al., 1998), peach (*Prunus persica* L.) (Cipriani et al., 1999), barley (*Hordeum vulgare* L.) (Donini et al., 1998), and beans (*Phaseolus vulgaris* L.) (Yu et al., 1999). Where SSR size polymorphisms can be resolved by high throughput and inexpensive technologies (e.g., agarose gel analysis), their codominant nature, and relatively high polymorphism rate make them amenable for many types genomic analyses (Donini et al., 1998;

Gupta et al., 1999). Likewise, the conversion of a RAPD marker to SCAR or SNP markers can enhance the marker's repeatability (Michelmore et al., 1991; Staub et al., 1996a).

The initial development of microsatellite markers requires the characterization of sequences flanking the repeat motif followed by the design of flanking PCR primers. Several methods have been devised for the initial characterization of the flanking sequences (Gupta and Varshney, 2000) including the use of a published gene sequence (Litt and Luty, 1989), database searches (Danin Poleg et al., 2000; Danin Poleg et al., 2001; Katzir et al., 1996), the construction of genomic or cDNA libraries with subsequent screening with labeled oligonucleotide probes (Bryan et al., 1997; Dayanandan et al., 1998; Huang et al., 1998; Roder et al., 1998), the construction of microsatellite-enriched libraries followed by PCR screening (Fischer and Bachmann, 1998), hybridization of biotinylated oligonucleotides to single stranded M13 libraries (Paetkau, 1999), and the use of bacterial artificial chromosome (BAC) libraries to target specific genomic regions (Cregan et al., 1999). The development of SSR marker technology for cucumber is a necessary step to increase map saturation for a more complete analysis of its genome and the application of this information for its improvement. A method for the discovery of microsatellites and their flanking sequences is described herein along with the identification of 2 SNP markers and the development of 110 microsatellite and 4 SCAR markers and their optimization in the PCR.

Materials and Methods

SSR marker development

The development of SSR markers (Fig. 1) involved the construction of a cucumber DNA genomic library, and subsequent capture of library inserts aided by GeneTrapper technology, followed by the sequencing of captured products (Fazio and Staub, 2000; Fazio, 2001). Sequence information was then used to develop primer pairs, which flanked target SSR regions.

DNA EXTRACTION, RESTRICTION AND SIZE FRACTIONATION. Genomic DNA from cucumber line GY7 (released by the University of Wisconsin, 1996, R.L. Lower; evaluated as experimental line G421) was extracted from young leaves (1 to 15 cm^2) and apical meristems according to a nuclear DNA extraction protocol (Sambrook et al., 1989). Extraction products were treated with RNAse ONE (Promega, Madison, Wis.). Recovered DNA was quantified using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). In separate reactions, 100 mg of DNA was restricted using *Eco*R I* restriction enzyme (Promega) and *Acs* I (Roche, Indianapolis, Ind.), which recognizes the sequence (A or G)/AATT(T or C), and creates compatible ends with *Eco*R I restriction site. The star activity in *Eco*R I was initiated by adding 10% DMSO to the restriction buffer (Ander-

son and McDonald, 1993; Robinson and Sligar, 1995). This activity generated additional restriction fragments ranging 200 to 1300 bp as opposed to *Eco*R I normal activity.

Since the then available sequencing techniques could only resolve ≈800 bp, fragment sizes between 200 to 1300 bp were needed to avoid unnecessary subcloning and to simplify the sequencing process. Restricted DNA was size-fractionated using a low pressure, 75-cm-long, 16-mm-diameter, chromatography column (Fisher Scientific, Pittsburg, Pa.) filled with Sephacryl S-500 (Pharmacia, Peapack, N.J.). The column was packed according to manufacturer's specifications for Sephacryl S-500 and equilibrated with 200 mL elution buffer (0.1 M Tris-HCL pH 8.0, 0.15 M NaCl, 0.001 M EDTA) at 0.3 mL·min^{-1} before fractionation. Both samples (*Eco*R I* and *Acs* I) of restricted DNA were fractionated (0.3 mL·min^{-1}) separately, and eluted DNA was collected in 75 2-mL aliquots. Eluted DNA was precipitated, washed and resuspended in TE. Fragments from eluted aliquots of both restrictions were then sized by agarose gel electrophoresis

Fig. 1. Methodology for the development of cucumber simple sequence repeat (SSR) markers using the GeneTrapper kit (Life Technologies, Gaithersburg, Md.).
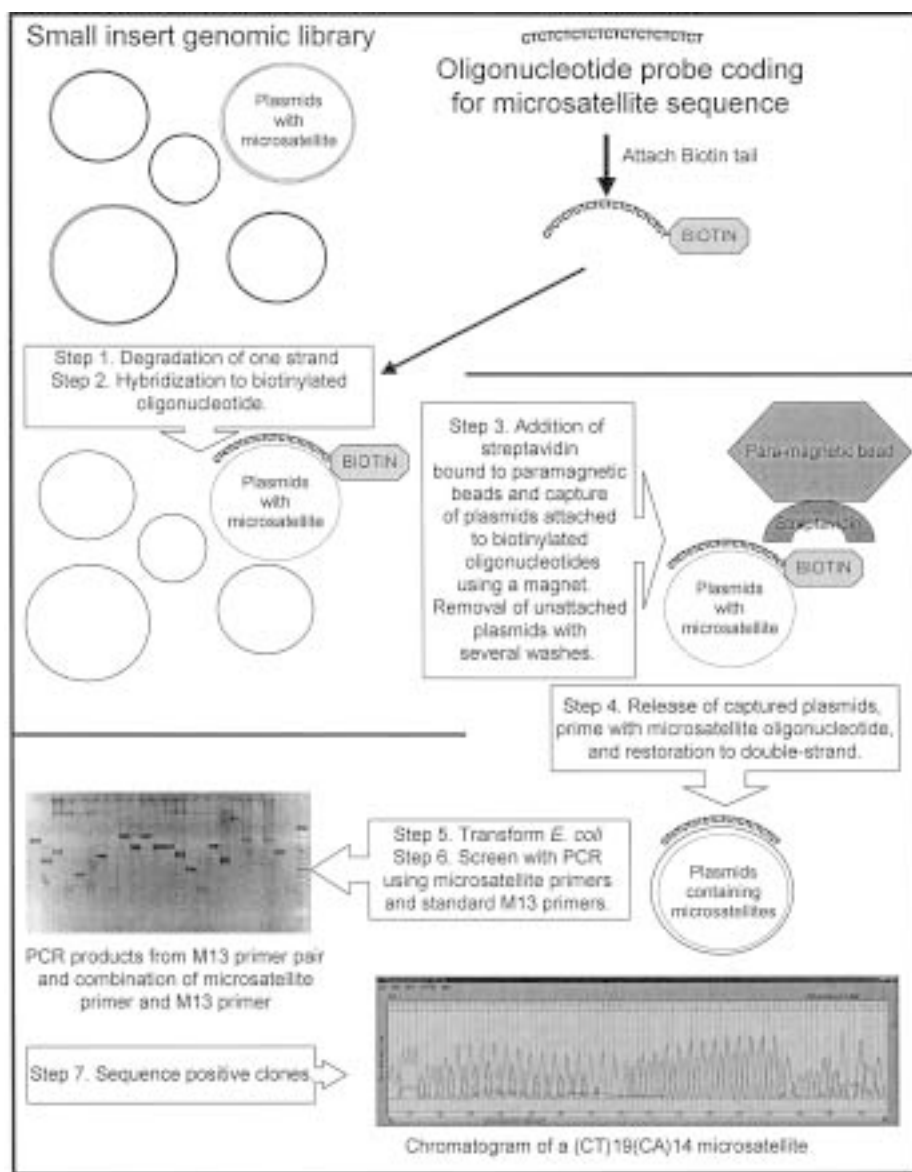
and simultaneously compared to the nonfractionated digestion products from each restriction. Fractions between 200 to 1200 bp were chosen to create a small insert genomic library, and then they were combined for ligation to a vector. These size fractions were chosen because of the increased difficulty of sequencing larger size inserts.

