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Identification and validation of microsatellite markers in strawberry tree (*Arbutus unedo* L.)

Pedro FAZENDA[1], Ricardo PEREIRA[2], Maria FONSECA[1], Jorge CARLIER[1], José LEITÃO[1,*]

[1]Laboratory of Genomics and Genetic Improvement, FCT, University of Algarve, Faro, Portugal.

[2]Cortevelada Investigação Lda, Odiáxere, Portugal

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Abstract: Strawberry tree (*Arbutus unedo* L.), an evergreen shrub/small tree of the family Ericaceae, is a main constituent of the Mediterranean basin flora; although it is also found in southwestern France, Macaronesia, and Ireland. The small fruits are edible but mostly used for preparation of preserves and jams, and for liquors such as the Portuguese traditional "aguardente de medronho". Traditionally cultivated by small farmers, often in consociation with *Quercus* sp., strawberry tree is presently emerging as a new important fruit crop cultivated in large orchards by modern export-oriented enterprises. This change of paradigm requires a growing role of plant breeding, upstream of the production process. Genomic tools for this species are mostly limited to the chloroplast genome sequence and to genomic data described in this work. In order to identify strawberry tree microsatellite (SSR) loci we performed partial genome next-generation sequencing using the Ion Torrent technology. The sequenced ~24.6M nucleotides resulted in the identification of 1185 microsatellite markers mostly constituted by dinucleotide motifs. The relative amount of microsatellite dinucleotide motifs (AG/CT – 71.7%, AC/GT – 20.5%, AT/AT – 2.9%, and CG/CG – 0.3%) is similar to the one observed in other Ericaceae species. Among a tested sample of 40 SSR primer pairs, 20 amplified well-defined PCR products, 12 (30%) were validated as polymorphic. Used in our collaborative project for molecular identification of selected and improved clones, the identified SSR loci constitute a strong tool for a large panoply of applied and fundamental studies of this emerging fruit crop.

Key words: *Arbutus*, Ericaceae, microsatellites, next-generation sequencing, strawberry tree

1. Introduction

Strawberry tree (*Arbutus unedo* L.) is a member of the family Ericaceae and a major component of the Mediterranean basin flora but is also found from the north of the Iberian Peninsula to southwestern France, in Macaronesia, and in Ireland.

This evergreen shrub/small tree produces small edible light to dark red fruits used for the preparation of preserves, jams, and distillates. Both fruits and leaves are good sources of bioactive compounds and have been used for human health and well-being for a long time (Miguel et al., 2014; Ruiz-Rodríguez et al., 2014).

In Portugal, where they are locally known as "medronheiro", strawberry trees can be found almost all over the territory, although less frequently in regions with more accentuated continental climate. In the Algarve region, where strawberry tree is traditionally cultivated by farmers in small orchards, often in consociation with *Quercus* sp., the fruits are mainly used for production of the liquor "aguardente de medronho".

The popularity of strawberry tree as an ornamental species is also growing, and some commercial varieties, in general compact and with reddish to red flowers, are available on the market. So far, strawberry tree fruits have almost been absent from the fresh fruit market due to their fragility, short consumption period, and short shelf life (Molina et al., 2011).

During the last few years, an increasing interest in this fruit crop led to radical changes in strawberry tree production with the establishment of large orchards and the emergence of a modern strawberry tree industry. This new paradigm requires the substitution of traditional seed propagation with clonal propagation, and selection and breeding of specific clones for different purposes, e.g. ornamental plants, processed fruit, fresh fruit, and liquor production.

The omics studies of *A. unedo* are still at an early stage. The available genomic data (www.ncbi.nlm.nih.gov) are still very scanty and mostly include the sequence of the chloroplast genome (Martinez-Alberola et al., 2013),

* Correspondence: jleitao@ualg.pt

the data from our partial random genome sequencing (SRA/SRX341237) project, and identified microsatellite sequences.

