PCR-based markers developed by comparison of complete chloroplast genome sequences discriminate *Solanum chacoense* from other *Solanum* species

Soojung Kim • Tae-Ho Park

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Abstract One of wild diploid *Solanum* species, *Solanum chacoense*, is one of the excellent resources for potato breeding because it is resistant to several important pathogens, but the species is not sexually compatible with potato (*S. tuberosum*) causing the limitation of sexual hybridization between *S. tuberosum* and *S. chacoense*. Therefore, diverse traits regarding resistance from the species can be introgressed into potato via somatic hybridization. After cell fusion, the identification of fusion products is crucial with molecular markers. In this study, *S. chacoense* specific markers were developed by comparing the chloroplast genome (cpDNA) sequence of *S. chacoense* obtained by NGS (next-generation sequencing) technology with those of five other *Solanum* species. A full length of the cpDNA sequence is 155,532 bp and its structure is similar to other *Solanum* species. Phylogenetic analysis resulted that *S. chacoense* is most closely located with *S. commersonii*. Sequence alignment with cpDNA sequences of six other *Solanum* species identified two InDels and 37 SNPs specific sequences in *S. chacoense*. Based on these InDels and SNPs regions, four markers for distinguishing *S. chacoense* from other *Solanum* species were developed. These results obtained in this research could help breeders select breeding lines and facilitate breeding using *S. chacoense* in potato breeding.

Keywords cpDNA, InDels, *Solanum tuberosum*, SNPs, *Solanum chacoense*

Introduction

Potato (*Solanum tuberosum* L.) is the fourth ranked crops in the world production and a host to diverse pathogens and insects. Therefore, achieving high level resistance to them is one of the most important targets in potato breeding and various wild *Solanum* species have been used (Pavek and Corsini 2001; Simko et al. 2007).

S. chacoense originating from Argentina is one of wild potato species (Micheletto et al. 2000) and is important as resistant resources to several important pests and pathogens such as Colorado potato beetle, *Phytophthora infestans*, potato leafroll virus, potato tuberworm, Verticillium wilt, etc. in potato breeding (Brown and Thomas 1993; Cooper et al. 2009; Lynch et al. 1997; Micheletto et al. 2000; Sinden et al. 1986). However, its ploidy level is diploid and endosperm balance number (EBN) is two that are different from those of potato. Potato is tetraploid and its EBN is four. These conditions cause sexual incompatibility between *S. chacoense* and potato and restrict the utilization of the wild species in classical potato breeding (Cho et al. 1997; Ortiz and Ehlenfeldt 1992). Therefore, protoplast fusion was used with the two different species for crop improvement to overcome this sexual barrier for transferring certain interspecific genes from wild species into potato (Bidani et al. 2007; Nouri-Ellouz et al. 2016). Interspecific somatic hybrids between wild species such as *S. nigrum*, *S. brevidens*, *S. phureja*, and *S. commersonii* and potato were successfully produced in potato breeding (Barsby et al. 1984; Binding et al. 1982; Kim-Lee et al. 2005; Putie et al. 1986) and this technique has also been applied in between *S. tuberosum* and *S. chacoense* (Chen et al. 2013). When the products were obtained from the somatic fusion, the identification of the fusion products regarding both nuclear and organelle DNA is essential to confirm the

S. Kim • T.-H. Park (✉)
Department of Horticulture, Daegu University, Gyeongsan
38453, South Korea
e-mail: thzoo@daegu.ac.kr

Table 1 Summary of cpDNA sequences of *S. chacoense* and five other *Solanum* species

Species	Total Length (bp)	Total No. of genes	No. of rRNA	No. of tRNA	GC content (%)	Accession no.	Reference
<i>S. chacoense</i>	155,532	136	4	36	38	MF471371	In this study
<i>S. berthaultii</i>	155,533	137	4	39	38	KY419708	Kim et al. (2018)
<i>S. nigrum</i>	155,432	139	4	39	38	KM489055	Cho and Park (2016)
<i>S. commersonii</i>	155,525	133	4	33	37.5	KM489054	Cho et al. (2016)
<i>S. tuberosum</i>	155,296	131	4	36	37.5	NC008096	Gargano et al. (2005)
<i>S. tuberosum</i>	155,312	130	4	30	37	KM489056	Cho et al. (2016)
<i>S. bulbocastanum</i>	155,371	133	4	30	38	DQ347958	Daniell et al. (2006)

*The data have been partially adopted from Kim et al. (2018).

hybridity with molecular markers because a large amount of nuclear and cytoplasmic DNA can be transferred during the somatic hybridization between two sexually incompatible species (Cho et al. 2016; Cho and Park 2016; Guo et al. 2004).

