Development of PCR-based markers for discriminating *Solanum berthaultii* using its complete chloroplast genome sequence

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Abstract *Solanum berthaultii* is one of the wild diploid *Solanum* species, which is an excellent resource in potato breeding owing to its resistance to several important pathogens. On the other hand, sexual hybridization between *S. berthaultii* and *S. tuberosum* (potato) is limited because of their sexual incompatibility. Therefore, cell fusion can be used to introgress various novel traits from this wild species into the cultivated potatoes. After cell fusion, it is crucial to identify fusion products with the aid of molecular markers. In this study, the chloroplast genome sequence of *S. berthaultii* obtained by next-generation sequencing technology was described and compared with those of five other *Solanum* species to develop *S. berthaultii* specific markers. A total sequence length of the chloroplast genome is 155,533 bp. The structural organization of the chloroplast genome is similar to those of the five other *Solanum* species. Phylogenetic analysis with 25 other Solanaceae species revealed that *S. berthaultii* is most closely located with *S. tuberosum*. Additional comparison of the chloroplast genome sequence with those of the five *Solanum* species revealed 25 SNPs specific to *S. berthaultii*. Based on these SNPs, six PCR-based markers for differentiating *S. berthaultii* from other *Solanum* species were developed. These markers will facilitate the selection of fusion products and accelerate potato breeding using *S. berthaultii*.

Keywords PCR-based marker, cpDNA, Potato, SNPs, *Solanum berthaultii*

Introduction

Potato (*Solanum tuberosum* L.) is one of the most important crops in the world. Its production has been jeopardized seriously by diverse pathogens, particularly the oomycete pathogen, *Phytophthora infestans*, the causal agent of late blight (Rauscher et al. 2006). Therefore, potato breeding has focused on achieving high level resistance to late blight. Moreover, several resistant genes have been introgressed to improve the resistance to late blight from wild *Solanum* species into cultivated potatoes in potato breeding.

S. berthaultii originating from Bolivia, South America is a wild diploid species and a relative of potato (Pavek and Corsini 2001; Spooner and Castillo 1997). The species has been known as one of the important resources for resistance to several important pathogens such as *Phytophthora infestans*, potato virus Y etc. in potato breeding (Ewing et al. 2000; Nouri-Ellouz et al. 2016; Park et al. 2009; Rauscher et al. 2010; Tan et al. 2010). On the other hand, sexual incompatibility caused by the different ploidy levels of the genome and endosperm balance number (EBN) between this wild species and potato have restricted the utilization of this wild species for improvements during classical breeding processes (Cho et al. 1997; Ortiz and Ehlenfeldt 1992). To overcome this sexual barrier for interspecific gene transfer, therefore, protoplast fusion has been attempted with the two different species for crop improvement (Bidani et al. 2007; Nouri-Ellouz et al. 2016). In potato breeding, this technique has also been applied and succeeded in producing interspecific somatic hybrids such as *S. tuberosum* (+) *S. chaconense* (Chen et al. 2013), *S. tuberosum* (+) *S. brevidens* (Barsby et al. 1984), *S. tuberosum* (+) *S. nigrum* (Binding et al. 1982), *S. tuberosum* (+) *S. phureja* (Putie et al. 1986) and *S. tuberosum* (+) *S. commersonii* (Kim-Lee et al. 2005). When somatic hybridization is performed successfully, molecular markers can be used for the identification and verification of the somatic hybrids to confirm the hybridity

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Table 1 Results of comparative analysis of the chloroplast genome sequence of *S. berthaultii* with those of five Solanaceae species

Species	Accession no.	Total Length (bp)	GC content (%)	Total No. of genes	No. of tRNA	No. of rRNA	Reference
<i>S. berthaultii</i>	KY419708	155,533	38	137	39	4	This study
<i>S. nigrum</i>	KM489055	155,432	38	139	39	4	Cho and Park (2015)
<i>S. commersonii</i>	KM489054	155,525	37.5	133	33	4	Cho et al. (2016)
<i>S. tuberosum</i>	NC008096	155,296	37.5	131	36	4	Gargano et al. (2005)
<i>S. tuberosum</i>	KM489056	155,312	37	130	30	4	Cho et al. (2016)
<i>S. bulbocastanum</i>	NC007943	155,371	38	133	30	4	Daniell et al. (2006)

of both nuclear and organelle DNA (Cho and Park 2016; Cho et al. 2016). This process is essential due to the fact that a large amount of nuclear and organelle DNA can be transferred between two sexually incompatible species (Guo et al. 2004).

