Development and Characterization of Microsatellite Markers for the Cape Gooseberry Physalis peruviana

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Abstract

Physalis peruviana, commonly known as Cape gooseberry, is an Andean Solanaceae fruit with high nutritional value and interesting medicinal properties. In the present study we report the development and characterization of microsatellite loci from a P. peruviana commercial Colombian genotype. We identified 932 imperfect and 201 perfect Simple Sequence Repeats (SSR) loci in untranslated regions (UTRs) and 304 imperfect and 83 perfect SSR loci in coding regions from the assembled Physalis peruviana leaf transcriptome. The UTR SSR loci were used for the development of 162 primers for amplification. The efficiency of these primers was tested via PCR in a panel of seven P. peruviana accessions including Colombia, Kenya and Ecuador ecotypes and one closely related species Physalis floridana. We obtained an amplification rate of 83% and a polymorphic rate of 22%. Here we report the first P. peruviana specific microsatellite set, a valuable tool for a wide variety of applications, including functional diversity, conservation and improvement of the species.

Introduction

Physalis peruviana commonly known as Cape gooseberry or golden berry is an Andean tropical fruit from the Solanaceae family native to South American countries including Colombia, Ecuador and Peru. Physalis peruviana grows wild in various parts of the Andes, typically 2,200 meters above sea level. The Cape gooseberry was known to the Incas but their origins are not clear, after Christopher Columbus the Cape gooseberry was introduced into Africa and India [1]. In Colombia, over the last three decades, P. peruviana went from being a neglected species to be the most promissory and successful exotic fruit for national and international markets; thus, since 1991, the Cape gooseberry market has been growing annually and in 2007 exports brought USD 34 million into the country. The main consumers of the Colombian Cape gooseberry are Europe with 97%, along with Asia and the United States with the remaining 3% [2]. The commercial interest in this fruit has grown due to its nutritional properties related to high vitamins content, minerals and antioxidants as well as its anti-inflammatory, anti-cancer and other medicinal properties [3,4,5,6,7,8].

Despite growing interest in the Cape gooseberry, little is known about its genetic diversity and population structure. The collections kept in germplasm banks have been partially evaluated for morphologic and agronomic traits [9,10,11]. Although it has been reported that Cape gooseberry is a diploid species with 2n = 48 [12]; different chromosome numbers might exist among genotypes since 2n = 24 has been reported for wild ecotypes, 2n = 32 for the cultivated Colombia ecotype and 2n = 48 for the cultivated Kenya ecotype [13]. The genetic diversity of the Cape gooseberry at the molecular level has been poorly studied, to our knowledge there is only one report applying dominant markers RAMs (Random Amplified Microsatellites) in 43 individuals from five geographical regions in Colombia suggesting high heterozygosity and genetic diversity [14]. Additionally, in our experience, the use of heterologous microsatellite markers previously developed for several other Solanaceae species have not been successful in identifying polymorphic markers in Cape gooseberry.

Microsatellites or SSRs are defined as highly variable DNA sequences composed of tandem repeats of 1–6 nucleotides with codominant inheritance which have become the markers of choice for a variety of applications including characterization and certification of plant materials, identification of varieties with agronomic potential, genetic mapping, assistance in plant-breeding programs, among others [15,16,17,18,19]. However, no SSR markers specific for P. peruviana have been developed. The genetic analysis with microsatellites is simple and robust, although their identification and development present significant challenges in emerging species [16,20]. According to the origin of the sequences used for the initial identification of simple repeats, SSRs are divided in two categories: Genomic SSRs which are derived...
from random genomic sequences and EST-SSRs derived from expressed sequence tags or from coding sequences. Genomic SSRs are not expected to have neither genomic function nor close linkage to transcriptional regions, while EST-SSRs and coding-SSRs are tightly linked with functional genes that may influence certain important agronomic characters. The de novo identification of simple sequence repeats has usually involved large-scale sequencing of genomic, SSR-enriched genomic or EST libraries, which are expensive, laborious and time-consuming. Next generation sequencing technologies have enabled rapid identification of SSR loci derived from ESTs which can be identified in any emergent species [17,19,21].