The chloroplast genome (cpDNA) has a circular double-stranded DNA molecule and chloroplast is a photosynthetic intracellular organelle. The size of cpDNA of angiosperm is approximately 115~165 kb and its organization is typically quadripartite consisting of two inverted repeats (IR), the large single copy (LSC) and small single copy (SSC) regions (Yurina and Odintsova 1998). Most cpDNA contain approximately 110~130 genes encoding diverse unique proteins, rRNAs, and tRNAs. Several cpDNA sequences of *Solanum* species such as *S. berthaultii* (KY419708, Kim et al. 2018), *S. tuberosum* (KM489056, Cho et al. 2016) and (NC008096, Gargano et al. 2005), *S. commersonii* (KM489055, Cho et al. 2016), *S. bulbocastanum* (NC007943, Daniell et al. 2006), and *S. nigrum* (KM489054, Cho and Park 2016) have previously been completed (Table 1). Their size and gene organization are very similar and their structures are exactly same with two copies of IRs, LSC, and SSC regions (Palmer 1991; Raubeson and Jansen 2005; Saski et al. 2005; Sugiura et al. 1998). Nevertheless, sufficient information can be obtained from the comparison of complete cpDNA sequences among plant species because a number of mutations caused by structural changes, for instance, rearrangements of gene order and inversions, single nucleotide polymorphism (SNPs), and insertions/deletions (InDels) in cpDNAs of many plants occurs (Calsa Junior et al. 2004; Cho et al. 2015; Jheng et al. 2012; Kim et al. 2005; Saski et al. 2005).

The complete cpDNA sequence of *S. chacoense* was previously announced (Cho et al. 2017). The detailed information of the cpDNA sequence, the comparison among the cpDNA sequence and those of other species in the

Solanaceae family, and PCR-based markers specific to *S. chacoense* were described in this study.

Materials and Methods

Plant Materials

18 genotypes of *Solanum* species such as *S. acaule* (SA), *S. berthaultii* (SB1), *S. brevicale* (SB2), *S. cardiophyllum* (SC1), *S. chacoense* (SC3), *S. hjertingii* (SH1), *S. hougassi* (SH2), *S. jamesii* (SJ), *S. kurtzianum* (SK), *S. microdontum* (SM2), *S. mochiquense* (SM1), *S. pinnatisectum* (SP), *S. stoloniferum* (SS), *S. tuberosum* (PT56 and ‘Tamra’), *S. tuberosum* subsp. andigenum (ST2), *S. verrucosum* (SV1), and *S. vernei* (SV2) were obtained from the HARI (Highland Agriculture Research Institute). Their genebank accession numbers are PI310970, PI310981, PI205394, PI341233, PI201846, PI186559, PI161174, PI578236, PI498422, PI310979, PI338616, PI160224, PI566805, PI160228, and PI230468, respectively, except the *S. tuberosum* breeding line ‘PT56’ and variety ‘Tamra’. They were used to compare and develop molecular markers.

DNA Extraction

Approximately 100 mg of fresh leaves were collected from *in vitro* or greenhouse plants and used for the total genome DNA isolation using a Genomic DNA Extraction kit for plants (RBC, New Taipei City, Taiwan).