LIBRARY CONSTRUCTION, MASS EXCISION, AND PLASMID DNA EXTRACTION. Fractionated DNA was ligated to Lambda Ziplox *Eco*R I arms (Life Technologies, Gaithersburg, Md.), and then it was packaged in a lambda vector with Gigapack III Gold packaging extract (Stratagene, La Jolla, Calif.). The resulting library was titered and packaging efficiency was determined by blue-white colony screening following the manufacturer's specifications.

Primary Ziplox libraries were mass excised *in vivo* into the plasmid vector, pZL1 (Life Technologies) in the strain of *E. Coli* DH10B Zip (Life Technologies) as per manufacturer's recommendation. The mass excision of the lambda vector into a plasmid and the expansion of the plasmid library were performed simultaneously in a semi-solid medium according to the manufacturer to minimize representational biases (some cell lines dividing faster than others) that can occur during colony expansion in liquid media. The growth media was prepared by adding 1.35 g of SeaPrep agarose (FMC Bioproducts, Rockland, Maine) to 500 mL of 2× LB in an autoclavable bottle using a stir plate to avoid the formation of agarose clumps. The bottle was autoclaved for 30 min and cooled in a water bath until the media reached 37 °C. Ampicillin (Fisher) was then added to the media (150 mg·L^{-1}) and 5×10^5 primary transformants from the ZipLox libraries were added to the bottle while being stirred. The bottle was subsequently placed in a 0 °C water bath for 1 h to solidify the media. The culture was incubated undisturbed at 30 °C for 45 h, and then centrifuged at 3000 g_n for 20 min. The supernatant was discarded, and the recovered cells were resuspended in 100 mL of 2× LB Glycerol (12.5%). Subsequently, 100 mL of LB media were inoculated with 2.5×10^9 cells resulting from the mass excision, and the mixture was then cultured at 30 °C until the culture reached an OD of 0.8. Plasmid DNA to be used in the GeneTrapper protocol was extracted by with the plasmid Maxi-Prep Kit procedure (Qiagen, Valencia, Calif.).

CAPTURE OF PLASMIDS CONTAINING MICROSATELLITES. In preparation for the GeneTrapper (Life Technologies) reaction, five oligonucleotides (20 to 30 bp) homologous for common microsatellite motifs [(CT)$_{12}$, (TG)$_{12}$, (CTT)$_9$, (TCC)$_9$, (ATT)$_9$] were modified at the 3' end by the addition of biotin (biotin-14-dCTP) with a terminal transferase enzyme provided with the GeneTrapper kit. Addition of the biotin to the oligonucleotides was verified by electrophoretic analysis of the products on a 15 × 17 × 0.7 cm-thick 16% (w/v) 19 acrylamide : 1 bisacrylamide gel, containing 8 M urea, and 1× TBE buffer, for 1.5 h at 400 V. The gel was stained in an ethidium bromide solution (0.5 mg·L^{-1}) for 15 min. Reaction products were visualized using the Dark Reader transilluminator system (Clare Chemical, Denver, Colo.). Technical protocols for use of GeneTrapper technology are summarized below as steps 1 through 7 and in Fig. 1.

1) Plasmid DNA from the cucumber genomic library was treated with Gene II protein and Exonuclease III to obtain single-stranded circular plasmid DNA. The products from the GeneII and Exonuclease III treatments and the untreated plasmid DNA were electrophoresed on a 0.7% agarose gel stained with ethidium bromide (0.5 µg·mL^{-1}) to verify the formation of single-stranded plasmid DNA.

2) Single-stranded DNA was then separately hybridized to each of the biotinylated oligonucleotides. GeneII/ExonucleaseIII-treated DNA was added to a solution containing hybridization buffer, and then subsequently denatured at 95 °C for 1 min and chilled on ice for 1 min. Then a 1-µL aliquot of the biotinylated oligonucleotide (20 ng·µL^{-1}) was added to the denatured DNA and incubated for 1 h at 37 °C.

3) Streptavidin bound to paramagnetic beads was used to capture the microsatellite containing single-stranded plasmid molecules from Step 2. The hybridization mixture was incubated with a streptavidin bead suspension for 30 min at 37 °C with gentle mixing. The streptavidin beads were then separated from the reaction by means of a microcentrifuge tube rack fitted with a magnet. The capture products were washed four times to eliminate any unbound single-stranded DNA or intact plasmid DNA.

4) The captured product was then treated with elution buffer to release the captured single-stranded (SS) DNA from the streptavidin-bound biotin (Step 3). The SS DNA was coprecipitated in the presence of glycogen by the addition of 7.5 M NH$_4$Oac and 100% ethanol, and immediately centrifuged at 4 °C (14,000 g_n) for 30 min. The SS DNA was subsequently repaired to form double-stranded DNA by adding 50 ng of unbiotinylated capture oligonucleotide to a mixture containing a repair enzyme, a dNTP mix (1 mM), captured SS DNA, and 10× reaction buffer. This reaction mixture was incubated initially at 90 °C for 1 min, then at 55 °C for 30 s, and lastly at 70 °C for 15 min.

5) The resultant double-stranded plasmids (Step 4) were used to transform DH10B *E. coli* by standard electroporation. A Cell-Porator system was used in conjunction with the Cell-Porator voltage booster (Life Technologies) (0.15 cm gap chamber; 400 V; 330 mF; low ohms; fast charge rate; voltage booster resistance 4,000 ohms) to transform 23 µL of ElectroMAX DH10B cells mixed with 2 mL of the double-stranded repaired capture reaction products (Step 4). Initially, electroporated products were rescued by incubation in 1 mL SOC. media at 37 °C for 1 h. In subsequent captures, the transformations were incubated for only 25 min to reduce the amount of duplication in the captured products. The rescued products were plated onto ampicillin (150 µg·mL^{-1}) LB agar selection media and incubated for 8 h at 37 °C. Theoretically, only *E. coli* harboring the intact pZL1 (putatively containing a microsatellite region) derived from the cucumber DNA library survived the selection media challenge.

6) Single colonies were picked, named sequentially, and used to inoculate 3 mL of LB Ampicillin (100 µg·mL^{-1}). The inoculated media was incubated at 37 °C overnight, and then plasmid DNA were extracted using the QIAPREP miniprep kit (Qiagen) according to the manufacturer's protocol. Plasmid DNA was amplified by PCR [3 mM MgCl, 0.2 mM dNTPs, 15 to 20 ng of DNA, 0.4 mM of each primer, Taq polymerase (3 units) and commercial 10× buffer (Promega)] containing the capture oligonucleotide along with the standard forward or reverse M13 primer to confirm the presence of a microsatellite in the plasmid.

7) Plasmid DNA (Step 5) (4 µL at 200 ng·mL^{-1}) from positive clones was added to a 16-µL sequencing reaction mixture containing 4 µL of 2.5× reaction buffer [5× is 400 mM Tris pH 9, 10 mM MgCl2], 4 µL of BigDye enzyme mix (Applied Biosystems, Foster City, Calif.), 1 mL of M13 forward or reverse primer (20 pmol·µL^{-1}), and 7 µL of distilled water. Sequencing reactions were run on a thermal cycler (9700;

Perkin-Elmer, Boston, Mass.) (45 cycles of 95 °C 20 s, 55° 30 s, 60° 4 min, and then 72° 7 min). Subsequently dye terminators were removed using MicroBioSpin columns (BioRad, Hercules, Calif.). The sequencing reaction products were resolved using a sequencer (ABI 377; Perkin Elmer).