The goal of the present study was to identify polymorphic SSR loci using the assembled leaf transcriptome sequences from a commercial Colombian ecotype of *P. peruviana* developed in our laboratory (http://www.ncbi.nlm.nih.gov/bioproject/67621). Perfect as well as perfect repeat searches in non-coding or untranslated regions (UTRs) were performed. From these loci, primers were designed for amplification of UTR SSR loci. The effectiveness of these primers was tested via PCR in seven *P. peruviana* accessions (including Kenya, Ecuador and Colombia ecotypes) and one *Physalis floridana* accession, a closely related species (Table 1). The following PCR conditions were used: 1X PCR buffer: 1.5 to 3 mM MgCl2 depending on the primer pair, 0.2 μM dNTPs, 0.2 to 0.3 μM of each primer (depending on the primer pair), 0.05 U/μl Taq polymerase and 25 ng of genomic DNA, in a 15 μl reaction volume. The temperature conditions were 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 50 to 52°C (depending on the primer pair) for 30 seconds and 72°C for 90 seconds, and a final extension of 72°C for 8 minutes. The PCR amplification products were analyzed by polyacrylamide gel electrophoresis (PAGE).

### Materials and Methods

#### SSR loci identification and marker development

A collection of *Physalis peruviana* leaf transcript sequences was used as the source for SSR detection (Transcriptome Shotgun Assembly (TSA) Database, GenBank Accession numbers JO124085-JO157957). The transcripts were compared for sequence similarity with the non-redundant protein sequences database from NCBI using BLASTX. SSR loci were searched in both coding and non-coding sequences. Candidate SSR loci were identified using Phobos [22] in both coding and non-coding sequences using perfect and imperfect repeat searches with a minimum length of 18 bp for dinucleotides, 24 bp for tri and tetranucleotides, 30 bp for pentanucleotides and 36 bp for hexanucleotide repeats.

#### Primer design and amplification of SSR loci by PCR

Primer3 version 0.4.0 [23] was used to design primers for microsatellite amplification in *P. peruviana*. In addition, the oligocalculator - SIGMA Aldrich [http://www.sigma-genosys.com/calc/DNACalc.asp] was used to predict secondary structures (i.e. hairpins, primer dimers) for each primer pair designed. To determine the success of the microsatellite primer design, we carried out PCR tests to amplify the SSR loci in seven *P. peruviana* accessions (including Kenya, Ecuador and Colombia ecotypes) and one *Physalis floridana* accession, a closely related species (Table 1). The following PCR conditions were used: 1X PCR buffer: 1.5 to 3 mM MgCl2 depending on the primer pair, 0.2 μM dNTPs, 0.2 to 0.3 μM of each primer (depending on the primer pair), 0.05 U/μl Taq polymerase and 25 ng of genomic DNA, in a 15 μl reaction volume. The temperature conditions were 95°C for 3 minutes followed by 35 cycles of 95°C for 30 seconds, 50 to 52°C (depending on the primer pair) for 30 seconds and 72°C for 90 seconds, and a final extension of 72°C for 8 minutes. The PCR amplification products were analyzed by polyacrylamide gel electrophoresis (PAGE).

### Gene Ontology analysis of SSR loci

A gene ontology (GO) analysis was performed using blast2go [24] with the assembled transcript sequences containing the 30 polymorphic SSRs described here. These sequences were compared with the UniProtKB/Swiss-Prot database with a cutoff e-value of 1×10⁻³.