The chloroplast, a photosynthetic intracellular organelle has a circular double-stranded DNA molecule on its own genome. Most chloroplast genomes contain 110–130 genes encoding up to 80 unique proteins, approximately 30 tRNAs and 4 rRNAs. The angiosperm chloroplast is usually 115–165 kb in size and has a quadripartite organization comprised of two 12–75 kb inverted repeats (IR) separating the 80–90 kb large single copy (LSC) and 16–27 kb small single copy (SSC) regions (Yurina and Odintsova 1998). As shown in Table 1, several complete chloroplast genome sequences of *Solanum* species including *S. tuberosum* (KM489056 and NC008096), *S. commersonii* (KM489055), *S. bulbocastanum* (NC007943), and *S. nigrum* (KM489054) have previously been reported (Cho and Park 2016; Cho et al. 2016; Chung et al. 2006; Daniell et al. 2006). Their chloroplast genomes are highly conserved in size, structure and gene organization including the presence of two copies of IRs separated by LSC and SSC regions as previously known (Palmer 1991; Raubeson and Jansen 2005; Saski et al. 2005; Sugiura et al. 1998). Nevertheless, diversification of chloroplast genome sequences derived from the comparison of complete chloroplast genome sequences among plant species provides sufficient information (Cho et al. 2015). In the chloroplast genomes of many plants, a number of mutations such as single nucleotide polymorphism (SNPs), and insertions/deletions (InDels) and structural changes such as inversions, and rearrangements of gene order have been reported (Calsa Junior et al. 2004; Jheng et al. 2012; Kim et al. 2005; Saski et al. 2005).

Previously, the chloroplast genome sequence of *S. berthaultii* was completed using next-generation sequencing

technology and shortly announced (Park 2017). In this study, we described the result of the sequence in detail, compared it with those of other *Solanaceae* species, and developed specific markers for *S. berthaultii*.

Materials and Methods

Plant Materials and DNA Isolation

Plant materials of 22 genotypes of *Solanum* species such as *S. tuberosum* (PT56), *S. berthaultii* (PI310981; SB1-1, -2, -4 and -6), *S. acaule* (PI310970; SA-2), *S. pinnatisectum* (PI190115; SP-12), *S. hjertingii* (PI186559; SH1-15), *S. mochiense* (PI338616; SM1-6), *S. cardiophyllum* (PI341233; SC1-2), *S. verrucosum* (PI160228; SV1-4), *S. kurtzianum* (PI498422; SK-5), *S. stoloniferum* (PI160224; SS-1), *S. hougassi* (PI161174; SH2-10), *S. microdontum* (PI310979; SM2-3), *S. commersonii* (PI558050; SC2-7), *S. chacoense* (PI201846; SC3-6), *S. demissum* (PI218047; SD-9), *S. brevicale* (PI205394; SB2-7), *S. vernei* (PI230468; SV2-4), *S. jamesii* (PI578236; SJ-1) and *S. tuberosum* subsp. *andigenum* (PI566805; ST2-1) provided by the Highland Agriculture Research Institute, South Korea were used to compare molecular markers. They were cultivated in a greenhouse and cultured *in vitro*. For DNA isolation, fresh leaves collected from the greenhouse or *in vitro* plants were directly used or freeze-dried, ground and stored at -80°C when they were needed. Approximately 100 mg of fresh leaves were applied for the extraction of the total genomic DNA using a Genomic DNA Extraction kit for plants (RBC, New Taipei City, Taiwan).

