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Asymmetric single-strand conformation polymorphism: An accurate and cost-effective method to amplify and sequence allelic variants[1]

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- *Premise of the study:* An efficient alternative strategy to conventional cloning was needed to generate high-quality DNA sequences from a variety of nuclear orthologs for phylogenetic studies. This method would facilitate studies and minimize technical problems typically encountered in cloning methodologies.
- *Methods:* We tested a variety of single-strand conformation polymorphism (SSCP) protocols including purified and unpurified symmetric and asymmetric PCR, loading buffers, and electrophoresis conditions (buffers, matrix, running time, temperature). Results obtained from direct SSCP band sequencing were compared to those obtained from cloning.
- *Key results:* Our optimized protocol uses asymmetric PCR, with the majority of the samples run in polyacrylamide gel electrophoresis (PAGE). It consistently separated PCR products from 450 to 1200 bp.
- *Conclusions:* Asymmetric PCR single-strand conformation polymorphism is an efficient alternative technique for isolating allelic variants of highly heterozygous individuals, with its greatest applications in sequencing allopolyploids. It eliminates two common problems encountered in cloning: PCR recombination and heteroduplex fixation. In addition, our protocol greatly lowers costs and time associated with procedures.

Key words: asymmetric PCR; PCR recombination; polyploids; single-strand conformation polymorphism; SSCP; symmetric PCR.

In evolutionary biology, population genetics, ecology, and many other fields, it is necessary to generate gene sequences in many species or individuals of a population. This procedure is relatively easy when the organism is diploid and homozygous, because sequences can be generated by direct sequencing. However, sequencing can become greatly complicated, time-consuming, and expensive when working with heterozygous individuals, especially with heterozygous polyploids. In such cases, more than one nucleotide is generated per site, making annotation difficult or impossible. Cloning is frequently used to circumvent this problem because it generates multiple simple sequences from all allelic variants. However, rare alleles or alleles that are not very well represented in the gene pool have less probability to be captured during cloning, a time-consuming and expensive procedure (Gasser, 1998). Furthermore, the number of clones that need to be sequenced to capture all alleles increases with ploidy level. Such constraints were faced by the International HapMap Consortium (2005), that is developing a haplotype map of the human genome, forcing a strategy to focus only on common single nucleotide polymorphisms (SNPs) with allelic variants occurring in at least 1% of the population.

Single-strand conformation polymorphism (SSCP) can alleviate such problems. SSCP is the electrophoretic separation of single-stranded nucleic acid, with differing tertiary structures formed by sequence differences as small as a single base pair (SNPs), with visual detection using silver staining, SYBR Gold, radioactivity, or other reagents (Orita et al., 1989; Hayashi, 1991; Fujita and Silver, 1994). One of its biggest limitations is the limited fragment length that can be separated. Because the sensitivity of SSCP is inversely proportional to the length of the fragment, one single base pair difference can be resolved 99% of the time for 100–300 bp fragments and more than 80% for 400 bp (Sunnucks et al., 2000). However, for phylogenetic studies sequences of this small size are limiting, and it is preferable to have much greater lengths, up to and exceeding 1200 bp that can be easily sequenced by one forward and one reverse primer.

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Another consideration in SSCP is co-migration, in which two or more different sequences occur in the same position in the gel. Alternatively, a single sequence can have different stable conformations, and two or more bands can be detected for the same sequence (Hayashi, 1991). Temperature, pH, and running time influence single-strand DNA mobility, and the best combination of all of these factors for a given gene or PCR product needs to be determined empirically for each primer (Orita et al., 1989; Hayashi, 1991; Fujita and Silver, 1994; Kukita et al., 1997).

Yet another consideration is the number of bands to be sequenced per individual. Two alleles are possible in a diploid organism, producing four single-stranded DNA bands for a single-copy gene. A tetraploid can have up to four alleles (eight single-stranded bands) and an octoploid up to eight (16 single-stranded bands).

Asymmetric PCR is used to preferentially amplify one strand of the original DNA (Mazars et al., 1991). Asymmetric PCR can be performed by using unequal concentrations of the two primers or by using a two-step amplification procedure. The first method is based on a large number of cycles that can incorporate a potential source of sequencing errors (Mazars et al., 1991). The second method initially uses a symmetric PCR, and a sample of that PCR product is used as a template for a subsequent asymmetric PCR; this method thus has the potential to generate procedural errors by its additional steps (Mazars et al., 1991). In the asymmetric PCR, the number of bands is reduced in half, because only the upstream (sense) or downstream (antisense) product is amplified. Despite the need for this additional step, asymmetric SSCP eliminates unwanted bands and produces clear bands with easily distinguishable homozygous and heterozygous individuals (Pokorny et al., 1997) when they are run in SSCP. As a consequence, the asymmetric PCR of genomic DNA followed by SSCP identification of sequence polymorphism reduces the cost of sequencing in half relative to symmetric SSCP. Another advantage of SSCP is the elimination of PCR artifacts such as PCR recombinants (Meyerhans et al., 1990; Brakenhoff et al., 1991) and heteroduplex molecules (Kanagawa, 2003).

Although presently there are yet other methodologies to generate genome sequences quickly and easily (Shendure and Hanlee, 2008), the cost cannot be reached for the majority of researchers, especially when hundreds of individuals are screened. Furthermore, even with the second and third generation of sequencing technologies, it is almost impossible to generate genome sequences for a highly heterozygous polyploid without generating higher genome coverage, which increases costs considerably.