Chloroplast Genome Sequencing of *Solanum chacoense*

For the cpDNA sequencing of *S. chacoense*, the total genomic DNA of the line SC3-12 of *S. chacoense* was extracted and sequencing was conducted using the Illumina

Hiseq2000 (Illumina, San Diego, CA, USA) platform at Macrogen (Macrogen, Seoul, South Korea). The cpDNA sequence assembly was performed by *de novo* assembly protocol via the Phyzen bioinformatics pipeline (<http://phyzen.com>) (Cho et al. 2015). De novo assembly was conducted using trimmed reads by a 4.06 beta version of CLC genome assembler (CLC Inc, Aarhus, Denmark) with parameters of a minimum (300–1,000 bp) autonomously controlled overlap size. Raw score reads of 20 or less from the overall reads were discarded using the CLC-quality trim tool. The principal contigs of the cpDNA were retrieved by Nucmer (Kurtz et al. 2004). As the reference sequence, the cpDNA sequence of *S. commersonii* (KM489054) was used for it (Cho et al. 2016). The representative cpDNA contigs were arranged in order with the results from BLASTZ analysis with the reference sequence (Schwartz et al. 2003). A single draft cpDNA sequence of *S. chacoense* was completed by joining the overlapping terminal sequences and manual editing via a comparison with the *S. commersonii* (KM489054) cpDNA sequence reported by Cho et al. (2016). Gene annotation was conducted using DOGMA (Wyman et al. 2004) and the circular map of the *S. chacoense* cpDNA was generated using the software OrganellarGenome DRAW (<http://ogdraw.mpimp-golm.mpg.de>) (Lohse et al. 2013).

Comparison of cpDNA Sequences with Other Solanaceae Species

The chloroplast coding sequences examined by MEGA 6.0 (Tamura et al. 2013) with eight species including *S. chacoense* (MF471371) identified in this study and seven *Solanaceae* species, *Capsicum annuum* (JX270811), *S. berthaultii* (KY419708), *S. bulbocastanum* (NC007943), *S. commersonii* (KM489054), *S. lycopersicum* (DQ347959), *S. nigrum* (KM489055), and *S. tuberosum* (KM489056 and NC008096) obtained from NCBI (the National Center for Biotechnology Information) were applied to phylogenetic analysis. The same parameters employed in the maximum parsimony methods under PAUP* v4.0b10 were used for the likelihood search (Swofford 2001). A total of 1,000 bootstrap replicates and a 50% cutoff value for the bootstrap consensus tree were applied. Multiple alignment was also conducted using the complete cpDNA sequences of six species such as *S. berthaultii* (KY419708), *S. chacoense* (MF471371), *S. commersonii* (KM489054), *S. nigrum* (KM489055), *S. stoloniferum* (MF471373), and *S. tuberosum* (KM489056) by ClustalW2 in EMBL (<https://www.ebi.ac.uk/Tools/msa/clustalw2>).

Development of specific PCR-Based Markers for *Solanum chacoense*

The *S. chacoense* specific InDels and SNPs were identified using the results of multiple alignment with five *Solanum* species: *S. stoloniferum* (MF471373), *S. berthaultii* (KY419708), *S. commersonii* (KM489054), *S. nigrum* (KM489055), and *S. tuberosum* (KM489056). The primers on each InDel region were designed to examine the allele specificity for *S. chacoense*. PCR was carried out with the 18 genotypes (PT56, ‘Tamra’, SC3, SA, SP, SH1, SB1, SM1, SC1, SV1, SK, SS, SH2, SM2, SB2, SV2, SJ, and ST2) in the 20 µl mixture (20 ng genomic DNA, 0.5 mM dNTP mixture, 10 pMol each primer and 1 U Taq polymerase (Genetbio, Daejeon, South Korea)) as described by Cho and Park (2016) in a thermocycler (Biomtra, Göttingen, Germany). The PCR products were analyzed using nucleic acid staining solution RedSafe (Intron Biotechnology, Seongnam, South Korea) on the 1% agarose gel. For the development of SNP-derived specific markers to *S. chacoense*, the primers on each SNP region were designed after checking whether the proper restriction enzymes, which could digest only a SNP region on the sequence of *S. chacoense*, but not on the sequences of aligned other five *Solanum* species or only on the sequences of other five *Solanum* species, but not on the sequence of *S. chacoense* existed using dCAPS Finder 2.0 (<http://helix.wustl.edu/dcaps/dcaps.html>). PCR and gel-electrophoresis were routinely performed as described above. When certain primer amplifies all 18 genotypes, the PCR products were treated with the selected restriction enzyme. Electrophoresis on 1% agarose gel separated the digested fragments and polymorphic bands were visualized on UV lights.

