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RESEARCH ARTICLE

# Isolation and characterization of microsatellite markers in *Garcinia gummi-gutta* by next-generation sequencing and cross-species amplification

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## Abstract

*Garcinia gummi-gutta* (L.) Roxb. (Clusiaceae) is an endemic, semidomesticated, fruit-yielding tree species distributed in the Western Ghats of India and Sri Lanka. Various bioactive phytochemicals, such as garcinol, benzophenones and xanthones are isolated from *G. gummi-gutta* and have shown antibacterial, antiviral and antioxidant activities. We sequenced the total genomic DNA using Illumina Hiseq 2000 platform and examined 241,141,804 bp high quality data, assembled into 773,889 contigs. In these contigs, 27,313 simple-sequence repeats (SSRs) were identified, among which mononucleotide repeats were predominant (44.98%) followed by dinucleotide and trinucleotide repeats. Primers were designed for 9964 microsatellites among which 32 randomly selected SSR primer pairs were standardized for amplification. Polymerase chain reaction (PCR) amplification of genomic DNA in 30 *G. gummi-gutta* genotypes revealed polymorphic information content (PIC) across all 32 loci ranging from 0.867 to 0.951, with a mean value of 0.917. The observed and expected heterozygosity ranged from 0.00 to 0.63 and 0.896 to 0.974, respectively. Alleles per locus ranged from 12 to 27. This is the first report on the development of genomic SSR markers in *G. gummi-gutta* using next-generation sequencing technology. The genomic SSR markers developed in this study will be useful in identification, mapping, diversity and breeding studies.

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## Introduction

*Garcinia gummi-gutta* (L.) Roxb., popularly known as 'Malabar Gamboge' (in English) '*uppage*' (in Kannada) belongs to the family Clusiaceae. It is a moderate-sized dioecious tree with round canopy, drooping branches and smooth dark barks. It is common in lower Shola forests of the Western Ghats, India, up to an altitude of 1800 mean sea level. *G. gummi-gutta* is recognized in Ayurveda, the traditional Indian system of medicine, for better digestion and is prescribed against abdominal disorders and heart diseases. The fruit rind is ground and used as a sour flavouring spice in the preparation of curries and to garnish fish preparations. The rind of the *uppage* fruit has been traditionally used in India and Sri Lanka as a culinary additive and fish preservative (Samarajeewa and Shanmugapirabu 1983; Bhagyavanth *et al.* 2010). Many studies showed that hydroxycitric acid (HCA), a secondary compound present in the rind of *uppage* fruit is effective in weight loss (Jena *et al.* 2002). The extract obtained from this fruits has exhibited the property of antiobesity. Recently, a number of studies have shown that *G. gummi-gutta* fruit extracts are rich in HCA and are effective in reducing body weight (Shara *et al.* 2004; Saito *et al.* 2005).

Germplasm utilization and conservation requires precise information on the genetics, genetic relationships and diversity. Earlier, a few studies attempted to examine diversity using random amplified polymorphic DNA (RAPD) and

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inter-simple sequence repeat (ISSR) markers (Mohan *et al.* 2012; Parthasarathy *et al.* 2013). However, development of microsatellite markers have helped in fingerprinting unique trees, assessing degree of diversity in the populations, and identifying marker tightly linked to the important agronomic traits like active ingredients, and resistance to biotic and abiotic stresses. Therefore, the development of markers has become a prerequisite for genetic studies (Bohra *et al.* 2011; Dutta *et al.* 2011). Keeping this in view, here, we report the development and standardization of microsatellite markers of *G. gummi-gutta* using next-generation sequencing (NGS) and their cross species transferability.

## Materials and methods

### *Plant materials*

The total genomic DNA from *G. gummi-gutta* was used for genome sequencing. We have also included *Garcinia indica* and *Garcinia morella* to examine cross species transferability of isolated microsatellite markers. The leaf material was obtained from the germplasm collection of the College of Forestry, Sirsi (University of Agricultural Sciences, Dharwad), India (see details in table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The leaf samples were obtained from Jaddi Gadde collection (14°44′13.2″N latitude and 74°43′03.2″E longitude with altitude of 498 m). The authenticated herbarium specimens were deposited to the herbarium of College of Forestry, Department of Forest Biology and Tree improvement.