In this study, we optimized SSCP protocols that represent a relatively easy, efficient, and economical method for generating DNA sequences from highly heterozygous or polyploid individuals. Even though it involves a moderate amount of manipulation, it offers the potential for automation to generate high-throughput sequencing. We describe an asymmetric-PCR SSCP technique to isolate all alleles from heterozygous individuals (diploid or polyploid) for direct sequencing. The cost and time needed to generate sequences is reduced by one-third (tetraploids) to one-fifth (hexaploids) relative to cloning. We test the utility of asymmetric-PCR SSCP in species of *Solanum* section *Petota*, a taxonomically difficult group, partly because this group has a history of interspecific hybridization at both the diploid and polyploid levels (Rodríguez and Spooner, 2009; Rodríguez et al., 2009; Spooner, 2009), showing the advantage of this fast and reliable method to isolate haplotypes.

MATERIALS AND METHODS

Two sets of experiments were carried out. First, a general SSCP protocol was determined, which was used to determine the best SSCP running conditions for six different sizes of PCR products generated from nuclear conserved ortholog set II (COSII) regions (Rodríguez et al., 2009) in diploid and polyploid wild potato species. Second, the protocol was tested for 16 wild potato polyploid accessions (six species) to assure that all the alleles in each accession were well isolated.

Plant material and DNA extraction—The first experiment tested four accessions, two diploids and two tetraploids; the second experiment tested 16 polyploid accessions, 11 tetraploids and five hexaploids (Appendix 1). Herbarium vouchers are deposited at PTIS. Total DNA extracted from young leaves grown from seeds in a greenhouse was isolated using a standard CTAB protocol (Ghislain et al., 1999). DNA quality and quantity were estimated by comparison with CsCl-purified λ DNA digested with *PstI* on ethidium bromide-stained agarose gels.

PCR amplification, SSCP protocol, reamplification protocol, and sequencing—Primers (Table 1) were designed to amplify regions with at least 60% intronic content for high discriminatory power among the closely related species of *Solanum* sect. *Petota*. Symmetric PCR amplifications were performed following Rodríguez et al. (2009) except that the final reaction volume was halved. The 10-μL reactions consisted of 0.1 μmol/L final concentration of each primer (UW-Biotechnology Center, Madison, Wisconsin, USA), 0.2 mmol/L of each dNTP (Promega, Madison, Wisconsin), 1.5 mmol/L of $MgCl_2$, 0.5 units of GoTaq DNA Polymerase (Promega), and 10 ng of template genomic DNA. Amplifications were carried out in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, California, USA) using an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and a final elongation at 72°C for 10 min. Four microliters of the reaction was run on a 1.5% agarose gel with 1× Tris-borate-EDTA (TBE) buffer for 3 h to verify the presence of a single, clear band. The remaining PCR products were used as template in the subsequent asymmetric PCR. The asymmetric PCR reaction had the same final volume (10 μL), components, and final concentration as the symmetric PCR except that it only contained one primer (0.1 μmol/L final concentration) and 1 μL of the symmetric PCR as template. This reaction was performed under the same cycling conditions as described above. Five microliters of this PCR reaction was run on a 1.5% agarose gel in 1× TBE buffer for 2 h to verify the success of the reaction.

Samples were denatured before being run in the SSCP by mixing 2 μL of the asymmetric PCR product with 8 μL of loading buffer I (95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) or loading buffer II (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), followed by heating at 95°C for 10 min and immediately placing on wet ice. Six microliters of each sample was loaded onto a Mutation Detection Enhancement gel (MDE; Lonza, Basel, Switzerland) or polyacrylamide gel (38 cm × 30 cm × 0.4 mm; IBI Scientific, Peosta, Iowa, USA) that had been pre-run at 3W for 20–30 min. Electrophoresis was carried out at constant power, 3 W, at 4°C in prechilled 0.6× TBE buffer, using a Sequi-Gen GT Nucleic Acid Electrophoresis Cell System from Bio-Rad (Bio-Rad). The gel was silver stained using the SILVER SEQUENCE DNA Sequencing System from Promega following the manufacturer's instructions except that the kit was used to stain two gels instead of one and all the water used in the staining was miliQ quality. After staining, the gel was photographed.

Bands were excised from the gel with a pipette tip or a scalpel and placed individually in one of the wells of a 96-well PCR plate with 40 μL of autoclaved water. This mixture was heated to 60°C for 10 min to elute the PCR product prior to reamplification. Reamplification was performed using the same symmetric PCR protocol outlined above using 4 μL of band eluate as template. Four microliters of the reaction was run on a 1.5% agarose gel with 1× TBE buffer for 3 h to verify the reamplification. The remaining PCR products were diluted with autoclaved water in a ratio of 1:8 PCR product to water, to be used as template in the following sequencing reaction.

One microliter of this product was cycle-sequenced in both directions with the same primers in a 5-μL reaction consisting of 0.5 μL of BigDye and 0.75 μL of dilution buffer from the BigDye Terminator kit v3.1 (Applied Biosystems, Foster City, California), 2.5 μL of 0.5 μmol/L of primer, and 0.25 μL of water. Cycle conditions were carried out in a Bio-Rad MyCycler thermal cycler (Bio-Rad, Hercules, CA) using an initial denaturation of 95°C for 3 min; followed by 40 cycles of 96°C for 25 s, 50°C for 20 s, 60°C for 5 min; and with a final elongation

TABLE 1. Details of the COSII markers tested and PCR and SSCP conditions (matrix type and running time).