These steps were repeated in five different experiments with different biotinylated oligonucleotides. The results of the biotinylated (CT)12 oligonucleotide capture procedure is reported herein.

SEQUENCE ANALYSIS AND PRIMER DESIGN. All sequences from the capture were analyzed and compared to each other using the assembly feature provided in the GeneTool 1.0 software package (Biotools, Edmonton, Canada). This allowed for the elimination of duplicates and identification of overlapping DNA regions. The resulting unique sequences were then entered into the primer design software OLIGO 6.0 (Life Science Software, Long Lake, Minn.). Initially, no restrictions were placed on the size of the oligonucleotides to be constructed, and thus the size of the oligonucleotides in any specific primer pair was determined by closely matching the Tm (melting temperature) of each primer in the pair. This construction strategy sometimes resulted in primers 14 to 16 bp in length, which were not amenable to infrared analysis protocols using the fluorescent M13 tailed primers technique (Oetting et al., 1995) then being implemented in some laboratories. Therefore, all subsequent primers were designed to have a minimum of 24 bp in length, be hairpin and duplex free, while possessing the possibility of a 3' GC clamp and no false priming sites. Although these general guidelines were followed in the main, in certain cases the flanking sequences available did not meet all the guideline prerequisites requiring a relaxation of the acceptance criteria.

Development single nucleotide polymorphism (SNP) and characterized sequence (SCAR) markers

Two inbred lines, G421 and H-19 (University of Arkansas-Fayetteville, 1993) used in a previous cucumber genetic mapping project (Serquen et al., 1997) were used as sources for template DNA in PCR to identify single nucleotide polymorphisms and/or polymorphisms caused by insertion/deletions (Indel) within SCAR markers (agarose analysis) monomorphic between G-421 and H-19. These markers were selected because of their potential usefulness in current mapping projects.

Two putative loci (L-18-2-SCAR, and N6-SCAR) were amplified using SCAR primers in both of the mapping inbred lines and two loci (RAPD L-18-3, AT1-2) were amplified with RAPD primers L-18 and AT-1 (Operon Technologies, Alameda, Calif.). L-18-2-SCAR, was monomorphic between G421 and H19 lines and N6-SCAR amplified bands of different sizes in H-19 and G-421 suggesting the possibility of a codominant genetic nature. Further analysis revealed that N6-SCAR marker under standard PCR conditions failed to amplify the higher molecular weight band in the F₁ hybrid as well as in the heterozygotes in an F₂ population thus making this potentially codominant marker behave as a dominant one. Because of these attributes, experiments were designed to improve the performance of those markers.

Amplicons were separated on a 1.6% agarose gel (Life Technologies) and stained with ethidium bromide (0.5 µg·mL⁻¹). Banding patterns were visualized using a Dark Reader transilluminator, which does not use UV light, and therefore does not damage the DNA during visualization ultimately increasing transformation efficiency during subsequent cloning procedures. DNA bands were cut out of the gel and extracted using the Qiagen Gel Extraction Kit (Qiagen). The protocol from the pGEM-T Easy Vector System II cloning kit (Promega) was followed to ligate PCR products into the pGEM-T vector and transform competent JM109 *E. coli* cells with the ligated vector–insert. Plasmid DNA of colonies with positive insertional events identified by blue-white visual screening were extracted, and then sequenced using the same methods described in the previous section (Fig. 1, Step 7, microsatellite capture). Likewise, sequence analysis and primer design were performed using software and procedures previously described. However, in this case sequences of each marker band from line H-19 were compared to corresponding band sequences in G421 to identify mismatch polymorphisms (SNPs) and insertion-deletion (Indel) polymorphisms. Sequence comparison information was then used strategically to design SNP and SCAR primers.

Optimization and customization of primer-pair-specific PCR conditions

An optimal PCR primer-pair design for amplification of specific DNA can be predicted by computer driven formulae and algorithms (Breslauer et al., 1986; Freier et al., 1986; Rychlik and Rhoads, 1989; Rychlik et al., 1990). Factors considered by these programs include 1) primer melting temperature (Tm), 2) product melting temperature, 3) primer sequence uniqueness with respect to the source sequence, 4) primer GC content, and, 5) 3' end GC base anchor motif. However, such calculated properties often do not coincide with the information obtained from empirical assessment of primer-pair product performance obtained under different reaction conditions. Thus, PCR optimization of primer pairs designed to amplify SSRs, SNPs, and SCAR markers was performed by subjecting such primers to annealing temperature gradient PCR (ATG-PCR). Temperature gradient PCR amplifications were performed in a Eppendorf Master Cycler (Eppendorf, Hamburg, Germany) having a standard 12 column (1 to 12) × 8 rows (A to H) microtiter format providing row-dependent response information across rows (i.e., 12 temperatures in each column). The PCR reactions were performed in 15-µL volumes of PCR mix [3 mM MgCl, 0.2 mM dNTPs, 15 to 20 ng of DNA, 0.4 mM of each primer, 2 units of *Taq* DNA polymerase and 10× commercial buffer (Promega)] overlayed with 10 µL of light weight mineral oil (Fisher). The ATG-PCR cycling conditions were conducted in split-well plates (Robbins Scientific, Sunnyvale, Calif.) as follows. A melting step (94 °C for 3 min), 40 PCR cycles (30 s at 94 °C, 1 min gradient annealing step 45 to 65 °C, and 90 s at 72 °C), and an elongation step (7 min at 72 °C).

Initially, DNA from H-19 and G-421 was used to detect locus-specific polymorphisms. A PCR master mix was initially prepared without DNA or primer, and then divided into two aliquots to which DNA was added. Each PCR master mix was then divided into eight aliquots to which primer pairs were added resulting in primer pair × DNA arrays having 12 temperature gradient replications. Split-well technology resulted in PCR reactions in each well containing identical primer pairs but different DNA (H-19 and G421) such that gradient temperature variations were minimized in each paired comparison. PCR reactions were resolved on 2% agarose gels (Life Technologies) with ethidium bromide staining (0.5 µg·mL⁻¹), and visualized using a Dark Reader transilluminator coupled with a closed circuit digital (CCD) camera.

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Results and Discussion

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Development of PCR markers based on simple sequence nucleotide repeats

DIGESTION, SIZE FRACTIONATION AND PLASMID LIBRARY CON-

Table 1. DNA sequences, optimal annealing temperatiure (°C), and expected product size (EPS) (bp) for 110 SSR and two SCAR markers characterized in cucumber (*Cucumis sativus* L.).