### *Genome sequencing and assembly*

High-quality genomic DNA was isolated from the leaves of 30 *G. gummi-gutta* genotypes, one genotype from each of *G. indica* and *G. morella* (table 1 in electronic supplementary material) following modified CTAB method (Ravishankar *et al*. 2000). Total genomic DNA was sequenced using NGS Illumina HiSeq2000 platform at M/s Genotypic Bengaluru facility following manufactures instructions. High-quality sequence reads (Q > 20; >70% bases in a read) were used for *de novo* assembly into contigs using SOAPdenovo2 software (Luo *et al.* 2012). Assembly with Kmer-63 was selected as it has the optimal readings for N50.

### *Survey, identification and design primers for genomic microsatellite markers*

The Perl Script software program MISA (http://pgrc.ipk-gatersleben.de/misa/) (Thiel *et al*. 2003; Ravishankar *et al*. 2015) was used for identification of SSRs from assembled contigs (Feng *et al.* 2009; Ravishankar *et al.* 2015). MISA files were transferred to Microsoft Excel where SSRs were classified into mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide repeats and compound repeats. Primer pairs flanking the repeats were designed using Primer3 software (Untergrasser *et al.* 2012) (table 2 in electronic supplementary material).

### *PCR and genotyping*

We selected 50 SSR primer sets randomly and synthesized with M13 tail. These M13 tailed primers were first screened for amplification using pooled total genomic DNA from five randomly selected genotypes. We used fluorescence-based M13 tailing PCR method following Schuelke (2000) to amplify the microsatellites in a quick, accurate and efficient manner. The forward primer tailed with 5′-GTAAAACGACGGCCAGT-3′ and reverse primer tailed with 5′-GTTTCTT-3′. PCR was carried out in 20 $\mu$L reaction volume containing 2 $\mu$L of 10× reaction buffer, 2.0 $\mu$L of 1 mM dNTPs, 0.9 $\mu$L (5 pmol) of forward, 0.9 $\mu$L reverse primers (5 pmol), labelled M13 probes (HEX, NED, VIC, TET) 1.2 $\mu$L (5 pmol), 5.0 $\mu$L (50–75 ng) of template genomic DNA, 0.8 $\mu$L (2 U) of *Taq* DNA polymerase and 7.2 $\mu$L of nuclease free water. The PCR cycling profile was: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 5 min. PCR reaction was carried out using thermocycler (Eppendrof Master Cycler Gradient, Germany). Amplified products were initially separated on 3% agarose gel to confirm the amplification. Finally, 32 SSR primers were selected based on amplification of clear PCR products. These primers were employed for amplification of 30 genotypes of *G. gummi-gutta* and one genotype each of *G. indica* and *G. morella*. The PCR products were separated using automated DNA Sequencer (Applied Biosystems, ABI 3730 DNA Analyzer) through capillary electrophoresis, at M/S Eurofins facility, Bengaluru.

### *Genetic analysis of SSR markers*

The raw data generated was analysed and compiled using Peak Scanner ver. 1.0 software (Applied Biosystems, Foster

**Table 1.** Details of sequenced genomic data.

| Sequencing details | |
|---|---|
| Total number of contigs | 773889 |
| Total number of examined sequences (bp) | 241141804 |
| Total number of identified SSRs | 27313 |
| Number of SSR containing contigs | 26663 |
| Number of SSR containing more than one SSR | 631 |

**Table 2.** Simple-sequence repeat types in the *G. gummi-gutta* contigs sequences.

| Motif length | Number of SSR | Frequency (%) |
|---|---|---|
| Mononucleotide | 12286 | 44.98 |
| Dinucleotide | 9638 | 35.29 |
| Trinucleotide | 3003 | 10.99 |
| Tetranucleotide | 552 | 2.02 |
| Pentanucleotide | 249 | 0.91 |
| Hexanucleotide | 58 | 0.21 |
| Complex/compound | 1527 | 5.59 |
| Total | 27313 | |