### Table 1. Plant material used for SSR development and characterization.

| Species       | Work Code | Accession/Common Name | Accession Code | Origin         | Country       |
|---------------|-----------|-----------------------|----------------|----------------|---------------|
| *P. peruviana*| 1         | ILS 3804*              | 09U086-1       | CORPOICA/Ambato| Ecuador       |
| *P. peruviana*| 2         | Ecotype Kenia         | 09U215-1       | Universidad de Nariño/NA | Colombia     |
| *P. peruviana*| 3         | Ecotype Colombia      | 09U216-1       | Universidad de Nariño/NA | Colombia     |
| *P. floridana*| 4         | ILS 1437*             | 09U139-1       | Botanical Garden of Birmingham/NA | U.K.       |
| *P. peruviana*| 5         | Novacampo (commercial) | 09U 274-1     | CORPOICA/Cundinamarca | Colombia      |
| *P. peruviana*| 6         | ILS 3807*             | 09U089-1       | CORPOICA/Antioquia | Colombia     |
| *P. peruviana*| 7         | ILS 3826*             | 09U108-1       | CORPOICA/Antioquia | Colombia     |
| *P. peruviana*| 8         | ILS 3817*             | 09U099-1       | CORPOICA/Caldas   | Colombia      |

ILS* = Introduction maintained at La Selva Research Center, CORPOICA; NA = Not available; "NA" = Not available (in vitro propagated material).

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### Table 2. SSR loci identified in *Physalis peruviana* leaf Expressed Sequence Tags (ESTs).

| Repeat Type | CDS UTRs | Total | Imperfect | CDS UTRs | Total | Frequency |
|-------------|----------|-------|-----------|----------|-------|-----------|
| Dinucleotide| 34       | 81    | 110       | 34       | 81    | 100       |
| Trinucleotide| 16      | 36    | 52        | 16      | 36    | 544       |
| Tetranucleotide| 12      | 36    | 48        | 12      | 36    | 544       |
| Pentanucleotide| 6       | 6     | 12        | 6       | 6     | 213       |
| Hexanucleotide| 46      | 64    | 110       | 46      | 64    | 530       |
| Total        | 34       | 81    | 110       | 34       | 81    | 100       |

The number of SSR loci identified at coding sequences (CDS) and Untranslated Regions (UTRs) by using perfect and imperfect repeat search criteria.

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Identification of SSR loci in *P. peruviana*

A total of 1,520 SSR loci were identified and a large fraction were located in UTRs (74%) as compared to coding sequences (CDS) with 26%. The highest number of SSR loci found contained trinucleotide and hexanucleotide repeats with 544 (36%) and 530 (35%) respectively (Table 2).

Microsatellite primer design and PCR analysis

The SSR loci selected for primer design were located at UTRs and identified with an imperfect repeat search to increase the probabilities for finding polymorphisms within the individuals analyzed. Using this strategy a total of 162 primers were designed. A successful PCR amplification was obtained for 138 (83%) of the 162 primers designed from microsatellite loci using seven *P. peruviana* and one *P. floridana* genotype (Table 1). Polymorphisms among the eight genotypes were observed for 30 (22%) loci whereas the remaining 108 loci were monomorphic (Figure 1, Tables 3 and 4).

Functional relationships of polymorphic SSR markers

A significant GO annotation was found for 10 of the 30 markers, which are related to 43 different ontology terms, of these 27 (67%) were related to biological process, 11 (25%) to molecular function and 5 (8%) to cellular component (Table 5).

| SSR Type     | Polymorphic | Monomorphic | Total |
|--------------|-------------|-------------|-------|
| Dinucleotide | 19          | 53          | 72    |
| Trinucleotide| 10          | 39          | 49    |
| Tetranucleotide| -         | 5           | 5     |
| Pentanucleotide| 1         | 1           | 2     |
| Hexanucleotide| -          | 10          | 10    |
| Total        | 30          | 108         | 138   |

Table 3. Polymorphisms in *Physalis peruviana* SSR loci.