Chloroplast Genome Sequencing

Sequencing was conducted using the Illumina HiSeq2000

(Illumina, SanDiego, CA, USA) platform at Macrogen (Macrogen, Seoul, South Korea) with the total genomic DNA of *S. berthaultii* (SB1-1). The chloroplast genome sequence was obtained by *de novo* assembly via the Phyzen bioinformatics pipeline (<http://phyzen.com>). A CLC assembler (ver. 4.06 beta, CLC Inc, Rarhus, Denmark) was used for *de novo* assembly. From the overall reads, reads with raw scores of 20 or less were discarded using the CLC-quality trim tool and the parameters of a minimum (300 ~ 1,000 bp) autonomously controlled overlap size were applied. From the total contigs, the principal contigs representing the chloroplast genome were retrieved by Nucmer (Kurtz et al. 2004) using the chloroplast genome sequence of *S. tuberosum* (KM489056) as the reference sequence (Cho et al. 2016). The results from BLASTZ analysis with the reference sequence arranged the representative chloroplast contigs in order (Schwartz et al. 2003) and they were connected to a single draft sequence by joining the overlapping terminal sequences followed by manual editing through a comparison with the chloroplast genome sequence of *S. tuberosum* (KM489056) reported by Cho et al. (2016). Gene annotation was conducted using DOGMA (Wyman et al. 2004) and the circular map of the *S. berthaultii* chloroplast genome was generated using the software OrganellarGenomeDRAW (<http://ogdraw.mpimp-golm.mpg.de>) (Lohse et al. 2013).

Comparison of Chloroplast Genome Sequences

The chloroplast genome sequence of *S. berthaultii* identified in this study was compared with those of 25 *Solanaceae* species including eight *Solanum* species, *S. tuberosum* (DQ386163), *S. commersonii* (KM489054), *S. bulbocastanum* (NC007943), *S. lycopersicum* (DQ347959), *S. pimpinellifolium* (NC026882), *S. peruvianum* (KP117026), *S. nigrum* (KM489055), and *S. melongena* (KU682719) obtained from the National Center for Biotechnology Information (NCBI). Sequence comparison and phylogenetic analysis were conducted using the chloroplast coding sequences of eight species by MEGA 6.0 (Tamura et al. 2013) and by a maximum likelihood and a maximum parsimony methods under PAUP* v4.0b10 (Swofford 2001), respectively. A total of 1,000 bootstrap replicates and a 50% cutoff value for the bootstrap consensus tree were applied for the analysis. Multiple alignment was also performed using the complete chloroplast genome sequences of six species by ClustalW2 in EMBL (<https://www.ebi.ac.uk/Tools/msa/clustalw2>).

Development of PCR-Based Markers

The *S. berthaultii* specific SNPs were identified with the

results of multiple alignment with five *Solanum* species: *S. berthaultii* (KY419708), *S. tuberosum* (KM489056 and NC008096), *S. commersonii* (KM489054), *S. bulbocastanum* (DQ347958), and *S. nigrum* (KM489055). After checking whether or not the proper restriction enzymes, which could digest only a SNP region on the sequence of *S. berthaultii* or on the sequence of other four *Solanum* species, existed using dCAPS Finder 2.0 (<http://helix.wustl.edu/dcaps/dcaps.html>), the primers covering each SNP region were designed. PCR was routinely performed with the 22 genotypes (PT56, SB1-1, SB1-2, SB1-4, SB1-6, SA-2, SP-12, SH1-15, SMI-6, SC1-2, SV1-4, SK-5, SS-1, SH2-10, SM2-3, SC2-7, SC3-6, SD-9, SB2-7, SV2-4, SJ-1, and ST2-1) as described by Cho and Park (2016). The DNA fragments in the PCR products were separated on 1% agarose gel and detected using the nucleic acid staining solution RedSafe (Intron Biotechnology, Seongnam, South Korea). When all 22 genotypes were amplified with certain primers, the selected restriction enzymes were applied to the PCR products. The digested fragments were separated by electrophoresis on 1% agarose gel and visualized under UV lights.