COSII marker	Forward primer	Reverse primer	Size (bp)	Annealing temp (°C) + Mg (mmol/L))	SSCP conditions
C2_At1g13380	AGGTGCTTTCTTGTTTCTTCTTTC	AGAGCATATCACGATACTTGGTGTG	700–800	55 + 1.5	PAGE, 24 h
C2_At1g20050	ATGATCTAAAATTGCCTGGTTTTG	AATAGCCCTCAAGGACCATGTGG	900–1000	55 + 1.5	PAGE, 24 h
C2_At1g32130	TCAACAAGAGTACACGGTTTGAAGAC	TTGCTCTAGCCCTGGCCCTAAC	400–500	55 + 1.5	PAGE, 13–16 h
C2_At4g10050	ATCACCCTTCTGCCTTTTCTTC	ATCTGGGATCTGAATGTCATCCTC	800	55 + 1.5	PAGE, 30 h
C2_At5g14320	TTCTCTTTCCCTTATCTGCAACAC	TCCTTCAATCATGTACTTAGAGACTTC	700–800	55 + 1.5	MDE, 48 h
C2_At5g47390	TGGTGGCTCTGTTGATGGTTATGC	ACATCCTATGCTCCTCCTCAGTCC	~1200	55 + 1.5	PAGE, 48 h

at 72°C for 7 min. Excess dye terminators were removed using Angencourt CleanSeq magnetic beads (Beckman Coulter Genomics, Danvers, Massachusetts, USA) following manufacturer's instructions except that the volume of the magnetic beads was halved (5 μL). Sequences were resolved on an ABI 3730xl capillary-based automated DNA sequencer (Applied Biosystems) with 50 cm POP-7 polymer capillaries at the Biotechnology Center of the University of Wisconsin-Madison.

Sequences were assembled and edited with Staden package windows version 1-7-0 (Staden, 1996) and aligned using the program Clustal X version 2.0.3 (Larkin et al., 2007) at default parameters, except for the "percentage of delay divergence sequences", which was set to 5% after testing various percentages. Alleles and potential PCR recombinants were identified by visual inspection using the program MacClade 4.08 (Maddison and Maddison, 2001).

Cloning procedures—After the symmetric PCR was performed, reactions were run on a 1.5% agarose gel with 1× TBE buffer for 3-5 h. Bands were cut out and cleaned using Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, California). The PCR product was eluted in 8 μL of nuclease-free water. Cleaned PCR products were cloned into Promega's pGEM-T Easy vector. Ligation, transformation, and plating were carried out following the manufacturer's instructions except that ligation and transformation reaction volumes were halved. Five positive colonies were sampled for diploid species, 10 for tetraploid species, and 20 for hexaploid species to have 95% confidence that all alleles were sampled. White colonies were picked using a sterile 20-μL pipette tip and transferred to a single well of a 96-well PCR plate that was previously filled with 20 μL of autoclaved water to allow the elution. Symmetric PCR was carried out following the protocol described using 5 μL of this eluate as template. Four microliters of the reaction was run on a 1.5% agarose gel with 1× TBE buffer for 3 h to verify the presence of the PCR product. The remaining PCR products were diluted with autoclaved water in a ratio of 1:8 (PCR product to water) to be used as template in the following sequencing reaction that was carried out following the protocol previously described.

Additional protocols used during testing—*Cleaning PCR products and testing four running buffers*—Before running in SSCP, the PCR products were purified using WizardSV Gel Clean-Up System (Promega) and resuspended in 50 μL of nuclease-free water, or they were digested with EXO-SAP-IT (USB Corp., Cleveland, Ohio, USA) following the manufacturer's instructions except that reaction volumes were halved. Four different running buffers were also tested: (1) TBE buffer pH 8.3, (2) TBE buffer with 5% of glycerol pH 7.7, (3) Tris-MES-EDTA (TME) buffer pH 6.8, (4) Tris-PIPES-EDTA (TPE) buffer pH 6.8. To prepare 1× TME buffer pH 6.8, 3.6342 g of Tris were mixed with 6.832 g of MES (2-[*N*-morpholino]ethanesulfonic acid from Fisher Scientific, Pittsburgh, Pennsylvania, USA) and 2 mL of 0.5 mmol/L EDTA with distilled water to a final volume of 1 liter; for 1× TPE buffer pH 6.8, 3.6342 g of Tris, 6.048 g of PIPES (1,4-piperazinediethanesulfonic acid from Fisher Scientific), and 2 mL of 0.5 mmol/L EDTA were mixed with distilled water up to final volume of 1 liter.

Tests to optimize SSCP protocols— To verify whether, under our PCR conditions, the residues from the components of the PCR reaction could interfere with the mobility of the DNA during SSCP, 50-μL volume symmetric PCR with COSII C2_At1g20050 was performed for four species. Ten microliters of this PCR product was run in a 1.5% agarose gel to verify amplification, 10 μL were cleaned using EXOSAP, 20 μL were cleaned with WizardSV Gel Clean-Up System, and the remaining 10 μL were kept uncleaned. All three treatments were run in a 0.7× MDE gel in 0.6× TBE buffer for 48 h at 4°C. The loading buffer II in a ratio of 1 : 4 PCR product to loading buffer was used; 6 μL was loaded in the MDE gel and silver stained.