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Discussion

Here we present the first collection of EST-derived microsatellite markers in *Physalis peruviana*. The highest number of SSR loci found contained trinucleotide and hexanucleotide repeats (Table 2), which is consistent with results reported in Solanaceae and other plant species [19,20,25,26,27,28,29,30,31]. 1,236 out of 1,520 SSR loci are composed of imperfect repeats increasing the probability of polymorphism among *Physalis* species. This inference is bolstered by the fact that 30 of the 162 imperfect SSRs (22%) were polymorphic in the panel of 8 accessions from *P. peruviana* and the related species *P. floridana* (Table 1), suggesting the potential utility of these genetic based SSR markers for future studies, i.e. germplasm diversity and breeding applications [17,19,32].

Our results show that most of the SSR loci were located at UTRs (Table 2) in agreement with the results reported by Morgante and others [27] who hypothesize that in plants most of the SSR loci from transcribed regions are distributed along the UTRs. Increased numbers of SSR loci at UTRs could be related to changes in transcription (5’UTRs) or RNA silencing (3’UTRs), which are sources of variation among species [18,19,20,29,30]. Cereal species appear to have a different SSR distribution; Yu and others [33] found that most of the 444 EST derived SSR markers (62%) were located at coding regions, while 38% were located at UTRs.

Since the SSR loci found in this study were derived from genes, they may be related to some traits of interest [18,20,27] such as resistance to *Fusarium oxysporum*, which is one of the main constraints for Cape gooseberry production at the commercial level. According to the functional annotation obtained by the GO analysis, two polymorphic SSR markers (SSR54 and SSR77 respectively) were related with proteins involved in defense responses to pathogens such as programed cell death and ethylene as well as jasmonic acid pathways. These two polymorphic SSR makers would be useful in *P. peruviana* breeding programs focused on *F. oxysporum* resistance.

The high rate of successful PCR amplification for the primer pairs designed (84%, Table 4) is related to the fact that these loci are specific to *P. peruviana* and they were also developed from genes, increasing the transferability within species of the same genus i.e. *P. floridana*. These results are in agreement with Zeng
Table 4. Allelic variation in 30 Physalis peruviana SSR loci.