**Table 3.** Genetic analysis of microsatellite markers developed for *G. gummi-gutta.*

| Locus name | Forward sequence 5′ → 3′ | Reverse sequence 5′ → 3′ | Repeat type | Number of allele (k) | Observed size range (bp) | Observed heterozygosity ($H_o$) | Expected heterozygosity ($H_e$) | Polymorphic information content | Probability of identity (PI) | Cross species amplification | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | GI | GM |
| GG_KVRf282 | TTTTGCACAAGCACACG CAGGT | TGTAGTCCTCCTTCAGGT TCGACG | (AG)6 | 24 | 107–163 | 0.296 | 0.957 | 0.936 | 0.0065 | NA | NA |
| GG_KVRf283 | TTTTCTCAATCTCCATGCA TTCCCCAA | AAAGGCCAAGAGCCTAA TATGATGAGC | (AC)6 | 24 | 101–255 | 0.375 | 0.925 | 0.900 | 0.0169 | NA | NA |
| GG_KVRf293 | TTTGCCAGGGGAAAAC ACAAATCCA | TGGGTTTTGGTTTATGCC CCAACTCG | (AG)7 | 19 | 100–191 | 0.05 | 0.965 | 0.938 | 0.0252 | NA | NA |
| GG_KVRf294 | TTTCTGCAGCATGGCC CTTGGC | TTGAACTAGGGAGGGCA CCCGT | (TG)7 | 19 | 100–186 | 0.000 | 0.969 | 0.942 | 0.0243 | NA | NA |
| GG_KVRf553 | TGTGAACATGCATGCCTT TGGATGTA | TCGGGTTGTTCCTCACAT CCACCT | (TG)8 | 24 | 330–460 | 0.208 | 0.969 | 0.946 | 0.0089 | A | A |
| GG_KVRf619 | TGTATATTTTGTGTGGCAG CAAGCCAT | TGGACTAGGGTGAGCC ACTACAGA | (AC)12 | 25 | 132–210 | 0.222 | 0.914 | 0.893 | 0.0133 | NA | NA |
| GG_KVRf659 | TGGTTGGCTTGCATATGT GGCATGT | CATTCACATTCATGTTGGCCA TGCTC | (AT)11 | 23 | 199–284 | 0.087 | 0.974 | 0.951 | 0.0105 | NA | NA |
| GG_KVRf716 | TGGTATGGATGGCATATGG TTTGGGT | GGGGGTGAGCAAACAAC AGGCT | (AT)6 | 27 | 126–278 | 0.36 | 0.965 | 0.943 | 0.0075 | NA | NA |
| GG_KVRf717 | TGGTATGGATGGCATATG GCTTGGT | GGGGTATCACTATAGC CCCCTN | (AT)8 | 23 | 160–264 | 0.292 | 0.951 | 0.928 | 0.0120 | NA | A |
| GG_KVRf796 | TGGGCTATACATGGTGACA GATGCC | GCCATGTGGATCCGCTTAG AACCA | (AT)6 | 22 | 192–286 | 0.208 | 0.964 | 0.941 | 0.0098 | NA | NA |
| GG_KVRg194 | TGCACCAGACAGTCGATT CCACC | TGGGGGTTTGATCCATGG AATGGG | (CA)7 | 20 | 143–241 | 0.364 | 0.964 | 0.939 | 0.0157 | NA | A |
| GG_KVRg313 | TGAGCATGCCATCTATTTGT GGGGA | GGACGGTCACTGGAAAG CACCT | (AT)6 | 17 | 302–451 | 0.143 | 0.954 | 0.927 | 0.0224 | NA | NA |
| GG_KVRj151 | AACAACGAGGGCGTCGT GGAAC | CATATCACCATCACCACC AACACGA | (TG)6 | 18 | 119–215 | 0.308 | 0.918 | 0.893 | 0.0151 | NA | A |
| GG_KVRj152 | AAATGGTGGCAAGACACAC ATACCTT | TGCCACCTTCCAGGCAC CATGT | (AC)6 | 15 | 146–243 | 0.091 | 0.943 | 0.916 | 0.0204 | A | NA |
| GG_KVRi741 | ACCACTGTTCCAACCAT GGGCA | AGAAGGTGGTGGAGGTGTA GGTGT | (CT)6 | 20 | 391–498 | 0.115 | 0.949 | 0.927 | 0.0089 | NA | NA |
| GG_KVRi010 | ACCACTCTGTCACGAC CCGACT | TGGTTGTGTAGGGATGTG GTAGCC | (TG)6 | 15 | 349–394 | 0.2 | 0.936 | 0.906 | 0.0324 | NA | A |
| GG_KVRi011 | AGGGGGAGGGGGTTTT GCTCAT | GTGTGTGCGTGTGTGG TCTTGC | (CA)6 | 17 | 241–351 | 0.37 | 0.943 | 0.921 | 0.0093 | NA | NA |
| GG_KVRg565 | TCCTATTACCCGCCCC ACCACt | GGAGGGCGGGATATATGTTG GAGGG | (AT)10 | 18 | 201–247 | 0.519 | 0.937 | 0.914 | 0.0103 | NA | A |
| GG_KVRg470 | TCGGGTGTCAGAACCGT AGCGA | GGTGTGGTATGCAGCTCTGT GATCT | (TA)6 | 17 | 106–188 | 0.37 | 0.945 | 0.923 | 0.0089 | A | NA |
| GG_KVRg670 | TCCACTCACAAGCCAAC AAGCCA | ACCCAAAAGAACCGGT GTGCGG | (TA)7 | 12 | 185–285 | 0.000 | 0.913 | 0.88 | 0.0384 | A | A |
| GG_KVRg756 | TCACGAAGAGAACCACTT ACCCCA | TCCTCGCCTCGTAACCTC CCAA | (GA)11 | 16 | 145–232 | 0.4 | 0.942 | 0.918 | 0.0120 | A | A |
| GG_KVRj174 | TTTGTCTGAGGCCTGTG AGGCA | GCAATGGAGGCAATGCT AGCCCA | (TTG)6 | 19 | 143–305 | 0.321 | 0.939 | 0.917 | 0.0097 | NA | A |
| GG_KVRj198 | TTGCCCCCAACCCAGA AATGGC | TGTTGCACTTGTGCCTGTT GTTGC | (CAA)9 | 18 | 102–158 | 0.077 | 0.937 | 0.913 | 0.0116 | A | A |