No.	Marker	Sense primer 5' to 3' (top) / Antisense primer 5' to 3' (bottom)	SSR motif	EPS (bp) (top) / Temp (°C) (bottom)
1	CS-AT1SCAR	CACTCTTTTGGGAGGGACATTGTG	IN-DEL, SNP	210
		AACCTCATTAACTAATAAAGAGATG		45–57
2	CS-L18-3SCAR	CTCTTTCAATCATCTTTCTTCTCT	IN-DEL	327
		ATCATAACAATGATATATTTTACG		45–57
3	CSWACC01	GATTACCGGACACTTTTGAACGAG	(ACC)5(A)8	305
		TGTTTTGCCTTTACAATAGATGCC		57–65
4	CSWACC02	TTGGCGTTGGAACTCCTGATGTTT	(ACC)7	227
		CCTCCTCAAGACTTGAGAGACGAT		58–60
5	CSWACC03	ACAATAGATGCCTTTTATGATAAT	(GGT)5	279
		TTTTGAACGAGAGGTGTTTCGATC		53–65
6	CSWATT01	ATATGTGTATCTAGGGGTAATTCA	(TG)4(ATT)3	218
		AATTCCAAAATCCATTTCGTCGTC		55–65
7	CSWATT02	CCAGTACAAATAACATCCCGAAGC	(ATT)5	312
		CAAACCTCTTTCAGTACCGGAATA		55–65
8	CSWATT04	TTTTGCCATCCCTAACAGTTAGAA	(TAA)4TTA	252
		CTTTTCCTGACGTCGATGGAG		45–50
9	CSWATT05	CGGTGAAAGAAAATCAAGA	(CTT)5(TAA)4	244
		TTTATGTATATTGTCCTTAGTTGT		45–57
10	CSWCOM03	TTGTCATACAGACGGTTTCGCTCG	(CCCAAA)3	278
		ACTTGGTTGAGGTGAATCCGCATC		45–65
11	CSWCOM04	CATTAACAAATATCAACTTGTTCG	((AC)2(GC)2)2(AC)4	291
		GGTAAAATTGGATAAGACCGCATT		45–50
12	CSWCOM05	CCACTAATTCCACAAATCCCGTCA	(TAAAAA)3(G)11	164
		GACTAGCCAAGAAGACCAATTACC		45–50
13	CSWCOM06	GAATCAAGTATACTAGTGAATATG	(GAAA) motif	275
		AGAGTTTAAAGGTATACACAATAC		45–62
14	CSWCT01	TTCTGATCAACGACGAAG	(CT)9(CT8)	242
		GAAACAAAAGCCTCCATTG		58–65
15	CSWCT02B	TTCTGCATACCCTCTCCT	(CT)21(TG)8	203
		CACACTTCCAGATGGTTG		55–58
16	CSWCT03	TTCTCAGAACTGCCACTG	(CT)16	390
		CACTCTTGAGGGGAAAAA		48–52
17	CSWCT04	ACTATTGGGTCTCTCCTA	(CT)6	556
		GACCCGAGGTTATTATT		48–54
18	CSWCT05A[z]	GTGTCGAAGTAGCCAACTTCAATG	(A)5,(A)11	321
		ATTCCTATAAAATAAAAGAGTTCG		47–62
19	CSWCT05B[z]	ATACGAACTCTTTTATTTTATAGG	(CT)14, (T)10	224
		ATTAAGGAGATAAGAATTGTGTTG		47–62
20	CSWCT06A	TTTAAATTCTTCCTAACC	(CT)9	200
		TTTGCTTTGCATTTGGAT		45–55
21	CSWCT07A[z]	GTACATCAATTGTTTCAATTAATG	(GTTTT)4	218
		TTGGCGTTGAAAGGATTAAATAAG		45–62
22	CSWCT07B[z]	CTCTCTTATTTAATCCTTTCAACG	(CT)10	196
		CCATATTATCATCTGGTAAAATTG		48–60
23	CSWCT07C[z]	CAATTTTACCAGATGATAATATGG	(ATTTT)3 AAATTT	316
		GATTTTTATAGTTCCATCTCTAAC		47–62
24	CSWCT08	ATTTCTTAAACCACCACA	(CT)11	594
		ACCGTGTGTTTAGAATGC		65–68
25	CSWCT09	TATCCTTTAATGCTTTCG	(CT)10	273
		CTAGCCTTGCATTTTGTT		45–55
26	CSWCT10	AGATCGGAATTGAAAAAG	(CT)9CTTTTCT	183
		AAAGGGGCTTCCTCTCTA		55–60
27	CSWCT11	ATAGGCAATATGGCTTCT	(CT)9(A)13	226
		CACTTCAATGGAGTTTCG		45–50
28	CSWCT12[z]	GAATTCCCGTCGGCCTTCCGAGCT	(GCTGGC)4 (CT)12	297
		GAAGCAAAACCTGTATCGCCGATG		61–65
29	CSWCT12A[z]	GAATTCCCGTCGGCCTTCCGAGCT	(GCTGGC)5	174
		ATGCAATGGAAGAGATGATGATTG		55–65

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Table 1 (continued). DNA sequences, optimal annealing temperatiure (°C), and expected product size (EPS) (bp) for 110 SSR and two SCAR markers characterized in cucumber (*Cucumis sativus* L.).

No.	Marker	Sense primer 5' to 3' (top) / Antisense primer 5' to 3' (bottom)	SSR motif	EPS (bp) (top) / Temp (°C) (bottom)
30	CSWCT12B[z]	TTCAATCATCATCTCTTCCATTGC	(CTT)2(CT)12	172
		TGAAGCAAAGATAGTATGGAATGG		63–68
31	CSWCT12C[z]	TTCAATCATCATCTCTTCCATTGC	(CTT)2(CT)12(CTT)3	332
		TCGATGGTGACCATGCGAGCACCA		63–68
32	CSWCT13B	TGTGATCAACCAACTTCA	(CT)17(TG)13	290
		GAATTATGGGTTCATTTT		45–58
33	CSWCT13Balt	ATAGGGCAATTTGTCTCT	(CT)17(TG)13	293
		CACTGGGATCCTAACAAC		45–57
34	CSWCT13C	AGAATCAATTTCAAACCA	(GAA)motif	278
		CATCATGCAGGCTTACAC		45–62
35	CSWCT14	TCCAATCGACCTAAATCCAAA	(CT)7	343
		TGGCCCTAACTGTTGATGC		58–65
36	CSWCT15[z]	GAATTCGCTCACTTCCGAAAAGAG	(CT)11	350
		GAATTCTCGCCGGAGGTCAGGTAG		53–65
37	CSWCT16B	CTTATGGTCGGAGAAG	(CT)14	226
		CTCAGATAACCCAAAATA		58–62
38	CSWCT17	TTGAATTATGGGTTCATTTTT	(CT)19(CA)6	193
		GACAATGATAAACTTCCCTGA		55–62
39	CSWCT18b[1]	AATAAATATATACGTGGCGTGATA	(CT)17(CA)6	316
		ATTCCAAAGCTTAATGGAAGAATC		55–65
40	CSWCT19	CAATTAAAAAAATCGACGAGAAAG	(CT)7	124
		GAATTCCCGTCGGCCTTCCGAGCT		63–68
41	CSWCT19alt	GCATTGGCCCTTTTTGACCCG	(CT)7	257
		GAATTCCACAAATTAAAAGTC		55–65
42	CSWCT20	GGAAACCTAATAAGAATATGG	(CT)7	286
		GAATTCGGTGATAGACATTGA		45–48
43	CSWCT22A	GGGAGTATCGAAACAAAGC	(CT)7GTT(CT)9	229
		TTCTGATCAACGACGAAGTAA		45–55
44	CSWCT23	GAGATATTATGTCTGCCATTACTG	(CT)9	334
		AAAAAGTATAATTTGACGGTAGAT		50–60
45	CSWCT24A	AGGACATTGGGAACTGCTATGGAT	(CTT)5(GTT)4(CT)10	172
		GAATTCTGTTCTTGGAAGAGTATC		59–61
46	CSWCT24B	ATCGCTTTATCTTCGGCTTTGATG	(CT)7(CT)11(CT)7(A)8	214
		AATCCATAGCAGTTCCCAATGTCC		60–65
47	CSWCT24C	AATTCTTGGGCCATTTAATTGTTG	(ATT)5(A)8(A)7	419
		TCTCTTCGGACATCAAAGCCGAAG		55–65
48	CSWCT25	AAAGAAATTAAGTCAATCAAACCG	(CT)5CTT(CT3)	357
		CCCACCAATAGTAAAATTATACAT		45–63
49	CSWCT26	GACAATGTTGCCGACCAAGTAAGG	(CT)11	346
		CTTCTTTAATCACTTGTCTAATCC		60–65
50	CSWCT27	TCAGGTTCATGAATGTAGGGAATG	(CT)11	273
		AGGGCTGTGGAGTGATATTGTAGA		56–60
51	CSWCT28	GAATTCAAAAGCATTTCAAAACTA	(CT)10(TA)9	207
		GAATTCAATTGGGTTTTTGAACCC		45–65
52	CSWCT29	TGGACGAGTTGCTCTTGTAAGCCT	(CT)8	270
		ATCAAACTTGGCATGTGGCATGAC		45–65
53	CSWCT30	CATGAATCTCAAGTCTTAAACCC	(CT)8	186
		AAAGGATTGAGAAAGAAATTAAGG		55–65
54	CSWCT31	GAATTTATTGATAGTTTAAAACAA	(CT)15	215
		GAATTTCATCATGACACGTTTCAG		45–62
55	CSWCT32	GCATTAAATTGGAAAGGGGAATCA	(CT)9(CTT)3(CCT)3	393
		TGTCCTTTAATTTGGAAATTGAAT		45–52
56	CSWCT33	ATCTACCATATTCCAACCTAAACA	(CT)6(TATTTC)2	218
		GTGGCTTTTGTGGAAAGCATCTTC		45–60
57	CSWCTT02A	AGATAACGCGGGTCAAAT	(CT)14(TA)7(CA)5(T)11	640
		GTAGGTTAATTACCAAAA		48–54
58	CSWCTT02C	TCCTTCTTTTCCTCGCTCTG	(CT)14(TA)7(CA)5	229
		TGCCCTAAAATCCTCACACTC		58–62