| Polymorphic loci | Forward primer (5’-3’) | Reverse primer (5’-3’) | PCR conditions | Alleles (pb) | Repeat type | Location |
|------------------|------------------------|------------------------|----------------|-------------|-------------|----------|
| SSR1             | AGAGGACTCCATTTGTGCT    | TGAGGGTGTTGGATTTCT    | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 206      | 170 210 AT | 3’ UTR  |
| SSR2             | CATTGAGTTTCGGATCAT     | AGACAAGCTCAGGGAAAGG   | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 237      | 230 250 AG | 3’ UTR  |
| SSR9             | TGTCCCGATTTTACGTTTC    | GCAGTTGGATAGTTGAAGACG | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 193      | 220 240 AG | 5’ UTR  |
| SSR10            | GCTCTCAATTTGTTGCTGTA   | ACTTTGGTTTCGGGAATTTG  | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 185      | 170 190 AT | 3’ UTR  |
| SSR11            | CAGCTGAAAATAGAGCTGATTTG | CCGTTTTTCTCTCTCAAGCTT | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 180      | 180 210 AG | 3’ UTR  |
| SSR13            | GCGGATATCTTGTCTCTCAA   | CCGATGAGATAGATCGAGAAA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 190      | 160 210 AC | 5’ UTR  |
| SSR14            | TGAAAACCATCTAGCTGAAAGC | TGCTTGTTCTCATAACTCATC | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 204      | 200 220 AT | 3’ UTR  |
| SSR15            | GCTTGTGATCACGCTTCTTTG  | TGATACGATCTGTCAATAGC  | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 172      | 160 180 AT | 3’ UTR  |
| SSR18            | CAGATGATTACCTTGGAGACA  | GTGATTTTACGCTGCCAAT   | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 179      | 180 230 AC | 3’ UTR  |
| SSR20            | GCACATCACTAAAGATTTACCTTC | TTGTCCCTGTTGCTGTTATG  | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 270      | 170 220 AT | 3’ UTR  |
| SSR36            | ATGAACACACATGTCTGAGGA  | GGGATACAGCAAGGTGATTCA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 211      | 170 240 AG | 3’ UTR  |
| SSR37            | CCACACACTGAAAAGGGATCTG | CCACACTGAAAAGGGATCTG  | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 212      | 260 330 AG | 3’ UTR  |
| SSR54            | CGGCTGGATGCTCTCAAAGAT  | GCACCTCCTACTTTTTTAATCTCC | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 197      | 190 210 AC | 3’ UTR  |
| SSR55            | CAACTACATAGGGAGCAGCAAAA | ATTTTGGGGGAGGAGAGGAG | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 183      | 200 210 AG | 5’ UTR  |
| SSR57            | AGTGAAGCCACAGCCTTCTC   | GGGAAAGCTGTAATTGAAAGAA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 183      | 200 210 AT | 3’ UTR  |
| SSR67            | GCTCTGTTCCTCATTATCACCA | GCAGTTGAGGATCTACTCATT | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 207      | 180 240 AG | 3’ UTR  |
| SSR68            | GAAGCACAACACTACACAAAAA | AAGCCCTCTGTTCTACAGGCA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 187      | 160 220 AG | 3’ UTR  |
| SSR72            | GTGTCGCGATTTTCTCTCAA   | CCGCCGTTACTCTCTAAA    | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 158      | 130 170 AG | 3’ UTR  |
| SSR77            | CATACATACACTCTCCTCCTC  | TGGCTACTGTTACCTTCCC   | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 216      | 170 200 AT | 5’ UTR  |
| SSR92            | TGGTTTGGAGATGCTAGAAAGAAGA | GTGGTCCATCAACGCAAGAGG | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 205      | 180 210 AAG | 3’ UTR  |
| SSR107           | CATCAAGACCAAGAATACGGC  | TCCAACTTTTACATCTTCTCC | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 205      | 220 250 AAG | 5’ UTR  |
| SSR110           | ACCCCTATCGGACATCTCTTC | GGGTTATTCTGACGGGAAT | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 198      | 170 200 CTT | 3’ UTR  |
| SSR112           | CTACGCTTACACTGGACA     | CAGTTGAAATGCTAAGATTC | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 203      | 200 220 TCT | 3’ UTR  |
| SSR119           | AATCAGGGTACAGAAAGATGGG | GCAAGATAGGATGGTGTTTGG | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 180      | 130 180 AAG | 5’ UTR  |
| SSR121           | AGAACACCCCCTACAGCTA   | TGTTGAGTAAATGGGGAAA   | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 189      | 170 190 ATC | 3’ UTR  |
| SSR123           | TCGAGGAGCGGCTATATCTC  | GGGCTGCACGCAACAACCTCTC | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 216      | 190 210 ATC | 5’ UTR  |
| SSR126           | TCCAAAAGGAAACAAACAATACT | TGAATGCTATGTTGTTAGGA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 202      | 190 200 AGC | 5’ UTR  |
| SSR127           | TTGGTTTGGTACATCTGCAA  | GGGTTGCAACCTGATCTGCTG | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 180      | 140 160 AAT | 5’ UTR  |
| SSR138           | TTCGACTACACTCCAGCAGG  | CAACTTGGGGTATGGAATGGGTT | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 138      | 130 160 AAT | 3’ UTR  |
| SSR146           | AGGCTAATGAGAGGAAGCGCA  | GGGTGCATTCAAAAGCAACTGA | Primer [µM] 2 | MgCl₂ [mM] 50 | Tm 187      | 160 210 AAGAG | 3’ UTR |
et al. and Csencsics et al. [19,21], who used full-length cDNA and ESTs and found rates of successful PCR amplification larger than 80%.