Results and Discussion

Complete Chloroplast Genome Sequence of *S. berthaultii*

The complete chloroplast genome sequencing of *S. berthaultii* was performed using NGS (next generation sequencing) technology. A library produced by the Illumina PE standard protocol generated 2,506,011,690 bp sequences in total and their average read length was 348 bp. Three representative contigs were identified using the *de novo* assembly of paired end sequences of the entire genome. They covered the entire chloroplast genome sequence of *S. tuberosum* (KM489056) reported by Cho et al. (2016) and arranged in order based on the results of BLASTZ analysis (Schwartz et al. 2003) (Fig. 1). The putative assembly errors curated by mapping 1,699.85x raw reads onto the final assembly, and PCR products of several regions including border sequences of a pair of inverted repeats (IRs), a small single copy region (SSC) and a large single copy region (LSC) validated by BigDye Terminator Cycle Sequencing with ABI3730 automated capillary sequencer finalized the completion of the chloroplast genome sequence. As a part of the results has shortly been announced (Park 2017), the size of the complete chloroplast genome sequence of *S. berthaultii* (GenBank accession no. KY419708) is 155,533 bp. It has the typical quadripartite structure including 25,593 bp IRs separated by SSC of 18,372 bp and LSC of 85,975 bp with a circular

Fig. 1 Assembly result of the complete chloroplast genome sequence of *S. berthaultii*. The four representative contigs (LSC, IRa, SSC, and IRb) for the chloroplast genome of *S. berthaultii* and comparison with the corresponding regions of the *S. tuberosum* chloroplast genome (KM489056) were combined. The blue and yellow bars indicate the contig matching with the reference sequence in the forward and reverse orientation, respectively and the corresponding positions are present at the sequence of *S. berthaultii*

Fig. 2 Gene map of the *S. berthaultii* chloroplast genome. The genes on the outside of the map are transcribed in the clockwise direction and the genes on the inside of the map are transcribed in the counterclockwise direction

double stranded DNA molecule as usual in most plant plastids. The total length is slightly longer than those of other *Solanum* species (Table 1). The chloroplast genome of *S. berthaultii* contains 137 unique genes and 11 of them

are duplicated in the IRs (Fig. 2). Forty-eight tRNAs and eight rRNAs exist and nine and four of them are duplicated and inversely oriented in the IRs (Fig. 2 and Table 1). 59.2% coding region of the genome was identified and an

Fig. 3 Maximum-likelihood phylogenetic tree of *S. berthaultii* and 25 species belonging to the *Solanaceae* family based on protein-coding sequences of each chloroplast genome. The numbers on each node indicate the bootstrap values from 1,000 replicates

average size of the coding sequences is 583 bp. It consists of 51.6% protein coding genes with an average size of 508 bp and 7.7% RNA genes with an average size of 214 bp. The GC content of the genome is 37.88% (Table 1). As reported by Cho et al. (2016) and Cho and Park (2016), the total length, the GC content, and the total number of predicted genes, tRNA and rRNA of the *S. berthaultii* chloroplast genome are highly conserved with those of other *Solanum* species.

Phylogenetic and Comparative Analysis of the Chloroplast Genome Sequences

As shown in Figure 3, the phylogenetic relationship was analyzed using chloroplast coding sequence of *S. berthaultii* and those of the 25 *Solanaceae* species. The maximum parsimony and maximum likelihood analyses produced the same topology and most nodes of the phylogenetic tree were strongly supported by the high bootstrap values. The results showed that *S. berthaultii* belonged to the same clade in *Solanum* species as expected and *S. berthaultii* formed the sister of *S. tuberosum*. Interestingly, *S. berthaultii* was the closest species to *S. tuberosum* even though it was *S. commersonii* (Cho et al. 2016).

The complete sequence of the *S. berthaultii* chloroplast genome was compared with those of the five other *Solanum*

species including two different genotypes of *S. tuberosum* to develop molecular markers for the selection of *S. berthaultii* cytoplasm as a reasonable number of InDels or SNPs was previously detected by the complete alignment of the chloroplast genome sequences including both the coding and non-coding regions of the *Solanum* species (Chung et al., 2006; Cho and Park, 2016). A result of multiple alignment with six species generated by ClustalW2 in EMBL (<https://www.ebi.ac.uk/Tools/msa/clustalw2>) showed that overall 39 and 1,788 regions for InDels and SNPs existed among the six *Solanum* species, respectively.