No.	Marker	Sense primer 5' to 3' (top) / Antisense primer 5' to 3' (bottom)	SSR motif	EPS (bp) (top) Temp (°C) (bottom)
59	CSWCTT02D	CATCCTCATTCATGGCGGAGTGTG	(T)6, (T)11, (CTT) motif	246
		GAATTTGTTAAAAATGTACATTAA		45–60
60	CSWCTT02D	CATCCTCATTCATGGCGGAGTGTG	(T)6, (T)11, (CTT) motif	246
		GAATTTGTTAAAAATGTACATTAA		45–60
61	CSWCTT03	GCATATCGATTTAGGTCTAAT	(CTT)4(T)11	184
		GGATGGAGCGTCTCTTA		49–52
62	CSWCTT04	GCGGAGATATGCCAATTTCAA	(CTT)4(A)13	231
		TTCAAGCGCGACGACAAT		50–65
63	CSWCTT05	AGTTAATGAGTGAATGTGCGAGTT	(CTT)5	424
		GAATTCCGTTCATATGTGGGATAC		45–50
64	CSWCTT06	TTTGAACTTTTTGATCCGCTTTTC	(GTT)2(CTT)5(ATT)2	423
		CAGAAGGCGTGTATTAGCTTGATA		52–65
65	CSWCTT07	TTGTTGCAGGCCAGTCTATACGGT	(CTT)6	275
		GAATTCAAATCAAGGTAGCCGATG		54–65
66	CSWCTT08	GATATAAGCGTTGTGAGGATATGC	(TAA)3(CTT)6	335
		CGTGCTCTATGAAGTAAATTAGTA		55–65
67	CSWCTT09	AAATTCTTTCCCATTCATCAACTT	(CTT)5	336
		AAGCCTTCTTGTACCTCTCAATAG		45–50
68	CSWCTT10	CTTCTCACCTCAAACCCCAATTTC	(CTT5)	371
		TGTTGACGCAGTTTAGCCGATTCT		52–55
69	CSWCTT11	ACAAAGGGCAGGGACGTAGTCAAC	(CT)6(CTT)4(CT)9	382
		AATTAACGAACCTGAACCGGAAAT		60–65
70	CSWCTT12	CTGCTCCATCTTGAAAACATGCTG	(CTT)6	227
		TATTACAGTTCGAGCAATTCTGCA		61–65
71	CSWCTT14	AAAATATGAAACCCATGGACATGA	(GAA)9(TA)4	263
		GATTAAATATTGGGAATTGCTAA		45–52
72	CSWGAAA01	TTAGACTTTTATCTATGATTG	(CT)4	218
		TTTATTTTATAACAGATTTCA		45–55
73	CSWGAAA02	AGGGCGTGTGAAAATTTGATATAA	(CTTT)8	316
		TTCGAGAGTGGAGGGCACTTTCGT		45–65
74	CSWGAAT01	GTCGGCTTGTGAAGAGAGATTGTG	(GAAT)5	276
		GTGGGCACTGGTCAGGCGTTGAGA		45–65
75	CSWGAT01	ACCCGTTCGTCTGTCTCT	(CTT)4	235
		CCGACCTCTAGGGGATAA		45–55
76	CSWGATT01A	ATTCAAGCAGTTTCTTTGAAAAGA	(A)12, (GA)10	377
		CAGAATTGCTTGTTGCTGATGACT		55–68
77	CSWGATT01B	TATCAGACGATCTCCATCCAGCAG	(GA)10	181
		CAGAATTGCTTGTTGCTGATGACT		65–68
78	CSWGATT01C	TATTGAAACAGAAATTAACATTGG	(GAA)10	155
		TCTTATCCACATTCCATTAAGAAG		55–68
79	CSWGATT01E	GTGCAATATGCATTTCTTC	(GGGTC)3, (AGCCC)3	235
		GAATTCCAAAGTTGGAGAAAG		55–68
80	CSWGCA01	AGTGATGGTGCAGGGCTATCTTAT	(GCA)8	235
		TTGTCTTCCCTCCTCTTCTCGTCT		55–65
81	CSWGTA01	TGTATGAATGTTTCTTACAAG	(GTA)5, (GAAA) motif	172
		AGGGTTTTATGTATTTGCATG		45–60
82	CSWTA01	AACATTCTCTATAAACATCTT	(TA)7	254
		GGTTTCCCTAATGATATAA		45–52
83	CSWTA03	ATTGTGATTATCATGGGAGCTATA	(TA)10(TA)20	413
		AAACCAAGAATGATTTGTAATATG		51–54
84	CSWTA04	TAAACATATGTGATTATACAGCAA	(A)11(TA)10	314
		GTGTTTTGGTGTTATGTGAATATC		57–60
85	CSWTA05	GCATGAGCTCGAGCTGGTGTAGTG	(TA)12	278
		CGCCTGTTTTCATTTTGATTGGTT		55–65
86	CSWTA06	GAATTTTAAATTTTTATGGGTCAT	(TA)7(TA)16	292
		AAACTTACCACTGGGGTGTAAATG		45–65
87	CSWTA07	TGCGATTTGAGACCACCTATTGAT	(TA)4(TA)7	355
		AAACAGGGACATAGCATGGATCTA		53–65
88	CSWTA08A	AAAGTGCGGGACTCACGCTTAATA	(T)8(A)13(TA)6	347
		CAGTAGCTTTTTATAGTACCTAAC		45–60

Table 1 (continued). DNA sequences, optimal annealing temperatiure (°C), and expected product size (EPS) (bp) for 110 SSR and two SCAR markers characterized in cucumber (*Cucumis sativus* L.).