Genetic studies of strawberry tree have been mostly based on random amplification of polymorphic DNA (RAPD) markers (Takrouni and Boussaid, 2010; Lopes et al., 2012) and cross-species *Vaccinium* spp. microsatellites (Gomes et al., 2013). More recently, Ribeiro et al. (2017) investigated the spatial distribution of the strawberry tree genetic variation in Portugal using microsatellites extracted from the chloroplast genome. In addition to *Vaccinium* (e.g., Boches et al. 2005; Liu et al., 2014; Schlautman et al., 2015), strawberry tree microsatellite (SSR) markers have been also developed for other Ericaceae genera such as *Rhododendron* (Delmas et al., 2011), *Erica* (Segarra-Moragues et al., 2009), and *Chimaphila* (Liu et al., 2012).

Herein, we report the identification of over one thousand microsatellite loci of *A. unedo* after partial next-generation genome sequencing of this species and the validation test of a sample of 40 microsatellite markers.

2. Materials and methods

2.1. Plant material

Small branches of 1 plant from the university campus and 15 plants from the enterprise Corte Velada (Lagos, Algarve, Portugal) were brought into the lab where leaves were used for DNA extraction.

2.2. Extraction of genomic DNA from leaf nuclei

Confirming the results of Sá et al. (2011), our first attempts to extract genomic DNA using common SDS- and CTAB-based protocols resulted in a low quantity of low-quality DNA (not shown). To circumvent this problem, we established a quick and reproducible protocol for DNA extraction from partially purified nuclei.

Briefly, leaf material was ground in the presence of liquid nitrogen in a mortar with a pestle and the leaf powder was transferred to 2 mL microfuge tubes containing 1 mL of isolation buffer: 50 mM Tris HCl pH 8.0, 1 M sucrose (gradient grade), and 2% Triton X-100. After first centrifugation at 80 × *g* for 2 min at 4 °C, the supernatant was transferred to a new microfuge tube and centrifuged at 900 × *g* for 5 min at the same temperature. The supernatant was discarded and the pellet, containing partially purified nuclei, was resuspended in 750 µL of previously heated to 75 °C modified buffer (300 mM Tris HCl pH 8.0, 25 mM EDTA pH 8.0, 2M NaCl, 1 mM DTT, 2% CTAB, and 2% PVP) (Doyle and Doyle, 1987) complemented with 250 µg/mL proteinase-K (Sigma) and 20 µg/mL RNase-A (Sigma). After 10 min incubation at 75 °C, the DNA was extracted with chloroform:isoamyl alcohol (24:1), precipitated, and stored in 75% absolute ethanol.

The extracted DNA was quantified by UV-spectrophotometry (NanoDrop 2000, ThemoScientific) and an evaluation of its amplifiability was performed by RAPD analysis as described in Elisiário et al. (1999) (Figure 1).

2.3. Preparation of sequencing library and Ion Torrent sequencing

The sequencing library was prepared from 2 µg genomic DNA of a plant from Campus de Gambelas, University of Algarve. Genomic DNA was digested with 20 U Csp6I (Fermentas, Life Sciences) for 5 h at 37 °C, followed by