**Table 3** (*contd*)

| Locus name | Forward sequence 5′ → 3′ | Reverse sequence 5′ → 3′ | Repeat type | Number of allele (k) | Observed size range (bp) | Observed heterozygosity ($H_o$) | Expected heterozygosity ($H_e$) | Polymorphic information content | Probability of identity (PI) | Cross species amplification | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | GI | GM |
| GG_KVRj225 | TGTTGGATCCGTTGTT GTGCCT | TGGGGAGAGTGGCATTG AGCCT | (TTG)6 | 18 | 103–192 | 0.593 | 0.943 | 0.92 | 0.0093 | A | NA |
| GG_KVRj345 | TGGGGCATGCTCTGATC CACCA | GTGGGGGTGGCAAATGAG GCAA | (TGG)6 | 12 | 105–178 | 0.115 | 0.916 | 0.89 | 0.0165 | NA | A |
| GG_KVRj447 | TGCTGAGATGGCAGCG ATGCTT | ACTGGTCGCCCTGAGGTG GAAA | (CCA)5 | 18 | 110–211 | 0.179 | 0.932 | 0.91 | 0.0114 | A | NA |
| GG_KVRj541 | TGAGGCATAAAATGCAC GAGGGGT | TGGACCTAGCCATGCCC ATCCT | (TCA)5 | 20 | 226–337 | 0.259 | 0.945 | 0.923 | 0.0089 | A | NA |
| GG_KVRj603 | TCTCATGCGAAAGCTCAT TGCTCA | TTGCCACCTAAGCATGC CATGAA | (TCA)5 | 14 | 227–302 | 0.208 | 0.896 | 0.867 | 0.0245 | A | NA |
| GG_KVRj938 | GCAACATCCCAAGTGT GGGGGT | TGGTGCCATCCAGTTAG GGCAT | (ACC)5 | 17 | 128–197 | 0.233 | 0.919 | 0.897 | 0.0163 | A | A |
| GG_KVRk715 | TCCCCATCACCTTCCAC AAACGA | AGGGTGAGTTTTTGGC CAGCCC | (AATA)6 | 13 | 175–268 | 0.118 | 0.923 | 0.888 | 0.0622 | NA | NA |
| GG_KVRk769 | ATGCCGCACACCATGC GCTATT | GGGGAGGGTGTGCGTTG CTTTT | (AATG)5 | 18 | 309–411 | 0.208 | 0.941 | 0.916 | 0.0144 | NA | NA |
| GG_KVRk831 | TGTATTTCGGTCCATTAGC GGCCA | CCATGAGCCCAAGTGC GGGTTT | (TTTTC)5 | 24 | 237–324 | 0.63 | 0.953 | 0.932 | 0.0070 | NA | NA |

GI, *Garcinia indica*; GM, *Garcinia morella*; A, amplified; NA, not amplified.

City, USA) for determining the exact allele size. Allele sizes for each SSR loci were used for genetic analysis using Cervus 3.0 software (Kalinowski *et al.* 2007). We have estimated the number of alleles, observed heterozygosity ($H_o$), expected heterozygosity ($H_e$) and PIC. Probability of identity (PI) was analysed for each SSR loci using Identity 1.0 software (Wagner and Sefc 1999).