Discovery and Verification of Specific Molecular Markers for Distinguishing *S. berthaultii* from Potato and Other Wild *Solanum* Species

The multiple alignment of the chloroplast genome sequences of six different *Solanum* species revealed a lot of InDels and SNPs among the species. On the other hand, the InDels and SNPs were caused mostly by the sequences of *S. nigrum* or *S. bulbocastanum* (detail data not shown). This result was supported by the fact that those two species were farther phylogenetically from *S. berthaultii* than *S. commersonii* and *S. tuberosum* as shown in the result of phylogenetic tree (Fig. 3). Therefore, the criteria to search for the InDels or SNPs in which the sequences should be specific to *S.*

Table 2 Information of the primers and restriction enzymes to generate *S. berthaultii* specific markers

Marker name	Region	Strand	Primer sequence	Size (bp) ^a	RE ^b
Sber_CAPS1	<i>rps16-trnQ</i> (Intergenic)	Forward	GATTCTGCATCTTCACGTG	488	<i>Clal</i>
		Reverse	TCTTAATCAATGAGGTAGGC		
Sber_CAPS3	<i>rpoC1</i> (Intragenic)	Forward	TGGAACCATGACATATTTGC	573	<i>ApoI</i>
		Reverse	TGTGAAAGAATTTTGGTCC		
Sber_CAPS5	<i>accD-psaI</i> (Intergenic)	Forward	AATAAACAGTACCCGAAGG	590	<i>MseI</i>
		Reverse	TGTCTTTTGTCTTGTCTTC		
Sber_CAPS6	<i>accD-psaI</i> (Intergenic)	Forward	TTGGGTGCGATTGCAACG	570	<i>XmnI</i>
		Reverse	GATACAAGTCCACGTTTTC		
Sber_CAPS7	<i>ycf4</i> (Intragenic)	Forward	TGGATGGCTGAAATGTAGTC	577	<i>TaiI</i>
		Reverse	ATCCCCAACGAAAAATACAG		
Sber_CAPS14	<i>ycf1</i> (Intragenic)	Forward	ATAAATGAATTTCCCCTTTC	373	<i>MnII</i>
		Reverse	ATCTGAAAGCAAACAAATGC		

^aSize (bp) indicates the expected sizes of PCR fragments determined based on the *S. berthaultii* sequence.^bRE indicates restriction enzymes generating *S. berthaultii* specific markers by cutting the *S. berthaultii* specific SNPs.**Sber_CAPS_1**

S. tuberos (KM489056) GGGAGGGTTTCCTATCTATCAATTCGATCAAACTAGACTGAGCAATTGTCAGTCTTTATAG 6711
S. tuberos (NC008096) GGGAGGGTTTCCTATCTATCAATTCGATCAAACTAGACTGAGCAATTGTCAGTCTTTATAG 6711
S. berthau (KY419708) GGGAGGGTTTCCT**ATCG**ATCAATTCGATCAAACTAGACTGAGCAATTGTCAGTCTTTATAG 6709
S. commers (KM489054) GGGAGGGTTTCCTATCTATCAATTCGATCAAACTAGACTGAGCAATTGTCAGTCTTTATAG 6730
S. bulboca (DQ347958) GGAAGGGTTTGTATCTATCAATTCGATCAAACTAGACTGAGCAATTGTCACCGTTTATAG 6651
S. nigrum (KM489055) GGGAGGGTTTCGTATCTATCAATTCGATCAAACTAGACTGAGCAATTGTCACCGTTTATAG 6711

Sber_CAPS_3

S. tuberos (KM489056) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23499
S. tuberos (NC008096) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23499
S. berthau (KY419708) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23508
S. commers (KM489054) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23529
S. bulboca (DQ347958) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23387
S. nigrum (KM489055) ATCCCAATTGGATTGACTGGGATGACAGTTTCTCCTCCGAGTCTGTAAATCA**GAATTT** 23446

Sber_CAPS_5

S. tuberos (KM489056) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60303
S. tuberos (NC008096) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60303
S. berthau (KY419708) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60551
S. commers (KM489054) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60571
S. bulboca (DQ347958) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60391
S. nigrum (KM489055) AGATAAATATGGCCTTTTCTTTGGTGATAGAAGATCGAATTGTAGAAAGAAATCAAAACGA 60636