According to previous reports, a good starting concentration of MDE for sizes longer than 500 bp is 0.7× (I. J. Maureira-Butler, University of Wisconsin-Madison, personal communication; Koopman and Baum, 2010). Samples from the same symmetric, forward-asymmetric, and reverse-asymmetric DNA amplification reactions were first applied to 0.7× MDE gels to determine the best SSCP conditions to separate all allelic variants and to identify which asymmetric PCR type produced the best separation among all alleles. Two different loading buffer compositions as mentioned in two different ratios (PCR : LB, 2 : 1 and 1 : 4) were tested to establish which combination was best for denaturation of samples before running in SSCP. Both symmetric and asymmetric PCR products longer than 500 bp were run for 48 h at constant power (3 W), and the remaining COSII (400 bp long) was run only for 24 h. Band position, number, and additivity were compared between symmetric and asymmetric PCR products. After the best running conditions were established in MDE at 4°C, asymmetric PCR products were run at room temperature to determine whether we could get comparable results without using cold running conditions.

Once the SSCP electrophoretic conditions for these six COSII were established in MDE, asymmetric PCR products longer than 600 bp were separated in an 8% polyacrylamide gel in an attempt to reduce costs. Two different ratios of acrylamide to bis-acrylamide were used (19 : 1 and 37.5 : 1), and samples were run at 4°C for 24 and 48 h. After these parameters were determined, three other buffer systems were tested for improving band separation: (1) TBE + glycerol pH 7.7, (2) TME pH 6.8, (3) TPE pH 6.8. Asymmetric PCRs of COSII longer than 600 bp were run at room temperature using these three buffers. See Appendix S1 (see Supplemental Data online at http://www.amjbot.org/content/98/7/1061/suppl/DC1) for amplified SSCP protocol procedures.

Testing the SSCP protocol in polyploid species—We tested our optimized SSCP protocol with 16 accessions of six polyploid potato species (Appendix 1) to assure that all the alleles in each accession were well isolated. For comparing results obtained by SSCP, symmetric PCR for all COSII were cloned into pGEM-T Easy vector. SSCP bands and clones were sequenced following the protocol described already. In a few cases, it was necessary to make dilutions (1 : 100 or 1 : 400) of the eluted SSCP band to get reamplifications. A maximum likelihood analysis using sequences from individual COS was performed using RAxML Web-servers (Stamatakis et al., 2008) with all sequences isolated by SSCP and by cloning and with sequences from 11 diploid species from a previous study (Rodríguez and Spooner, 2009) to determine and compare the placement of all alleles determined by cloning and SSCP. The individual analyses were run with a GTR model and allowing gamma-distributed rate variation among sites, and all free model parameters were estimated by RAxML. A bootstrap analysis with 100 replicates was also performed for each individual COS.

RESULTS

Tests to optimize SSCP protocols—No differences regarding band number or mobility were found among the three treatments, so unpurified PCR products were run in SSCP in all subsequent tests. Bands using loading buffer II showed slightly darker and sharper bands in comparison with loading buffer I. To assure good denaturation and quality of the bands, we used loading buffer II in a 1 : 4 ratio for subsequent experiments. Band position, number of bands, and additivity between the symmetric and both asymmetric bands patterns were completely concordant (Fig. 1). Cases of co-migration were not detected, but cases of more than one stable conformation for a

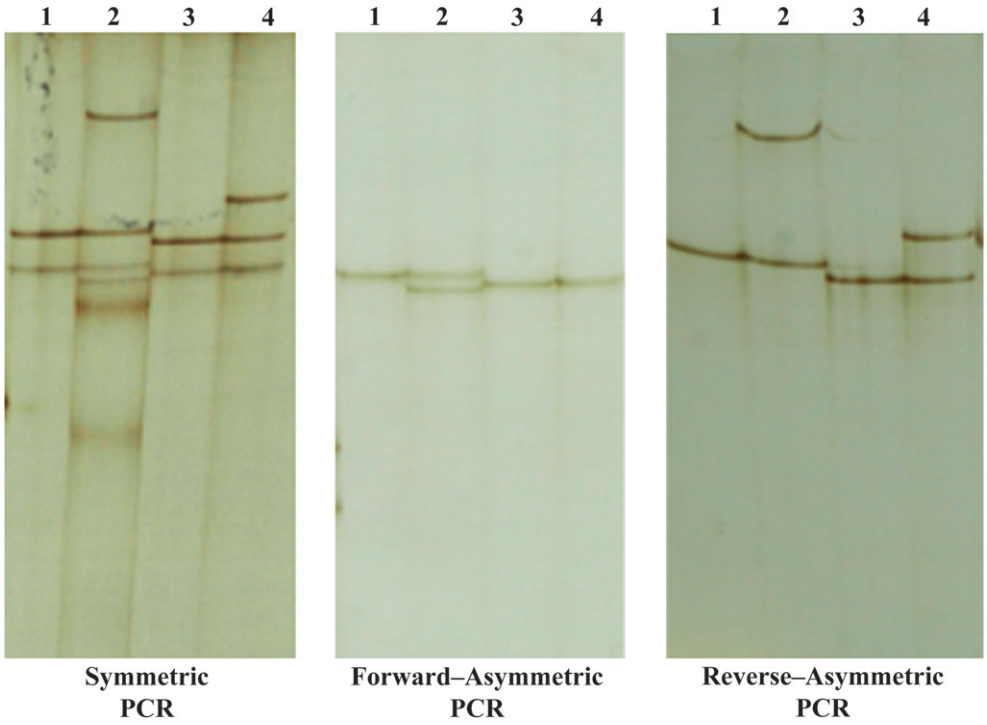

Fig. 1. Symmetric and asymmetric PCR for C2_At1g20050 (950 bp) in four species: (1) *S. acroscopicum* PI 230495 (4*x*), (2) *S. flahaultii* PI 570620 (4*x*), (3) *S. urubambae* PI 763314 (2*x*), and (4) *S. santolallae* PI 760350 (2*x*).

sequence were detected. In five cases, the asymmetric PCR performed with the reverse primer produced the best separation among bands in comparison with the forward and was selected for the next experiments. Only for one COSII (C2_At5g14320) did the asymmetric PCR performed by the forward primer produce better separation among bands in comparison with the reverse, but bands were smeared under all the conditions that were tested (two loading buffers and two ratios of PCR and loading buffer). Consequently, the asymmetric PCR performed by the reverse primer was selected as well.