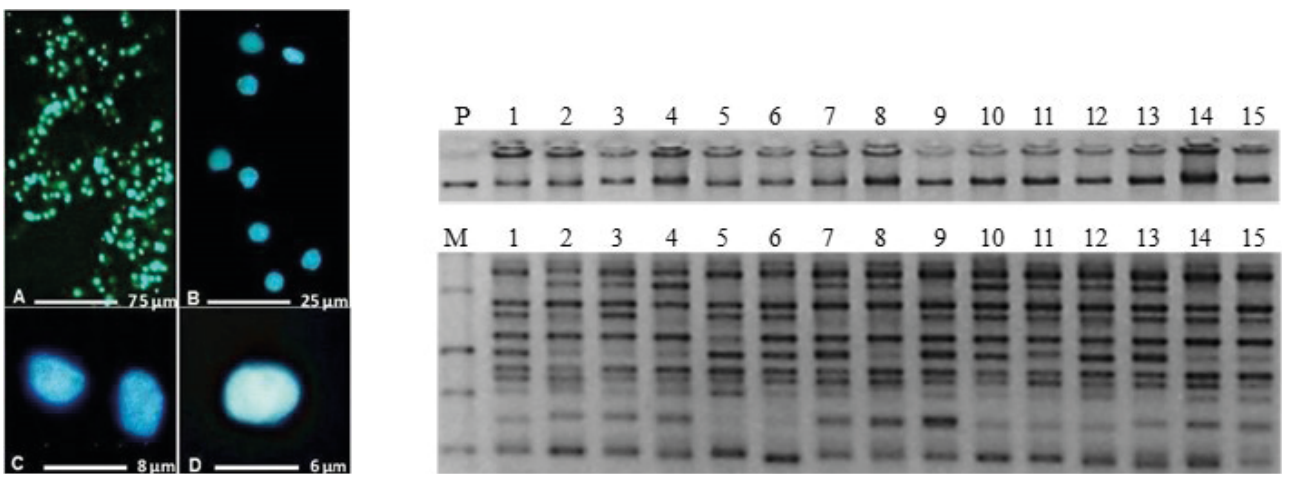

Figure 1. Left – Isolated cell nuclei of strawberry tree (*Arbutus unedo* L.) leaves stained with DAPI. Right (top) – extracted DNA from tree leaf cell nuclei of 15 strawberry tree (*Arbutus unedo* L.) plants. Notice the n absence of degradation in the extracted DNA. Right (bottom) – notice the good amplifiability of the extracted DNA tested by RAPD amplification (Primer AA02, Operon Technologies). P – Control DNA from pea (*Pisum sativum* L.); M – 1 Kb DNA ladder (Fermentas).

digestion (5 h at 65 °C) with 20 U TasI (Fermentas, Life Sciences). The restriction fragments, mostly in the range of 200–300 bp, were purified using GeneJET PCR Purification Kit (Fermentas, Life Sciences). End repair of fragments, ligation to adapters, and "nick-repair" were carried out with 100 ng digested DNA using the Ion Plus Fragment Library Kit (Life Technologies) according to the "User Bulletin – Preparing Short Amplicon (<250 bp) Libraries Using the Ion Plus Fragment Library Kit" (Life Technologies). After 8 cycles of PCR amplification, the 250–350 bp fragments were purified from agarose gels using GeneJET Gel Extraction Kit and the concentration of the library was estimated by fluorometry (Qubit ® 2.0, Life Technologies). Twenty microliters of the diluted library 26 pM library were ligated to the nanospheres, amplified by emulsion PCR, and the library was enriched in positive spheres using Ion One Touch System and Ion One Touch 200 Template Kit (Life Technologies). Finally, the enriched library was loaded onto an Ion 314TM chip and sequenced using the Ion PGMTM platform (Life Technologies) at the University of Algarve.

2.4. Data analysis

Raw data were saved in "fastq" format and the quality of the sequences was assessed using the FastQC v. 0.10.0 tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequences were selected by size using FastQ v. 1.0.0 filter and trimmed using Trim sequences v. 1.0.0 (http://hannonlab.cshl.edu/ fastx_toolkit/) of the Galaxy platform (Goecks et al., 2010). FASTQ to FASTA v.1.0.0 tool was used to convert the sequences to "fasta" format and sequence redundancy was eliminated using the cd-hit-est tool of the CD-HIT Suite platform (Ying et al. 2010).

2.5. Identification of microsatellite sequences

Sequences containing microsatellite motifs were identified using the software MsatCommander v. 0.8.2 (Faircloth, 2008) for detection of 6 or more repeats of di-, tri-tetra-, penta-, and hexanucleotide motifs. Sequences containing microsatellites were further aligned with raw data sequences using the GS Reference Mapper tool (Newbler v.2.6, http://454.com/products/analysis-tools/gs-de-novoassembler.asp) for identification of deeper alignments. Raw sequences were "de novo" assembled using GS De Novo Assembler available in the software Newbler v.2.6 set to default parameters except for "large or complex genome" and "heterozygous mode". Visualized using software Tablet 1.13.05.17 (Milne et al., 2010), the resulting contigs were used for selection of additional microsatellite loci.