No.	Marker	Sense primer 5' to 3' (top) Antisense primer 5' to 3' (bottom)	SSR motif	EPS (bp) (top) Temp °C (bottom)
89	CSWTA08B	TTGCATTAATGCTATAAACTTACC GAAATTAATATTTAGGCATTG	(T)7(TA)7 IN-DEL	630 54–56
90	CSWTA08C	TAATATTGCATTAATGCTATAAAC TCCTTAATTTCCCGCAATGCCTAA	(T)7(TA)7	556 53–65
91	CSWTA09	CTACAAAACCTCTCATTCCTTATT TCTACTTTTAAATTTAGCACAACT	(TA)4(TG)3(TA)6	246 55–65
92	CSWTA10	TATAAGAGGACGCCATTAGCGACG ACTCACTGATCAATCATCCAGAGA	(TA)4(TTAA)3(T)9	435 55–65
93	CSWTA11B	GGTAGGCAATCAAAGAGTGGATGG AACATATAGGAATCTAACAAAGTG	(TA)10(CA)7(TA5)	390 53–60
94	CSWTA11C	GTTAAAGTATAAGTATAGTCTCCC ACTTCCACTTCTCGCTTGACTCTC	(TA)8	564 45–56
95	CSWTA13	AGATGGGCAGTTAGAGTTGATGCT CATTTAAAGCCTCATCAACACCTC	(TA)8 (GGTT)4	303 55–65
96	CSWTA14	GAATTCTAAACGATTCTTGGATTG GAATTCCGAACATTTCCTATAAAC	(TA)6	301 45–65
97	CSWTA15	GAATTCTACGAATCATTAAACAAT AATTCCTTTTCTTGTTCTTGTGTC	(TA)8	239 47–65
98	CSWTA16	TTATCAACCCTTCGTTTGCAAGTT CATTGCCATTCAAGCAAGTGATTC	(TA)6,(TA)3	332 45–60
99	CSWTA17	CTTTGATGAGACTTCTAATCCCAA CCATGATTCAAGAAGATCAAATGA	(TA)6(CTT)3(TA)6(T)11	198 45–60
100	CSWTAAA01	CAATGCCTCAATCTGATAGGAATG ACTGGCTCTCTACATATTGTGAGG	(TAAA)4	306 57–65
101	CSWTG01	ATTGTCTGATTCGGGTATG GGCAGCAAGAGCACTACC	(TG)5	178 45–65
102	CSWTG02	CTTTTGGACCGTTTTTGG AAGAACCGCTCGGAGATG	(TG)3-(TG)4	240 58–65
103	CSWTG03	GAATTCAAGAGAAATTTCCAAGGT GAATTCAACTTCATAAGCATACGA	(T)13(TG)7(T)7	273 52–65
104	CSWTG04	GAATTCCAAAATGTTGAATACGAG GAATTCCGTATATTAATGTATTAT	(TG)9	201 52–57
105	CSWTG06	ATCCAAGTTTTCTTAGTTGTTACA GAAAGAAATTGACTTGACTACTAC	(CA)5(GAAA)4	344 45–65
104	CSWTG07	GAATTCAAATTCAACATGCGTGCG ATTTCAAAATTAAAAAATATCAAC	(CA)5N5(CA)4N(CA)3	249 68–72
105	CSWTG08	ATTCAACATGCGTGCGTTCGTGCG AATATCAACTTGTTCGATATCTTT	(TG)5N3(TG)4(TGCG)5	246 45–65
106	CSWTG09	GAAGATACATAAATGTAGAAATGA CCGCAATGAATAAGATCCAACACA	(TA)5(CA)11	262 45–60
107	CSWTG10	TCTTATGACAAAGAGTGTGGGTGG TTCACTTCACATGCGCATGCACAC	(TGGG)2(TG)9	252 68–72
108	CSWTGCG01	CAACATGCGTGCGTTCGTGCGTTT TCAAAATAATATTAATGGCTTGAA	(TG)6(TG)4(TGCG)6	216 64–71
109	CSWTT01	CTTGGCGCGACCTAC GTAAGCGCGAGTCATCAG	(CT)7	372 55–60
110	CSWTT02	ATTTTTGCTCTGCGATGT AATTCTCCAAGCGTCCTT	(CT)4(T)6	193 45–65
111	N6-1RTRANS	TCTATGATTTTCAACAATTGGAAG GGTTTTCTTAAATAGAAGAACACC AAACAATTTTCCAGATTCCACAGC	Retrotransposon 5' flanking region 3' flanking region	45–60
112	L18-2 H19A	CCATCATAGTCAAATAAGAAATGA GGTAGATATGTGTGCGCTATTTTG	SNP	50–65

[z]Primer redesign based on emprical results from previous primer sets.

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STRUCTION. Agarose gel analysis of digested (*Eco*RI* and *Acs*I) G-421 DNA revealed distinct bright bands about 200 bp, 400 bp, and 600 bp in length. These bands belong to two cucumber satellite DNA families (Type I and Type II), which consist of repeated units ≈180 bp in size having one restriction site sequence for both *Acs*I and *Eco*RI* digests (Ganal et al., 1986). These highly repetitive satellite DNA families represent about 20% to 30% of the total nuclear DNA in cucumber.

The DNA was digested with *Acs* I (4 bp recognition site) and *Eco*R I* (4 to 5 bp recognition site) to 1) match the *Eco*R I ligation sites of the Lambda ZipLox arms for library construction and 2) increase the number of restrictions when compared to *Eco*R I. Digestion, thus, decreased the average fragment size in the digest to simplify the sequencing process. Size fractionation of restriction digests resulted in the separation of digests into 28 size pools (0.1 to 20 kb). Agarose gel analysis showed that the 200, 400, and 600 bp size pools contained bright DNA bands, which were later identified as Type I and II cucumber satellite DNA. The products of both restriction digests (ranging 200 to 1200 bp) were used in ligations leading to the construction of the genomic libraries used for the microsatellite capture. The inclusion of the 200, 400, and 600 bp products in the ligation pool created a representational bias in the library toward satellite DNA sequences.

Sequencing and characterization of 355 clones derived from the (CT)12 GeneTrapper capture revealed numerous chimeric plasmid inserts (where at least one of the sequences was a Type I or Type II satellite DNA), and numerous single insertion events of Type I and Type II satellite DNA sequences. Similar results were obtained with the (CA)12 and the (CTT)8 captures. Therefore, the representational bias documented during restriction analysis was present in the library and ultimately in captured clones that contained putative microsatellite sequences. This indicates that the attempt to increase representation of the cucumber genome in digested products ranging between 200 to 1200 bp decreased the effectiveness of the capture by increasing the presence of repetitive DNA in the library.

MICROSATELLITE CAPTURE. The capture experiment used a biotinylated (CT)12 oligonucleotide to probe genomic libraries and identify clones containing (CT)n microsatellites. As identified by PCR, this capture yielded 457 colonies harboring plasmids with positive insertional events. Characterization of plasmid inserts through PCR amplification with standard M13 forward and reverse primers, and a (CT)12 primer revealed that 65% (297/457) of the clones were redundant (i.e., more than one clone per insert). Further characterization of 312 clones from the first capture by sequence analysis revealed three sources for the redundancy observed in the first capture. First, the capture hybridization step of the GeneTrapper protocol was more efficient in retaining plasmids with microsatellites having relatively long motifs (>24 bp) that matched or exceeded the length of the biotinylated capture oligonucleotide (24 to 26 bp) than plasmids with shorter microsatellite motifs (<24 bp). Thus, a specific sequence containing a (CT)17 repeat would be more prevalent in redundant clones than a sequence containing a (CT)9 repeat. Second, the rescue following transformation called for the incubation of electroporation products in SOC media for >60 min allowing for one to two cell divisions in DH10B *E. coli* cells. This time interval may have resulted in the duplication to quadruplication of a single capture product. This hypothesis is supported by the fact that in a second capture experiment using the same library and oligonucleotide where the incubation time was shortened to 25 min, redundancy was reduced from 65% to 10%. Last, single-

stranded contaminants which were not eliminated during washing steps after the streptavidin capture were likely retained in the final solution used to electroporate competent cells. Given the representational bias of Type I and Type II satellite DNA in genomic libraries, this satellite DNA was likely also represented in the captured clones. Moreover, the redundancy of clone sequences was observed in subsequent captures (data not presented).