In MDE gels, the shorter PCR product (C2_At1g32130, 400-500 bp) separated very well in a 24-h run; for four of the five remaining COS, the running time of 48 h was sufficient to get good separation among bands (C2_At1g13380: 700 bp; C2_At1g20050: 900 bp; C2_At5g47390: 1200 bp; C2_At4g10050: 800 bp). For C2_At5g14320 (700 bp), it was necessary to increase the running time to 60 h to get good separation. SSCP runs at room temperature did not produce better separation or quality of bands in comparison to those run at 4°C.

The ratio 37.5 : 1 worked much better than the ratio 19 : 1 in PAGE gels. Only for one of the COSII (C2_At5g14320) was the separation of the bands under both running times (24 and 48 h) significantly better in MDE than in PAGE. In the other cases, MDE and PAGE gave almost the same results with the only difference that the running time was usually reduced to 24 h instead of 48 and to 16 h instead of 24 h.

Results did not show a significant improvement in the separation and quality of the bands in the other three buffer systems. For some samples, bands were more separated than in TBE, but for others less, and in some cases the bands were fuzzy. Hence, TBE buffer pH 8.3 was selected as the best buffer because it gave sufficient separation and is cheaper.

Testing the SSCP protocol with polyploid species—Our SSCP protocol was able to isolate all the variant sequences present in an individual in all COSII in all 16 polyploid accessions that we tested. Some sequences from cloned PCR products showed Taq errors and PCR recombinants, and there were three cases in which an allele from an accession was not picked by cloning.

Table 2 shows only the smallest number of SNPs and the difference in size of the alleles in one accession, found among all samples tested. In five of the six COSII, the number of SNPs between alleles was more than 11 (12–23), and the difference in size was from 7–36 bp. On the other hand, for C2_At5g47390, that had a length of 1200 bp, it was possible to separate alleles that differed in only three SNPs.

Sequences generated by SSCP were compared with those generated by cloning. Possible PCR recombinants were identified in two polyploid accessions in C2_At1g32130, one accession in C2_At5g14320, four accessions in C2_At1g13380, and one accession in C2_At1g20050. When a maximum likelihood analysis was run with those samples, the majority of the PCR recombinants were placed outside of the clade where their SSCP-isolated

TABLE 2. The minimal number of single nucleotide polymorphisms (SNPs) and deletion that were found in the samples tested.

COSII marker	Size bp	No. of SNP	Deletion difference bp
C2_At1g32130	453	12	none
C2_At5g14320	700–800	16	14
C2_At1g13380	700–800	20	7
C2_At1g20050	900–1000	15	16
C2_At5g47390	~1200	3	1
C2_At4g1005	800	23	36

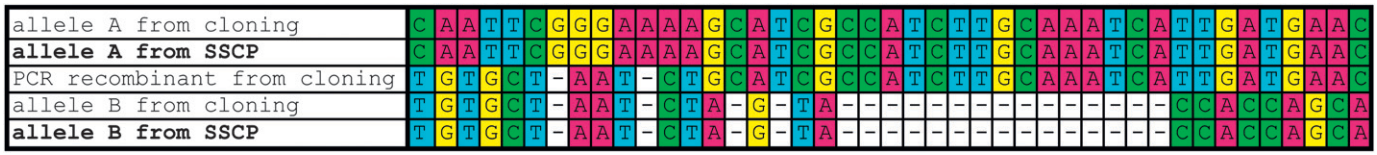

Fig. 2. The 43 polymorphic sites (coded as A, red; C, green; G, yellow; T, blue) from the two homeologs in allotetraploid *Solanum hjertingii* PI 186559 from C2_At1g13380 generated by cloning and by SSCP, and a PCR recombinant generated by cloning.

alleles were placed (not shown). Figure 2 shows one of these examples, for C2_At1g13380, in which PCR recombinants were found for *S. hjertingii* PI 186559. In *S. iopetalum* PI 558405, three alleles were found by SSCP, and six likely PCR recombinant sequences were obtained by cloning. All six were different, but resulted from recombinants between only two alleles. One of these six showed a more complicated pattern, in which the beginning and the end of the sequence belonged to one allele and the middle of the sequence to the other allele (Fig. 3). These results showed the utility of SSCP to isolate alleles from heterozygous individuals that eliminated PCR recombinants and heteroduplexes that are fixed during cloning.

DISCUSSION

The utility of SSCP to separate single nucleotide polymorphisms is well documented. It has been used to detect mutant cancer alleles (Orita et al., 1989) and for many other applications (Martins-Lopes et al., 2001; Hodgkinson et al., 2002; Cronn and Adams, 2003). In most of these applications, the final result demonstrated a difference in band mobility but in our application we use SSCP as a separation technology to replace cloning.