2.6. SSR markers evaluation

The software FastPCR v. 4.0.27 (Kalendar, 2007) was used to design primers for 40 SSR markers. These markers were amplified in 15 µL final volume reaction mixtures containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl, 0.15 mM of each dNTP, 0.4 µM of each primer, 0.6 U of Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and 10 ng of genomic DNA using a thermocycler (VWR UnoCycler) programmed for: an initial step of 1 min and 30 s at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 57–60 °C (depending on the primer pair), 1 min at 72 °C, and a final extension step of 10 min at 72 °C.

Amplification products were analyzed in 3% agarose gels at 8 V/cm and in 10 cm long 10% polyacrylamide gels at 13 V/cm, stained with ethidium bromide, and photographed under UV transillumination.

3. Results

3.1. Extraction and amplifiability of genomic DNA

The developed protocol for DNA extraction based on partially purified nuclei resulted in a good quantity of high-quality DNA, with good amplifiability demonstrated by RAPD analysis (Figure 1).

3.2. Next-generation sequencing and identification of microsatellite loci

The partial Ion Torrent next-generation sequencing of strawberry tree genome resulted in 24.6M bases corresponding to 198,856 sequences with an average length of 123 nucleotides (www.ncbi.nlm.nih.gov/sra/SRX341237). The number of sequences was reduced to 132,681 sequences with ≥100 nucleotides after raw data

Table 1. Identified microsatellite (SSR) loci.

Repeats	Number of identified loci	Fraction (%)	Estimated genomic distance between SSRs (kb)
Dinucleotides	1131	95.44	10.70
Trinucleotides	48	4.05	252.17
Tetranucleotides	4	0.34	3026.02
Hexanucleotides	2	0.17	6052.04
Total	1185	100	-

filtering, and additionally reduced to 99,786 after trimming of the sequences at 125 nucleotides length and elimination of redundancy.

Using the Msatcommander v. 0.8.2 software, 1085 sequences were identified as harboring 1185 microsatellite loci and uploaded to the genomic databases (www.ncbi.nlm.nih.gov) with accession numbers: KF023636.1 to KF024720.1.

3.3. Analysis of microsatellite sequences and potential SSR markers

Analysis of the microsatellite motifs revealed that the most common repeated motifs are dinucleotides (~95%) with a strong contribution of the AG/CT motif (71.7%), followed by AC/GT (20.5%), AT/AT (2.9%), and CG/CG (0.3%). The AAG/CTT motif represents approximately half (4%) of the trinucleotide microsatellites (Tables 1 and 2).

To assess the general usefulness of the identified microsatellite and to select a set of SSR markers for identification of selected and genetically improved strawberry tree clones, primers were designed for a sample of 40 loci.

Two primer combinations did not produce amplification products; among the remaining 38 primer combinations, 20 produced clear and easily scorable amplification products. Twelve markers (30%) were found polymorphic among the tested strawberry tree plants (Table 3, Figure 2).

4. Discussion

The development of next-generation sequencing technologies combined with shotgun sequencing approach have created the conditions for a very efficient, rapid, affordable, and straightforward identification of large thousands of microsatellite loci.

The Ion Torrent technology, based on electronic detection of the incorporation of nucleotides with no fluorescence or luminescence and optical detection, and the successively increasing size of the sequencing fragments, has made next-generation sequencing particularly affordable, especially for relatively small projects as the one described here.

The extraction of good quantities of high-quality DNA from strawberry tree leaves using current protocols was found to be difficult by Sá et al. (2011) and was confirmed in our preliminary experiments.

A new protocol including an initial step of quick partial purification of cell nuclei was established which allows extraction of good quantities of high-quality DNA from a large number of leaf samples.