This study reports the first set of microsatellite markers developed for *P. peruviana* and related species. A total of 1,520 SSR loci were identified, including 932 imperfect SSRs located at

| SSR Marker | GO Category: ID | Functional Annotation |
|------------|-----------------|-----------------------|
| SSR2       | P:0006350       | Transcription         |
| SSR37      | F:0001301       | Kinase activity       |
|            | C:0005886       | Plasma membrane       |
| SSR54      | P:0006952       | Defense response      |
|            | P:0012501       | Programmed cell death |
|            | C:0044464       | Cell part             |
|            | F:00001166      | Nucleotide binding    |
| SSR55      | P:0051865       | Protein autoubiquitination |
|            | F:0004842       | Ubiquitin-protein ligase activity |
|            | P:0048437       | Floral organ development |
|            | P:0046621       | Negative regulation of organ growth |
| SSR77      | P:0009789       | Positive regulation of abscisic acid mediated signaling pathway |
|            | P:0006979       | Response to oxidative stress |
|            | P:0052544       | Callose deposition in cell wall during defense response |
|            | P:0009753       | Response to jasmonic acid stimulus |
|            | P:0031348       | Negative regulation of defense response |
|            | P:0008219       | Cell death             |
|            | P:0009651       | Response to salt stress |
|            | P:0042742       | Defense response to bacterium |
|            | P:0009926       | Auxin polar transport  |
|            | P:0010119       | Regulation of stomatal movement |
|            | P:0009408       | Response to heat       |
|            | F:0005515       | Protein binding        |
|            | P:0010150       | Leaf senescence        |
|            | P:0048765       | Root hair cell differentation |
|            | P:0009871       | Jasmonic acid and ethylene-dependent systemic resistance, ethylene mediated signaling pathway |
|            | P:0001736       | Establishment of planar polarity |
|            | P:0050832       | Defense response to fungus |
|            | P:0010182       | Sugar mediated signaling pathway |
| SSR92      | F:0004674       | Protein serine/threonine kinase activity |
|            | P:0045449       | Regulation of transcription |
|            | P:0007169       | Transmembrane receptor protein tyrosine kinase signaling pathway |
|            | F:0005524       | ATP binding            |
|            | F:0003700       | Transcription factor activity |
|            | P:0010030       | Positive regulation of seed germination |
|            | P:0006468       | Protein amino acid phosphorylation |
| SSR110     | C:00044444      | Cytoplasmic part       |
| SSR126     | F:0005488       | Binding                |
|            | F:0003824       | Catalytic activity     |
| SSR138     | F:0016740       | Transferase activity   |
| SSR146     | C:0005730       | Nucleolus              |
|            | C:0016020       | Membrane               |
|            | F:0003677       | DNA binding            |

Gene ontology (GO) functional Categories: C = Cellular component, F = Molecular function, P = Biological process.

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UTRs. From these loci a total of 162 SSR primers were developed to assay their utility as microsatellite markers in a panel of seven accessions of *P. peruviana* and one accession of *P. floridana* by PCR amplification. A total of 138 (83%) primer markers amplified, with a polymorphism rate of 22%. The markers developed here can be used in plant breeding programs that may ultimately lead to reduction in the tendency to split during transport, reduction in the plant susceptibility to pests and diseases, and improvement of fruit quality.

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**Author Contributions**

Conceived and designed the experiments: LM-R LSB MIC VMNZ. Performed the experiments: JS ES PS. Analyzed the data: LM-R LSB MIC JS ES PS. Contributed reagents/materials/analysis tools: LM-R LSB MIC VMNZ. Wrote the paper: LM-R LSB JS. Conceived the SSR in silico component: LM-R. Conceived the SSR in silico component: LM-R. Conceived and oversaw the project and its components: LSB. Conceived the SSR analyses in the laboratory: MIC. Contributed to the original concept of the project: LSB LM-R MIC VMNZ. Jointly performed the in silico identification of SSRs: JS ES. Performed SSR analyses in laboratory: PS. Jointly prepared the manuscript: JS LSB MIC LM-R. Selected plant material for analyses: MIC LSB.