Results and Discussion

cpDNA Sequence of *S. chacoense*

The sequencing of the *S. chacoense* complete cpDNA was conducted using the NGS technology. The Illumina PE standard protocol produced a library generating a total of 632,775,161bp sequences with an average read length of 297bp. Three representative contigs were generated by assembling *de novo* paired end sequences of the whole genome that covered the entire cpDNA sequence of *S. commersonii* (KM489054, Cho et al. 2016). BLASTZ analysis arranged them in order (Schwartz et al. 2003) (Fig. 1).

The putative errors in the assembled sequence were

Reference chloroplast genome: *S. commersonii* (KM489054: 155,525bp)

Fig. 1 Result of assembly of the complete cpDNA sequence of *S. chacoense*. The four representative contigs of the *S. chacoense* cpDNA and the corresponding regions of *S. commersonii* cpDNA (KM489054) are present. Yellow and blue bars indicate contig matching with the reference sequence in reverse and forward orientation, respectively

Fig. 2 Gene map of the *S. chacoense* cpDNA. Genes on the inside and outside of the map are transcribed in the counterclockwise and clockwise direction, respectively

curated by mapping 154.34x raw reads onto the final assembly. PCR and BigDye Terminator Cycle Sequencing with ABI3730 validated further on several regions including border sequences of a pair of inverted repeat regions (IRs), a small single copy region (SSC) and a large single copy region (LSC). As a part of the results has shortly been announced (Cho et al. 2017), the final cpDNA assembly reveals that the cpDNA has double-stranded DNA molecules and is circular as usual in most plant chloroplasts and the

size of the complete cpDNA sequence of *S. chacoense* is 155,532 bp (GenBank accession no. MF471371). Its structure is typically quadripartite and includes 25,592 bp IRs connected with SSC of 18,376 bp and LSC of 85,972 bp. The length is somewhat longer than those of other species in the *Solanum* genus, but 1 bp shorter than that of *Solanum berthaultii* (Table 1). The *S. chacoense* cpDNA contains 156 genes, 23 of which are duplicated in the IRs (Fig. 2). They included 106 protein-coding genes, 48 tRNAs

Fig. 3 Maximum-likelihood phylogenetic tree of *S. chacoense* with seven other *Solanaceae* species. The analysis was conducted with chloroplast protein-coding sequences of each cpDNA. The numbers indicate the bootstrap values from 1,000 replicates on each node

and eight rRNAs. Ten, nine and four of the protein coding genes, the tRNAs and rRNAs are inversely duplicated in the IRs, respectively (Table 1 and Fig. 2). 59.5% of the cpDNA occupied coding regions with an average size of 582.4 bp. There are 51.9% protein coding and 7.6% RNA genes with an average size of 761.7 bp and 223.7 bp, respectively. The GC content of the cpDNA is 37.89% that is highly conserved with those of other *Solanum* species (Table 1) and the predicted genes, tRNA and rRNA of the *S. chacoense* cpDNA are almost same in number and order with those of other *Solanum* species (Cho et al. 2016; Cho and Park 2016).

Phylogenetic and Comparative Analysis of the cpDNA Sequences

The phylogenetic analysis was conducted using the *S. chacoense* cpDNA coding sequences and those of the seven species in the *Solanaceae* family (Fig. 3). The maximum likelihood and parsimony analyses produced the same topology and the high bootstrap values supported strongly most nodes in the phylogenetic tree. The results interestingly showed that the closest *Solanum* species with *S. chacoense* was *S. commersonii* originating from Argentina.

To develop molecular markers which could discriminate *S. chacoense* cytoplasm, the complete *S. chacoense* cpDNA sequence was aligned with those of the five other species in the *Solanum* genus. A number of InDels or SNPs was previously reported by the alignment of the cpDNA sequences in the *Solanum* species (Cho and Park, 2016; Chung et al., 2006; Kim et al., 2018). Overall 280 and 1,490 InDels and SNPs through the whole cpDNA sequences were detected

by the multiple alignment with six species generated using ClustalW2 in EMBL (<https://www.ebi.ac.uk/Tools/msa/clustalw2>), respectively.