Comparison of purified and unpurified PCR products—Prior studies reported that the purification of a symmetric PCR product before asymmetric PCR helped eliminate unwanted amplicons and simplified analysis (Cai and Touitou, 1993; Fujita and Silver, 1994; Pokorny et al., 1997; Zhu et al., 2006). Free oligonucleotides can anneal to PCR product strands and alter their mobility (Fujita and Silver, 1994) or may interfere with the amplified sequence and inhibit the discriminative potential of the SSCPs (Cai and Touitou, 1993). We failed to detect differences between the unpurified and two purified procedures regarding number of bands or their mobility in both symmetric and asymmetric PCR samples. This favorable result may be caused by the initial low concentration of primers (0.1 μmol/L) in the PCR reaction and the amount that remained after all PCR cycles (unincorporated primers) was very low and did not interfere with band mobility. In the case of asymmetric PCR, it may be because the unincorporated primers cannot anneal with single-strand DNA because they have the same nucleotide sequence as single-strand DNA (Zhang et al., 2008).

Comparison of loading buffers and ratios of dilution—The use of several denaturants for SSCP has been investigated before (Fujita and Silver, 1994; Pokorny et al., 1997; Hennessy et al., 1998; Zhu et al., 2006). The choice of denaturant and ratios of loading buffer and PCR product had a minimal effect on the SSCP pattern for the six COSII tested. The slightly better performance of loading buffer II than loading buffer I may be because loading buffer II is a stronger denaturant. Nevertheless, if overdenaturation of the DNA occur and smeared bands appear, it is advisable to test loading buffers and ratios.

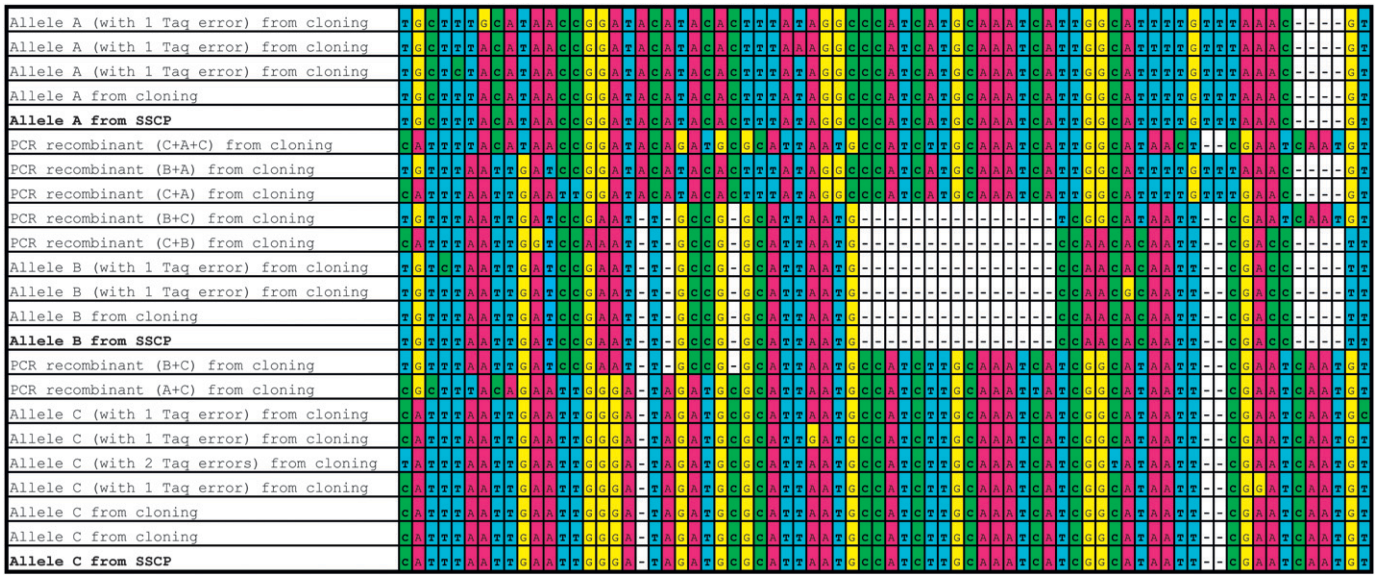

Fig. 3. The 74 polymorphic sites (coded as A, red; C, green; G, yellow; T, blue) in allohexaploid *Solanum iopetalum* PI 558405 from C2_At1g13380 generated by cloning and by SSCP, and PCR recombinants generated by cloning.

Comparison of symmetric and asymmetric PCR—The secondary structure of single-strand DNA is labile. Many physical and chemical factors can change its structure, and different SSCP results may appear even from the same samples under different PCR-SSCP conditions. Bands can appear closely spaced and fuzzy, which may increase the difficulty of picking out a single band. Asymmetric PCR reduces the number of bands by half in comparison to symmetric PCR. It reduces the number of bands to be picked up and sequenced and facilitates the isolation of the alleles because there is a better chance to get better separation in the gel. It is always advisable to test which of the primers give better results because this can be only determined empirically.

SSCP running conditions in MDE and in PAGE gels—Our longer PCR products (700–1200 bp) separated well in 48 h and the shorter product (450 bp) in 24 h; longer runs for the latter produced fuzzy bands. Time and running conditions always need to be established empirically for each gene. In a few cases, we had problems eluting DNA from MDE gels; we solved the problems by vortexing the gel and water mixture before and after the treatment at 60°C for 10 min.