The application of the GeneTrapper system made it possible to isolate and characterize several cucumber microsatellite loci in a relatively short time without the use of radiolabeled products. This GeneTrapper protocol first described by Fazio and Staub (2000) in a preliminary report was also used to isolate sequences containing tandem repeats in a catfish (*Ictalurus punctatus*) brain cDNA library (Nonneman et al., 2001) where a redundancy of only 30% was observed. This capture reportedly yielded 64% (299/467) nonredundant clones, all containing microsatellites. Their experiments yielded a higher number of microsatellite containing clones (299) than what was obtained in the capture of cucumber microsatellites. This difference is likely due to efforts to increase genome representation in the 200 to 1200 bp range, which consequently decreases efficiency in cucumber. Future attempts to use this methodology should take into consideration the presence of repetitive sequences when constructing a genomic library.

The capture and identification protocol described herein was used to develop 110 microsatellite markers (Table 1). Of these markers, 58 (52%) contain dinucleotide repeats (CT, CA, TA), 21 (19%) possess trinucleotide repeats (CTT, ATT, ACC, GCA), 3 (2.7%) contain tetranucleotide repeats (TGCG, TTAA, TAAA), 4 (3.6%) enclose pentanucleotide repeat (ATTTT, GTTTT, GGGTC, AGCCC), 3 (2.7%) contain hexanucleotide repeats (CCCAAA, TAAAAA, GCTGGC), and 21 possess composite repeats. All primers amplified the expected product and were subjected to ATG-PCR to identify optimized conditions for PCR.

OPTIMIZATION OF PRIMER-PAIR-SPECIFIC PCR CONDITIONS. The potential of microsatellites and other PCR based markers in high throughput applications has been widely discussed (Donini et al., 1998; Gupta and Varshney, 2000; Powell et al., 1996). Multiplexing (i.e., the addition of multiple markers in a single PCR amplification) has been successfully used for parentage testing in goats (*Capra hircus* L. Bovidae) using two multiplex systems, each containing 11 microsatellite loci (Luikart et al., 1999). Multiplexing has also been used in several plant species including wheat (*Triticum* ssp.) (Donini et al., 1998; Gupta et al., 1999), apple (*Malus ×domestica* Borkh.) (Hokanson et al., 1998), and grape (*Vitis* ssp.) (Lamboy and Alpha, 1998). However technical problems can arise during multiplexing. Donini et al. (1998) reported that multiplex PCR was very dependent on the choice of primer combinations and seldom produced amplifications as consistently as when primer pairs were used individually, and that background (nonspecific) amplification was common to many primer pairs, thus hindering the use of multiplex PCR. Furthermore, preferential amplification of smaller alleles over larger alleles as well as the variable plus A modification (nontemplated adenylation of the 3' end of the amplified sequence by *Taq* polymerase) resulted in misidentification of certain rainbow trout genotypes (*Oncorhynchus mykiss,* Salmonidae, Walbaum 1792) (Fishback et al., 1999). During PCR each primer pair may yield several products depending on reaction conditions (i.e., PCR mix, thermal cycler, and cycling conditions) (Staub et al., 1996a), and the nature of the DNA region that is being amplified (i.e., repeated
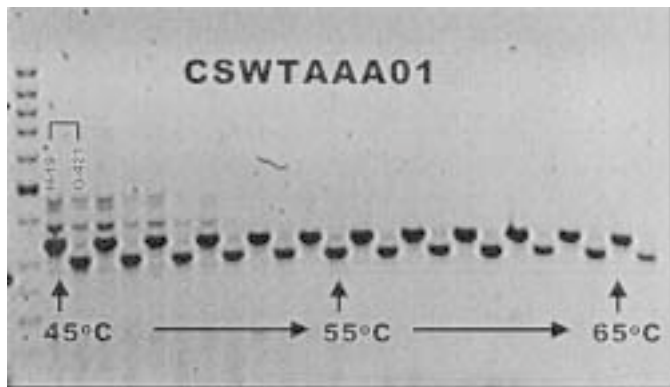
Fig. 2. Annealing temperature gradient (ATG) profile of CSWTAAA01 SSR marker using DNA of cucumber lines H-19 and G421 alternating in paired comparison. Lane 1 contains a 100-bp ladder and annealing temperature increases from 45 °C (far left) to 65 °C far right with a step of 1.8 °C every two wells.

or unique) (Paglia and Morgante, 1998). Primer-pair characteristics may be such that they hinder PCR (i.e., reaction kinetics) resulting in inadequate resolution of predicted multiplexed primer products.

This and other problems associated with multiplexing reactions (i.e., reaction optimization) could result in inconsistent and
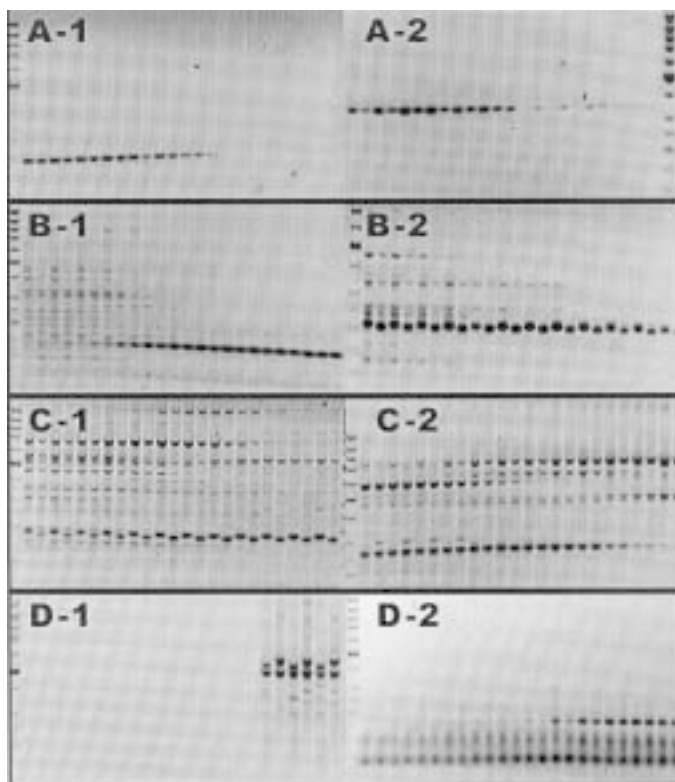
Fig. 3. Types of annealing temperature gradient (ATG) profiles observed in 214 simple sequence repeat (SSR) and sequence characterized amplified regions (SCAR) cucumber marker optimization experiments (Panels A–D). (**A**) Amplification of a single PCR product; (**B**) amplification of multiple PCR products at lower temperatures and of a single product at higher temperatures; (**C**) amplification of multiple PCR products across the gradient; (**D**) amplification of single or multiple PCR products only at higher temperatures. Lane 1 contains a 100-bp ladder, and remaining lanes contain paired DNA of lines H-19 and G421 which underwent PCR in ascending 1.8 °C intervals from 45 to 65 °C. A1, A2, B1, B2, C1, C2, D1, and D2 represent results from different sets of SSR primers.

inefficient implementation of this PCR application where equipment and reagents are not standardized. Implementation difficulties may be minimized by performing ATG-PCR profiling for each primer using standard DNA preparations (Fig. 2). The application of ATG-PCR as a preparatory step to PCR, assists in reaction optimization by identifying low risk temperature dependent annealing regions in which only a single target amplicon is present after PCR. Thus, the ATG-PCR profiling of potential PCR-based markers such as those developed herein (Table 1) is necessary for their universal acceptance and utility.

Observation of 214 SSR primer-pair annealing temperature optimizations (45 to 65 °C) using cucumber DNA (lines H-19 and G421) revealed four consistent visual band grouping types: 1) amplification of a single ATG-PCR product (≈25% of the primer pairs) (Fig. 3, panel A); 2) amplification of multiple products at lower temperatures and the presence of a single PCR product at higher temperatures (≈40% of the primer pairs) (Fig. 3, panel B); 3) amplification of multiple PCR regardless of annealing temperature (≈25% of the primer pairs) (Fig. 3, panel C), and; 4) amplification of single or multiple PCR products only at relatively high temperatures (≥60 °C; ≈10% of the primer pairs) (Fig. 3, panel D).