Discovery and Verification of *S. chacoense*-Specific Molecular Markers

The alignment of the cpDNA sequences revealed many InDels and SNPs. However, it was mainly caused by *S. nigrum* species that was farther phylogenetically from other *Solanum* species (Fig. 3). Therefore, the InDels or SNPs should be limited with criteria. The sequences on the InDels or SNPs should be specific to *S. chacoense*, but conserved in the five other *Solanum* species. SNPs were almost equally positioned on coding and non-coding regions through the whole cpDNA sequence, however, InDels on the non-coding regions were positioned more than on the coding regions in the cpDNA of *Solanum* species (Cho and Park 2016; Chung et al. 2006).

PCR with primer designed on InDel regions could be effectively applied to develop molecular markers that reveals polymorphism and the markers can be used to distinguish plant species (Garcia-Lor et al. 2013; Cho et al. 2015; Yamaki et al. 2013). Only two of the 280 InDels detected on the coding regions, *rps4* and *accD*, were comparable enough to develop markers specific to *S. chacoense*. Fourteen and four base pairs on the *S. chacoense* sequence differ from those of other five *Solanum* species on the first (SC3_InDel_11) and the second (SC3_InDel_16) InDel regions, respectively (Fig. 4). The InDel specific primers were designed on those two regions to develop PCR-based markers (Table 2) and used for PCR with a *S. tuberosum* breeding line (PT56), a potato variety ‘Tamra’, *S. chacoense* (SC3), *S. acaule* (SA), *S. pinnatisectum* (SP), *S. hjertingii* (SH1), *S. berthaultii* (SB1), *S. mochiquense* (SM1), *S. cardiophyllum* (SC1), *S. verrucosum* (SV1), *S. kurtzianum* (SK), *S. stoloniferum* (SS), *S. hougassi* (SH2), *S. microdontum* (SM2), *S. brevicaulis* (SB2), *S. vernei* (SV2), *S. jamesii* (SJ) and *S. tuberosum* subsp. *andigenum* (ST2). The results of PCR showed that the two InDels-based primers amplified specifically only *S. chacoense* as expected (Fig. 5A and 5B).

In total, 37 of the 1,490 SNPs were specific to *S. chacoense*. Molecular markers can effectively be developed by the application of PCR on the regions covering SNPs followed by the digestion of PCR product with the appropriate restriction enzymes that called the cleaved amplified polymorphism sequence (CAPS) marker (Konieczny and Ausubel 1993). The CAPS markers were widely used to compare with polymorphism in different genotypes of *Solanum*

SC3_InDel_11 (*rps4*)

SS16	CTTAATTACCAATTTTACTAACAACA-----AGGGCTCAAATAGCATTGG
SC3-12	CTTAATTCACCAATTTTACTAACAACA TTTACTAACAACA AGGGCTCAAATAGCGTTGG
Sber	CTTAATTCACCAATTTTACTAACAACA-----AGGGCTCAAATAGCATTGG
Scom	CTTAATTCACCAATTTTACTAACAACA-----AGGGCTCAAATAGCATTGG
Snig	CTTAATTCACCAATTTTACTAACAACA-----AGGGCTCAAATAGCAATGG
Stub	CTTAATTCACCAATTTTACTAACAACA-----AGGGCTCAAATAGCATTGG

SC3_InDel_16 (*accD*)

SS16	TAATAATCCTAGTGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
SC3-12	TAATAATCCTAGTGA AACA CTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Sber	TAATAATCCTAGTGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Scom	TAATAATCCTAGTGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Snig	TAATAATCCTAGTGA---TTGGATTCTATGGTTAGTCTGATAGGAAATAAGATATTCA
Stub	TAATAATCCTAGTGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA

SC3_SNP_7 (*rpoB*)

SS16	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG
SC3-12	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG
Sber	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG
Scom	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG
Snig	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG
Stub	GGAAATGATACCTTTATTTCCGTG TCTTC CAGCTACTTTATCGCCTACTTTGATTTCACG

SC3_SNP_15 (*accD*)

SS16	TAATAATC CTAGT GGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
SC3-12	TAATAATC CTAGT GGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Sber	TAATAATC CTAGT GGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Scom	TAATAATC CTAGT GGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA
Snig	TAATAATC CTAGT GGA---TTGGATTCTATGGTTAGTCTGATAGGAAATAAGATATTCA
Stub	TAATAATC CTAGT GGA---TTGGATTCTATGCTTAGTCTGATAGGAAATAAGATATTCA