Ninety-five percent of the identified 1185 microsatellite loci in strawberry tree exhibits dinucleotide motifs. The relative frequency of microsatellite motifs in this species (Table 2) was found to be very similar to the one exhibited by other Ericaceae species, such as *Vaccinium macrocarpon* Ait. (Zhu et al., 2012) and *Vaccinium corymbosum* (Rowland et al., 2012), in which the motif AG represents, respectively, 73.0% and 76.0% of all dinucleotide microsatellites, and the motif GC is less represented, less than 1%.

The most common motifs, representing over 90% of all strawberry tree microsatellites, are AG/CT and AC/GT. This relatively equilibrated presence of A's and T's vs. G's and C's place Ericaceae between dicots, in which microsatellite motifs are richer in A's and T's, and monocots, in which G's and C's prevail (cf. Cavagnaro et al. 2010).

Although relatively small in number, the tested sample of 40 SSR loci allowed the identification of 12 SSR markers (30%) that produced well defined and polymorphic products.

In general terms, our results fit between those found by other authors in other plant species. If in *Jatropha*

Table 2. Type and frequency of identified microsatellite loci.

Motifs	Number of identified loci	Fraction (%)	Motifs	Number of identified loci	Fraction (%)
AG/CT	850	78.34	ATC	3	0.28
AC/GT	143	13.18	ACC	2	0.19
AT/AT	34	3.13	AAAG	2	0.19
AAG	19	1.75	AGT	1	0.09
AAC	8	0.74	CGG	1	0.09
AGG	6	0.55	AAAT	1	0.09
AGC	5	0.46	ACTC	1	0.09
CG/CG	4	0.37	AAAAAC	1	0.09
AAT	3	0.28	ATCTCT	1	0.09
Total				1085	100

Table 3. Validated microsatellite markers.

Locus / Genbank accession number	Primer sequence (5'–3')	SSR motif	Poly morphic
AU1427 / KF023647	F: gaaatataagcccaaaatcagc R: gcagaaacctatgctcatc	(AG)7	Yes
AU10205 / KF023705	F: caaactgtggcagtatgag R: tctaattcttcagcatgatatgg	(TA)6	No
AU18473 / KF023768	F: caatcggataaaaaattaatacctc R: ggttctttgacgagttactat	(CT)6	Yes
AU18938 / KF023771	F: aattttaggagaaagtg R: acgatacgaacaacaataataag	(CT)7	No
AU25325 / KF023821	F: ggataacggattcttcctag R: cattatactttcatcttgaaaaagg	(AG)7	Yes
AU32030 / KF023875	F: atttgaggtatccacaacatg R: gcagtattcgccatctaag	(GT)6	Yes
AU65100 / KF024173	F: taagaacgtatcaatgggc R: ttcaagatggtgttcctaatac	(GT)6	Yes
AU69656 / KF024218	F: attgagcgacagaactagt R: ctgtaactcatgcacgaa	(AG)9	Yes
AU81604 / KF024305	F: aatttgatcgaacttcacac R: tacttatccaaactctgaagg	(AG)8	Yes
AU93953 / KF024411	F: tggtaaacagtattaaggacag R: gtaggttttgccctacag	(AG)6	Yes
AU95244 / KF024426	F: gaatcaaaggtttggagt R: gtcagatcttccggtca	(AG)11	Yes
AU118386 / KF024616	F: gcgaaacaacgcagatc R: cagagagtggttgtagagag	(CT)6	No
AU47361 / KF024010	F: aacatttcatttctctctctct R: tcttgaagaggttgagtg	(CT)10	No
SSR_Au_ctg73	F: ttcataggatctcctctta R: aaggtagttcacaatttagaaatc	(TA)5	No
SSR_Au_ctg320	F: ccaaacattaatcggtcg R: ctcctacaagtaaagggag	(AT)5	No
SSR_Au_ctg608	F: tgtgactggtcacagag R: aattgcatgattggtcaaac	(CA)4	Yes
SSR_Au_ctg621	F: aacataacatccccttctc R: cgtagaacgtaaatatggtc	(CT)7	Yes
SSR_Au_ctg668	F: aatccaaggtaacccgaa R: ctctatagcactgccgaa	(GA)6	No
SSR_Au_ctg1191	F: gaatcaagggtttggagt R: aattttctgggcgattttaaag	(AG)11	Yes
SSR_Au_ctg1220	F: aagtggaaagctctctct R: atctaagttaagtttacggaagat	(TC)17	No