These ATG-PCR profiling observations can be used to strategically multiplex SSR or SCAR primers, identify new polymorphisms, and avoid mislabeling of amplicons. For instance, ATG-PCR profile outcome information can be used to group primer pairs to set optimized temperature conditions for singlet or multiplexed PCR. For multiplexing, markers of differing molecular weight can be grouped by optimal annealing temperature to increase the success of the PCR. Moreover, ATG-PCR profiling provides evidence of primer PCR temperature complexity that can be used by laboratories wishing to use particular markers. New polymorphisms were observed that were not expected based on primer ATG-PCR profiles (data not presented). These polymorphisms may be due to duplicated DNA regions in the cucumber genome.

Development of PCR markers based on single nucleotide polymorphisms (SNP) and characterized sequences (SCAR)

The conversion of randomly amplified targeted marker bands (RAPDs) to sequence specific PCR markers results in more stable markers (Staub et al., 1996c). Such conversion is often accomplished by cloning and sequencing polymorphic target bands. Resulting primers are designed specifically for a particular sequence (e.g., SCARS, STS) (Michelmore et al., 1991; Paran and Michelmore, 1993). This procedure was used in cucumber (Horejsi et al., 1999) in an attempt to convert 75 RAPD into SCAR

Fig. 4. Comparison of single nucleotide polymorphism (SNP) primers developed to differentiate cucumber lines H-19 and G421. Primers designed on a 5-bp polymorphism (L18-H19A) vs. a 2-bp polymorphism (L18-H19B). Lane 1 contains a 100-bp ladder, and the remaining lanes contain paired DNA of lines H-19 and G421, which underwent PCR in ascending 1.8 °C intervals from 45 to 65 °C.

markers. Conversion resulted in 48 (64%) SCARs, in which 11 (15%) retained their polymorphic nature.

Molecular markers tightly linked to economically important traits such as disease resistance, yield or quality are of particular importance to cucumber improvement programs. Given the low level of polymorphism in cucumber (3% to 8%), the problematic nature of RAPD markers (reproducibility across laboratories; Staub et al., 1996a), and the low percentage of RAPD to SCAR conversion (Horejsi et al., 1999), the identification and development of methodologically stable markers is of consequence. Moreover, concomitant assessment of ATG-PCR profiles of putatively useful PCR markers increases their potential implementation.

CONVERSION OF SCAR L18$_{600}$ TO AN SNP MARKER. The SCAR, L18$_{600}$, is of particular importance since it is associated (LOD 10.4) with quantitative trait loci (QTL) for multiple lateral branching (MLB), an important yield component trait in cucumber (Serquen et al., 1997). In contrast to its source RAPD, however, this marker was monomorphic with respect to the mapping parents H-19 and G421. Its RAPD counterpart is linked in repulsion phase to the MLB QTL limiting its usefulness in marker-assisted backcross selection. Sequencing of monomorphic L18$_{600}$ SCAR products in this study revealed several single nucleotide polymorphisms (SNPs) within the amplicon (data not presented). Consequently, both sense and antisense primers were designed so that the 3' end of the oligonucleotides matched only the SNP allele evident in H-19. Since alternative SNPs were identified, two unique markers (L18-2-H19A and L18-2-H19B) were created. Marker L18-2-H19A was designed on a five-base polymorphism (i.e., two-base identity, and two-base Indel in the forward primer and one-base identity in the reverse primer). Marker L18-2-H19B SNP was based on one-base polymorphisms in both forward and reverse primers. These primer constructions were examined by the ATG-PCR protocol (Fig. 4) to determine their reliability in differentiating H-19 and G421 DNA, and to optimize reaction conditions. Results indicate that the SNP marker construction based on multiple polymorphisms (L18-2-H19A) was more reliable in a broad PCR temperature range than the marker based on a single base pair change (L18-2-H19B) (data not presented). Thus, L-18-H19A was successfully used in marker-assisted selection and quantitative trait analysis experiments with cucumber (Fazio, 2001). Sequencing of RAPD bands L18-3 and AT1-2 resulted in the construction of two codominant SCAR markers, L18-3SCAR and AT1-2SCAR (Table 1).

CONVERSION OF RAPD N6$_{200}$ TO SCAR N6$_{200}$. The RAPD to SCAR conversion of a codominant RAPD product, N6$_{800}$, resulted in the characterization of a SCAR marker designated SCAR N6$_{800}$ (Horejsi et al., 1999). Although this SCAR yielded different size bands in both H-19 (584 bp) and G421 (1046 bp), analysis of a F$_2$ mapping population (Fazio, 2001) revealed that this marker behaved in a dominant fashion exhibiting preferential amplification of the 584 bp band in heterozygous individuals. An ATG-PCR profile of the original N6$_{800}$ SCAR primers revealed novel polymorphisms between 45 °C to 48 °C. These were subsequently mapped (Fazio, 2001), and found to be distant (50 cM) from the N6$_{800}$ SCAR.

Given the size difference between the two N6$_{800}$ alleles (462 bp), it is possible that competition in the PCR reaction caused the higher molecular weight band to be amplified less efficiently than its smaller weight counterpart in heterozygous DNA samples. Sequence analysis (data not presented) of both alleles revealed an insertion of a transposable element belonging to the SINE-like family of retrotransposons (Kumar and Bennetzen, 1999; Kunze et al., 1997). These elements share features with SINE and *Alu* sequences in mammals, and are characterized by flanking 14 to 15 bp target-site duplications (TSDs) and a GC rich region in the 5' half of the insert. The element from cucumber contains a 90-bp GC rich region in one of the termini as well as 17-bp long TSDs (data not presented). The GC rich region might have contributed to the failed amplification of the 1046-bp band of N6$_{800}$ SCAR marker in heterozygous individuals by raising the Tm requirement of the amplicon during PCR.

A strategy was used to convert this pseudodominant N6$_{800}$ SCAR marker into a codominant marker. Three primers were constructed resulting in a forward and reverse primer which amplified the putative nonretrotransposon allele and an additional reverse primer 30 bp within the putative retrotransposon insertion further away from the GC rich region. Genetic analysis of these primers using an F$_2$ population (H-19 x G421) demonstrated that the new SCAR marker (N6A-SCAR) segregated in a codominant fashion (Fazio, 2001). Sequences from plant retrotransposons elements have been used in *Pisum* species and conifers to construct PCR-based marker systems (Flavell et al., 1998; Pearce et al., 1999) for use in high throughput analysis. The development of markers based on retrotransposon sequences such as N6$_{800}$ will provide additional markers for genetic analysis in cucumber.

The application of molecular markers may increase the efficiency of plant breeding (Gupta and Varshney, 2000; Lande, 1990; Lee, 1995; Staub et al., 1996c; Young, 1999). Marker systems developed for the genetic analysis of cucumber have provided valuable information about genetic diversity (Dijkhuizen et al., 1996; Horejsi and Staub, 1999; Staub et al., 2002), have enhanced genetic map construction (Bradeen et al., 2001), and have been used in marker-assisted selection (Fazio, 2001). The level of polymorphism in a preliminary assessment of SSR variation between *C. sativus* var. *sativus* and var. *hardwickii* and among elite germplasm (European and U.S.) is ≈35% and ≈15%, respectively (unpublished data). Thus, the addition of 110 SSR, 4 SCAR and 2 SNP markers to the array of previously identified markers will enhance the use of genetic markers in breeding, diversity analysis, variety identification, and in the protection of cucumber germplasm. Moreover, these markers will likely provide a means for greater integration of genetic information during mapping experiments in cucumber (e.g., map merging) and in elucidating synteny relationships between cucumber and melon (*Cucumis melo* L.).

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