To reduce costs, we tested two different running times and ratios of acrylamide : bis-acrylamide. As reported previously by other researchers (Teschauer et al., 1996), these variables also need to be established empirically. Under our conditions, a ratio of 37.5 : 1 of acrylamide to bis-acrylamide worked much better than 19 : 1. Only in one case (for C2_At5g14320) were MDE results significantly better than PAGE. In the majority of cases under our conditions, separating alleles in PAGE decreased the running time from 48 to 24 h, and in the case of the shorter PCR product from 24 to 16 h. The use of PAGE instead of MDE also has the advantages of reducing the price of each gel by 40%. PAGE gels allowed easy SSCP band elution, and keeping SSCP bands in water at 4°C overnight was enough to elute the DNA from the gel.

The three new buffer systems suggested by Kukita et al. (1997), run at room temperature, did not significantly improve the separation and quality of the bands. We chose a TBE buffer system because the prices of the TME and TPE buffer are five and 10 times more expensive than TBE buffer, and the discriminatory power of the SSCP with TBE system allowed us to isolate all alleles in the samples we tested.

Testing the established SSCP protocol in 16 potato polyploid accessions—The ultimate goal of our research was to determine a procedure that could separate all alleles from polyploid accessions. Our optimized protocol isolated all alleles in 16 accessions of six polyploid species and with all six COSII. This high success rate for sizes more than 700 bp could be expected because the allelic differences in our targeted intronic regions contain several SNPs and indels. Our comparison of SSCP and cloning from the same accessions showed PCR recombinants only in the cloned samples, and a maximum likelihood analysis placed these sequences outside of the clade of their correct clade. These results demonstrate the utility of asymmetric-PCR SSCP to isolate alleles from heterozygous individuals, eliminated PCR recombinants and heteroduplexes that are fixed during cloning, and reduced costs. This approach has wide applicability for a variety of other applications such as population genetics and ecology.

Cost comparisons—Costs to sequence one gene in 100 tetraploid individuals by asymmetric-PCR-SSCP using MDE under the assumption that the organism has four different alleles is US$1100, by PAGE $1040, by cloning (taking 10 or 20 colonies per accession for sequencing) $3281 and $5774, respectively (a 3–5.3× cost reduction). Presently, we have optimized the SSCP protocol for 24 COSII, from 450–1500 bp, and those are being used to isolate alleles in potatoes, tomatoes, sweet potatoes, and carrots. Asymmetric PCR-SSCP is a very convenient alternative to cloning for heterozygous individuals. The limitations of the technique reported by others will not play a fundamental role when intronic regions are mainly being amplified, because the difference between alleles usually is much more than one base pair.

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APPENDIX 1. Voucher information for taxa and accessions used in this study. GenBank accessions are grouped by COSII allelic sequences. For information on experiment I or II, see text. Country and first level administrative division are given for the accessions examined. All voucher specimens are deposited at PTIS.

***Taxon*;** USDA plant introduction no.; GenBank accessions; Ploidy; Experiment no.; Country; Division.

Solanum acroscopicum Ochoa; 230495; C2_At1g13380 - JF832417, JF832418, C2_At1g20050 - JF832424, C2_At1g32130 - JF832431 JF832432, C2_At5g14320 - JF832438 JF832439; 4*x*; I; Peru, Tacna.

S. agrimonifolium Rydb.; 243350; C2_At4g10050 - HQ641761, HQ641762, C2_At5g47390 - HQ641932, HQ641933, C2_At1g13380 - HQ642102, HQ642103, C2_At1g20050 - HQ642274, C2_At1g32130 - HQ642435, HQ642436, C2_At5g14320 - HQ642605; HQ642606; 4*x*; II; Guatemala, Huehuetenango.

S. colombianum Dunal; 583325; C2_At4g10050 - HQ641789, HQ641790, C2_At5g47390 - HQ641961, HQ641962, C2_At1g13380 - HQ642132, HQ642133, C2_At1g20050 - HQ642299, HQ642300, C2_At1g32130 - HQ642462, HQ642463, C2_At5g14320 - HQ642632; 4*x*; II; Venezuela, Tachira.

S. flahaultii Bitter; 570620; C2_At1g13380 - JF832415, JF832416, C2_At1g20050 - JF832422 JF832423, C2_At1g32130 - JF832429 JF832430, C2_At5g14320 - JF832437; 4*x*; I; Colombia. Cundinamarca.

S. hjertingii Hawkes; 186559; C2_At4g10050 - HQ641823, HQ641824, C2_At5g47390 - HQ641996, HQ641997, C2_At1g13380 - HQ642168, HQ642169, C2_At1g20050 - HQ642332, HQ642333, C2_At1g32130 - HQ642497, HQ642498, C2_At5g14320 - HQ642666; HQ642667; 4*x*; II; Mexico, Coahuila. ***S. hjertingii***; 251067; C2_At4g10050 - HQ641822, C2_At5g47390 - HQ641995, C2_At1g13380 - HQ642166, HQ642167, C2_At1g20050 - HQ642330, HQ642331, C2_At1g32130 - HQ642495, HQ642496, C2_At5g14320 - HQ642664; HQ642665; 4*x*; II; Mexico, Nuevo León. ***S. hjertingii***; 498050; C2_At4g10050 - HQ641820, HQ641821, C2_At5g47390 - HQ641993, HQ641994, C2_At1g13380 - HQ642165, C2_At1g20050 - HQ642328, HQ642329, C2_At1g32130 - HQ642493, HQ642494, C2_At5g14320 - HQ642662; HQ642663; 4*x*; II; Mexico, San Luis Potosí ***S. hjertingii***; 545713; C2_At4g10050 - HQ641827, HQ641828, C2_At5g47390 - HQ642000, HQ642001, C2_At1g13380 - HQ642172, HQ642173, C2_At1g20050 - HQ642336, HQ642337, C2_At1g32130 - HQ642501, HQ642502, C2_At5g14320 - HQ642670; HQ642671; 4*x*; II; Mexico, Coahuila.