Sber_CAPS_6

S. tuberos (KM489056) CTCAGTTAGATTAGATATATCGATACTTCGATCTATACTAAGAATTTTAAATCTTCTCAA 60788
S. tuberos (NC008096) CTCAGTTAGATTAGATATATCGATACTTCGATCTATACTAAGAATTTTAAATCTTCTCAA 60788
S. berthau (KY419708) CTCAGTTAGATTAGATATATCGATACTTCGATCTATACTAAGAATTT**GAATTTCTTCTCAA** 61036
S. commers (KM489054) CTCAGTTAGATTAGATATATCGATACTTCGATCTATACTAAGAATTTTAAATCTTCTCAA 61056
S. bulboca (DQ347958) CTCAGTTAGATTAGATATATCGATACTTCGATCTATACTAAGAATTTAAATCTTCTCAA 60876
S. nigrum (KM489055) CTCAGTTAGATTAGATATATAGATACTTAGATCTATACTAATAATTTAAATCTTCTCTA 61115

Sber_CAPS_7

S. tuberos (KM489056) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61502
S. tuberos (NC008096) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61502
S. berthau (KY419708) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61750
S. commers (KM489054) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61770
S. bulboca (DQ347958) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61591
S. nigrum (KM489055) CAATTCCAATAAAATGCAATCGAGTAAAGTATGACTTGGCGATCAGACGATATATGGATA 61829

Sber_CAPS_14

S. tuberos (KM489056) TCAGTCACAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT124961
S. tuberos (NC008096) TCAGTCACAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT124961
S. berthau (KY419708) TCAGTCACAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT12508
S. commers (KM489054) TCAGTCACAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT125210
S. bulboca (DQ347958) TCAGTCACAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT124998
S. nigrum (KM489055) TCATTCAAAAAGCGAATAATTGAAACCTAAATCCTAAAT**GAAGA**AGATTCTGTTGAT125068

Fig. 4 Multiple alignment of the sequences on the intergenic and intragenic regions containing SNPs used to develop the CAPS markers. The chloroplast genome sequences of *S. tuberosum* (KM489056 and NC008096), *S. berthaultii* (KY419708), *S. commersonii* (KM489054), *S. bulbocastanum* (DQ347958), and *S. nigrum* (KM489055) were used and listed from top to bottom in each region of the CAPS markers. The regions of the SNPs detected on that of *S. berthaultii* are highlighted and the restriction sites are indicated in italic and bold

Fig. 5 PCR-based markers for the discrimination of *S. berthaultii* from other *Solanum* species. A: Sber_CAPS1. B: Sber_CAPS3. C: Sber_CAPS5. D: Sber_CAPS6. E: Sber_CAPS7. F: Sber_CAPS14. Sber_CAPS1, Sber_CAPS5, Sber_CAPS6, and E: Sber_CAPS7 are positively specific to *S. berthaultii* and Sber_CAPS3 and Sber_CAPS14 are negatively specific to *S. berthaultii*. The names of the markers correspond to those listed in Table 2. M, PT56, SB1(-1, -2, -4 and -6), SA-2, SP-12, SH1-15, SM1-6, SC1-2, SV1-4, SK-5, SS-1, SH2-10, SM2-3, SC2-7, SC3-6, SD-9, SB2-7, SV2-4, SJ-1 and ST2-1 indicate a 100bp marker ladder (Bioneer, Daejeon, South Korea), a breeding line of *S. tuberosum*, four different lines of *S. berthaultii* (PI310981), *S. acaule* (PI310970), *S. pinnatisectum* (PI190115), *S. hjertingii* (PI186559), *S. mochiuense* (PI338616), *S. cardiophyllum* (PI341233), *S. verrucosum* (PI160228), *S. kurtzianum* (PI498422), *S. stoloniferum* (PI160224), *S. hougassii* (PI161174), *S. microdontum* (PI310979), *S. commersonii* (PI558050), *S. chacoense* (PI201846), *S. demissum* (PI218047), *S. brevicale* (PI205394), *S. vernei* (PI230468), *S. jamesii* (PI578236), and *S. tuberosum* subsp. *andigenum* (PI566805), respectively

berthaultii, but mostly conserved in the five other *Solanum* species.