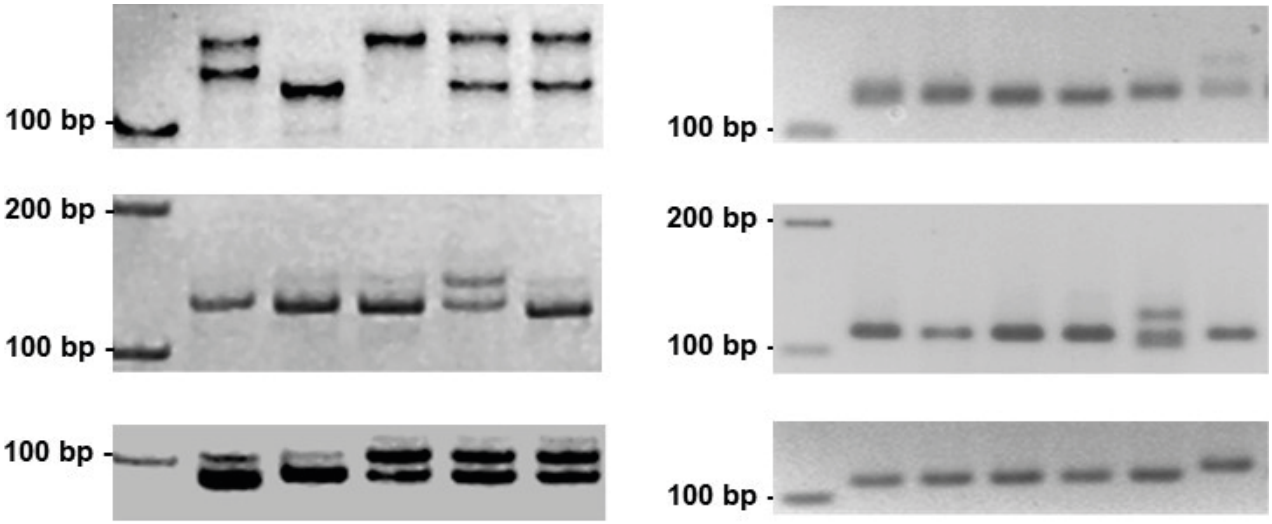

Figure 2. Confirmation of polymorphic microsatellite markers. (Left) 10% polyacrylamide gels. From top to bottom: markers AU81604, AU93953, and AU1427; (Right) 3% agarose gels. From top to bottom: markers AU32030, AU25325, and AU69656.

curcas Tian et al. (2017) were able to successfully amplify 77.1% of SSR markers selected for validation, in calla lily (*Zantedeschia rehmannii* Engl.) only 38.5% of SSR primer pairs produced clear PCR amplicons (Wei et al., 2016), and in maqui (*Aristotelia chilensis* [Molina] Stunz) only 22% (11 out of 50) primer pairs produced scorable polymorphic bands (Bastías et al., 2016).

Our preliminary analyses suggest that among over 1000 microsatellite markers identified in this study it can be expected that hundreds of markers are amplifiable and polymorphic, providing the research community with a strong analytical tool for the most diverse genetic and genomic strawberry tree studies.

Using fluorescent labelling and capillary polyacrylamide electrophoresis, the identified 12 polymorphic markers are being used for molecular characterization of some selected and improved clones aiming at the protection of intellectual property and for inclusion in the DUS data for registration of new strawberry tree varieties in the national catalogue of plant varieties.

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