S. iopetalum (Bitter) Hawkes; 498021; C2_At4g10050 - HQ641845, HQ641846, HQ641847, C2_At5g47390 - HQ642018, HQ642019, HQ642020, C2_At1g13380 - HQ642190, HQ642191, HQ642192, C2_At1g20050 - HQ642354, HQ642355, C2_At1g32130 - HQ642519, HQ642520, HQ642521, C2_At5g14320 - HQ642688; HQ642689; HQ642690; 6x II; Mexico, Veracruz. ***S. iopetalum***; 558405; C2_At4g10050 - HQ641857, HQ641858, HQ641859, C2_At5g47390 - HQ642030, HQ642031, HQ642032, C2_At1g13380 - HQ642202, HQ642203, HQ642204, C2_At1g20050 - HQ642362, HQ642363, C2_At1g32130 - HQ642531, HQ642532, HQ642533, C2_At5g14320 - HQ642700; HQ642701; HQ642702; 6x II; Mexico, Michoacán. ***S. iopetalum***; 607850; C2_At4g10050 - HQ641863, HQ641864, HQ641865, C2_At5g47390 - HQ642036, HQ642037, C2_At1g13380 - HQ642208, HQ642209, HQ642210, C2_At1g20050 - HQ642366, HQ642367, C2_At1g32130 - HQ642537, HQ642538, HQ642539, C2_At5g14320 - HQ642706; HQ642707; HQ642708; 6x II; Mexico, Hidalgo.

S. santolallae Vargas; 760350; C2_At4g10050 - HQ641878, C2_At5g47390 - HQ642049, C2_At1g13380 - JF832411 JF832412, C2_At1g20050 - JF832419 JF832420, C2_At1g32130 - JF832425 JF832426, C2_At5g14320 - JF832433 JF832434; 2x I; Peru, Cusco.

S. schenckii Bitter; 545733; C2_At4g10050 - HQ641894, HQ641895, C2_At5g47390 - HQ642065, HQ642066, HQ642067, C2_At1g13380 - HQ642238, HQ642239, HQ642240, C2_At1g20050 - HQ642397, HQ642398, HQ642399, C2_At1g32130 - HQ642568, HQ642569, HQ642570, C2_At5g14320 - HQ642735; HQ642736; HQ642737; 6x II; Mexico, Puebla. ***S. schenckii***; 558456; C2_At4g10050 - HQ641889, HQ641890, C2_At5g47390 - HQ642060, HQ642061, C2_At1g13380 - HQ642233, HQ642234, C2_At1g20050 - HQ642392, HQ642393, C2_At1g32130 - HQ642563, HQ642564, C2_At5g14320 - HQ642730; HQ642731; 6x II; Mexico, Oaxaca.

S. stoloniferum Schltdl.; 497994; C2_At4g10050 - HQ641916, HQ641917, C2_At5g47390 - HQ642088, HQ642089, C2_At1g13380 - HQ642262, HQ642263, C2_At1g20050 - HQ642421, HQ642422, C2_At1g32130 - HQ642592, HQ642593, C2_At5g14320 - HQ642759; HQ642760; 4*x*; II; Mexico, Chihuahua. ***S. stoloniferum***; 558395; C2_At4g10050 - HQ641903, HQ641904, C2_At5g47390 - HQ642075, C2_At1g13380 - HQ642248, HQ642249, C2_At1g20050 - HQ642407, HQ642408, C2_At1g32130 - HQ642578, HQ642579, C2_At5g14320 - HQ642745; HQ642746; 4*x*; II; Mexico, Baja California Sur. ***S. stoloniferum***; 558453; C2_At4g10050 - HQ641905, HQ641906, C2_At5g47390 - HQ641906, HQ642077, C2_At1g13380 - HQ642250, HQ642251, C2_At1g20050 - HQ642409, HQ642410, C2_At1g32130 - HQ642580, HQ642581, C2_At5g14320 - HQ642747; HQ642748; 4*x*; II; Mexico, Jalisco. ***S. stoloniferum***; 558454; C2_At4g10050 - HQ641907, HQ641908, HQ641909, C2_At5g47390 - HQ642078, HQ642079, HQ642080, C2_At1g13380 - HQ642252, HQ642253, HQ642254, C2_At1g20050 - HQ642411, HQ642412, HQ642413, C2_At1g32130 - HQ642582, HQ642583, HQ642584, C2_At5g14320 - HQ642749; HQ642750; HQ642751; 4*x*; II; Mexico, Queretaro. ***S. stoloniferum***; 558466; C2_At4g10050 - HQ641910, HQ641911, C2_At5g47390 - HQ642081, HQ642082, HQ642083, C2_At1g13380 - HQ642255, HQ642256, HQ642257, C2_At1g20050 - HQ642414, HQ642415, HQ642416, C2_At1g32130 - HQ642585, HQ642586, HQ642587, C2_At5g14320 - HQ642752; HQ642753; HQ642754; 4*x*; II; Mexico, Michoacán.

S. urubambae Juz.; 763314; C2_At1g13380 - JF832413, JF832414, C2_At1g20050 - JF832421, C2_At1g32130 - JF832427 JF832428, C2_At5g14320 - JF832435 JF832436; 2*x*; I; Peru, Cusco.