## Results and discussion

Off the wide range of DNA markers in use, microsatellites or SSR markers are extensively employed in plant studies. SSR markers are highly reproducible, multiallelic, PCR based, highly polymorphic, easy to use and amenable to automation. Thus, SSRs markers are widely used for mapping, crop breeding programmes and population genetics (Varshney *et al.* 2005). However, the use of microsatellite markers for studying nonmodel species like *G. gummi-gutta* has been impeded by lack of available genomic resources. A few years ago, identification of genomic SSRs and subsequent conversion to markers were expensive and time-consuming, involving construction and screening of microsatellite-enriched genomic DNA libraries (Glenn and Schable 2005). Compared to this hybrid capture method using probes, the present NGS based method is fast, simple, and overcomes a number of technical difficulties. The advent of NGS technologies, such as pyrosequencing, has made this process less complicated and easy (Zalapa *et al.* 2012). As a result, a large number of SSR markers can be developed in a short span of time and at a lower cost. This approach is especially useful for many tree crops where there is no sequence information available.

We used high-throughput IlliminaHiSeq 2000 platform to develop genomic SSR markers in *G. gummi-gutta*. The number of assembly of reads of the long sequences was 773,889 contigs of total length 241 Mb (table 1). An SSR survey of genomic sequences using MISA software (http://pgrc.ipk-gatersleben.de/misa) revealed that the 773,889 contigs contained 27,313 SSRs. Mononucleotide repeats are predominant (44.98%) followed by dinucleotide (35.29%) and trinucleotide (14.9%) repeats (table 2). Mononucleotide repeats are present in high number in some monocots (rice, sorghum and *Brachypodium*) and also in some dicots (*Arabidopsis*, *Medicago* and *Populus*) (Sonah *et al.* 2011). In the present study, apart from mononucleotide repeats, dinucleotide repeats were the most prevalent accounting for 35.29% of all SSRs identified, followed by trinucleotide repeats (10.29%; table 2). While the mononucleotide, dinucleotide and trinucleotide repeats contribute to the major proportion of SSRs (90.56%) and the rest was contributed by tetranucleotide, pentanucleotide and hexanucleotide repeats (table 2). The molecular mechanism of the origin and evolution of microsatellite markers are not clearly understood. The relative dominant occurrence of repeat motif of a particular sequence types and its length in plant genome might be the outcome of selection pressure applied on that specific motif during evolution. The most common mutation mechanisms assumed to be operating are replication slippage, and unequal

**Table 4.** Summary of genetic analysis.

| | Mean | Range |
|---|---|---|
| Polymorphic information content | 0.9173 | 0.867–0.951 |
| Observed heterozygosity | 0.2474 | 0.000–0.63 |
| Expected heterozygosity | 0.942 | 0.896–0.974 |
| Allele per locus | 18.8 | 12–27 |
| Probability of identity | 0.01631 | 0.0065–0.0622 |

Total number of alleles is 606. Total probability of identity is 4.538906e-060.

crossing over leading to addition or removal of one or more motifs and variation in the length (Buschiazzo and Gemmell 2006; Sonah *et al.* 2011).

### *Genetic analysis and transferability of genomic SSR markers*

In this study, 32 primers amplified clear PCR products in *G. gummi-gutta*. A high rate of successful amplification can be due to high-quality sequence data and the appropriate primer parameters, such as high GC content. Genetic analysis using 32 SSR markers in 30 accessions showed PIC values ranging from 0.867 to 0.951 with a mean value of 0.917. The mean values of observed and expected heterozygosity are 0.2474 and 0.942, respectively. The allele per locus ranged from 12 to 27 with a mean value of 18.8. The PI values ranged from 0.0065 to 0.0623 with a mean value of 0.0163 (tables 3 and 4). The total PI was $4.538906 \times 10^{-60}$.

Higher average PIC value (0.917) and average alleles (18.8) per locus (tables 3 and 4) was observed. This may be due to the high heterozygosity in the species, which helped to capture a large number of alleles. In our study, 12 SSR markers (36%) had more than 20 alleles per locus, indicating the high heterozygosity and diversity of accessions used (tables 3 and 4). The PI (the probability that two randomly selected diploid genotypes would be identical, assuming observed allele frequencies and random assortment) is very low for many loci. These low PI values confirm their applicability to DNA fingerprinting. Thus, these SSR markers can be easily employed for genotyping individuals.

Of the 32 primer sets, 11 (34%) yielded amplification products in *G. indica* DNA and 12 (36%) in *G. morella* DNA (table 3) indicating successful cross amplification of SSR markers in *Garcinia*.

The present study describes the isolation and characterization of microsatellites isolated from whole-genome sequence data of *G. gummi-gutta.* The NGS and mining of the *G. gummi-gutta* genome helped in identification of thousands of SSR markers. The information in this study will be an important repertoire of molecular tools available for genetic studies, genotyping and conservation strategies in *G. gummi-gutta.*

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