Only two of the 39 InDels detected were specific to *S. berthaultii*, but PCR-based molecular markers could not be developed using InDel specific primers. One base pair in

the sequence of *S. berthaultii* was different from those of *S. tuberosum* and *S. commersonii*, 19 base pairs from that of *S. bulbocastanum* and six base pairs from that of *S. nigrum* on the first InDel region. Two base pairs in the sequence of *S. berthaultii* were different from those of *S.*

tuberosum and *S. commersonii*, five base pairs from that of *S. bulbocastanum* and 438 base pairs from that of *S. nigrum* on the second InDel region. Therefore, this study focused on the SNPs identified among six species to develop PCR-based markers. A total of 25 of the 1,788 SNPs detected were specific to *S. berthaultii*. Five SNPs including four consecutive base pairs existed on the inverted repeat region. The regions on which the SNPs existed were distributed almost equally in both coding regions and non-coding regions throughout the entire chloroplast genome sequence although the number of InDels in the non-coding regions was reported to be detected much more than that in the coding regions of the chloroplast genome of *Solanum* species (Cho and Park 2016; Chung et al. 2006).

The application of PCR to SNPs can be used effectively to develop molecular markers after the PCR products are digested with the appropriate restriction enzymes on the site of the SNPs revealing polymorphisms, which is called the CAPS (cleaved amplified polymorphism sequence) marker (Konieczny and Ausubel, 1993). The CAPS is applied broadly to develop polymorphic markers that can be used to compare different genotypes on *Solanum* species (Park et al. 2005; Smilde et al. 2005; Uribe et al. 2014) and other plant species (Komori and Nitta 2005; Uncu et al. 2015; Wang et al. 2017). Therefore, 11 primers pairs covering each SNPs site onto which the proper restriction enzymes had been identified were designed. The restriction enzymes could digest only *S. berthaultii* or other *Solanum* species except *S. berthaultii*. The primers were used for PCR with four different lines of *S. berthaultii* (SB1-1, -2, -3, and -4), *S. tuberosum* (PT56), *S. acaule* (SA-2), *S. pinnatisectum* (SP-12), *S. hjertingii* (SH1-15), *S. mochiense* (SM1-6), *S. cardiophyllum* (SC1-2), *S. verrucosum* (SV1-4), *S. kurtzianum* (SK-5), *S. tuberosum* line, *S. stoloniferum* (SS-1), *S. hougassi* (SH2-10), *S. microdontum* (SM2-3), *S. commersonii* (SC2-7), *S. chacoense* (SC3-6), *S. demissum* (SD-9), *S. brevicale* (SB2-7), *S. vernei* (SV2-4), *S. jamesii* (SJ-1), and *S. tuberosum* subsp. *andigenum* (ST2-1) and the restriction enzymes for each SNPs were applied to the PCR products. Finally, six CAPS markers were identified (Table 2 and Fig. 5). Four primer combinations and restriction enzymes that produced distinct bands in the four *S. berthaultii* lines, but not in the other *Solanum* species including *S. tuberosum* (PT56) were selected (Table 2, Fig. 5A, 5C, 5D and 5E) and two others were vice versa (Table 2, Fig. 5B and 5F).

Characterization of the chlorotype using the molecular markers is important for not only potato breeding, but also evolutionary studies in *Solanum* species (Bohs and Olmstead 1997; Hosaka and Sanetomo 2012). In previous studies, a

random distribution of the chloroplast genome from a certain genotype during *in vitro* plant regeneration and somatic fusion was commonly observed in plant species including *Solanum* species, even though a high frequency of recombination occurred in the mitochondrial genome (Chen et al. 2013; Cho et al. 2016; Lössl et al. 2000; Mohapatra et al. 1998; Smyda-Dajmund et al. 2016; Xiang et al. 2004). Overall, the results suggest that the SNP-derived CAPS markers developed in this study can be used to discriminate *S. berthaultii* reliably from other *Solanum* species, to select the proper chlorotype from the fusion products between *S. berthaultii* and *Solanum* species and to facilitate potato breeding using *S. berthaultii*.

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