Fig. 4 Sequence alignments on the each InDel and SNP regions. The cpDNA sequences of *S. stoloniferum* (SS16: MF471373), *S. chacoense* (SC3-12: MF471371), *S. berthaultii* (Sber: KY419708), *S. commersonii* (Scom: KM489054), *S. nigrum* (Snig: KM489055), and *S. tuberosum* (Stub: KM489056) were included. The sequences on the InDels and SNPs in the sequence of *S. chacoense* are highlighted. The restriction sites are red-colored and bold

Fig. 5 PCR-based markers for the discrimination of *S. chacoense* from other *Solanum* species. A: SC3_InDel_11. B: SC3_InDel_16. C: SC3_SNP_7. D: SC3_SNP_15. The four markers are all positively specific to *S. chacoense*. M, PT56, TR, SC3, SA, SP, SH1, SB1, SM1, SC1, SV1, SK, SS, SH2, SM2, SB2, SV2, SJ, and ST2 indicate size marker ladder, a *S. tuberosum* breeding line, a potato variety (Tamra), a line of *S. chacoense*, *S. acaule*, *S. pinnatisectum*, *S. hjertingii*, *S. berthaultii*, *S. mochiquense*, *S. cardiophyllum*, *S. verrucosum*, *S. kurtzianum*, *S. stoloniferum*, *S. hougassi*, *S. microdontum*, *S. brevicale*, *S. vernei*, *S. jamesii* and *S. tuberosum* subsp. andigenum, respectively

Table 2 Primers and restriction enzymes used to develop *S. chacoense* specific markers

Marker name	Region	S ^a	Primer sequence	Size (bp) ^b	RE ^c
SC3_InDel_11	<i>rps4</i> (Intragenic)	F	TTGTATCTTTATCCCGGAGC	300	as
		R	GTTGTTAGTAAATGTTGTTAG		
SC3_InDel_16	<i>accD</i> (Intragenic)	F	CTTTGTTCCGTGTGAAATA	334	as
		R	AAGCATAGAAATCCAGTGTT		
SC3_SNP_7	<i>rpoB</i> (Intragenic)	F	CTAGCAGACTCCCTGCTAAC	397	<i>MboII</i>
		R	GTGAAAGAATCTTCGTATGC		
SC3_SNP_15	<i>accD</i> (Intragenic)	F	TATAGGAGAGGACAAATCTC	406	<i>MaeI</i>
		R	TTCAATAGGACCAAGACTG		

^aF and R indicate forward and reverse strand of primers.

^bThe expected size of PCR fragments are measured based on the sequence of *S. chacoense*.

^cRestriction enzymes generating *S. chacoense* specific markers. ‘as’ indicates allele specific marker.

species (Cho and Park 2016; Cho et al. 2016; Kim et al. 2018; 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, 17 primer pairs which can amplify regions including each SNPs were designed and the proper restriction enzymes had been identified. The enzymes should digest only *S. chacoense* but not other *Solanum* species or digest all of other *Solanum* species, but not *S. chacoense*. PCR was performed with the primers on 18 different lines of *Solanum* species as above and the each PCR product was treated with the each proper restriction enzyme. Finally, two CAPS markers were confirmed (Table 2 and Fig. 5). Two primer combinations and restriction enzymes produced unique bands in the four *S. chacoense* lines, but not in the other *Solanum* species (Table 2, Fig. 5C and 5D).

The molecular markers characterizing chlorotypes are crucial for not only evolutionary studies, but also potato breeding in *Solanum* species (Bohs and Olmstead 1997; Hosaka and Sanetomo 2012). During somatic fusion and *in vitro* plant regeneration, a random distribution of cpDNA was reported in certain genotypes of plant species including *Solanum* species, although a high frequency of recombination occurred in mtDNA (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 markers developed based on the InDels and SNPs identified in this study can be used to reliably distinguish *S. chacoense* from other *Solanum* species, to select the proper chlorotype from the somatic hybrids between *S. chacoense* and *S. tuberosum* and to accelerate potato breeding using *S. chacoense*.

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