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*Original Research Paper*

# Isolation and characterization of microsatellite markers from *Garcinia morella* using next generation sequencing technology and cross-species amplification

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## ABSTRACT

The fruit-bearing medicinal tree, *Garcinia morella*, grows in the tropical rain forests of India's Western Ghats, Indo-Chinese Himalayan regions and Sri Lanka. Its fruit rinds are used as a garnish and in seasoning during food preparation. In order to genetically exploit it and assess diversity, development of microsatellite markers was attempted. We partially sequenced genomic DNA using the Illumina Hiseq 2000 platform and examined sequence data for microsatellite loci. We obtained high-quality 10653 Mbp data and was assembled into 1613263 contigs. A total of 121199 SSRs were discovered, Di nucleotide repeats were predominant (42.5%), followed by mono and tri nucleotide repeats (30.4 and 7.9%, respectively). We were able to design primers for 52901 microsatellites. Genetic analysis of 48 SSR loci, showed PIC values ranging from 0.067 to 0.939 with a mean value of 0.7547. The allele per locus ranged from 2 to 24 with a mean of 13. These microsatellite markers can be employed for genetic diversity analysis, molecular characterization and mapping different traits.

## INTRODUCTION

*Garcinia morella* (Gaertn.) Desr., family Clusiaceae, is commonly recognized as 'Indian gamboges' or 'Ceylon gamboges.' This tree is found in the tropical rain forests of the Western Ghats in India, Sri Lanka, and the Indo-China Himalayan regions. Its habitat spans various regions, including the forests of Eastern Bengal, Khasi Mountains, and both Western and Eastern Peninsular India. It particularly thrives in the rain forests of Karnataka state within the Western Ghats (Parthasarathy et al., 2013a). *G. morella* is a multipurpose tree grown as a plantation crop along with *Garcinia indica* (Kokum) and *Garcinia cambogia* (Malabar tamarind). The fruit rinds are used as a condiment and a garnish. From *G. morella,* many bioactive compounds are isolated from the fruits and the bark such as moreollin (Subba Rao et al., 1978) and gambogic acid (Tang et al., 2011), respectively, which are evaluated for their antibiotic and anticancer properties. The yellow color from the gamboge is used as dye to draw paintings on traditional house walls.

To ensure the efficient utilization and conservation of germplasm, it is essential to gain precise understandings of genetic relationships and diversity. In light of this, the development of markers, especially microsatellite markers, emerges as a valuable technique to evaluate genetic diversity, interpopulation relationships, and molecular characterization. The development of molecular markers would help in the construction of genetic linkage map, enable the study of trait inheritance, and subsequently enable the pinpointing of markers closely linked to vital agronomic traits. (Bohra et al., 2011). Till date, there are no reports on development of microsatellite markers for *G. morella*. Here, we report the partial sequencing of *G. morella* genome, *de novo* assembly and identification of SSR markers.

## MATERIALS AND METHODS

### Plant material

The total genomic DNA from *G. morella* was used for genome sequencing. The plant material was obtained from the germplasm collection of the College of Forestry, Sirsi, Karnataka, India.

**Genome sequencing and assembly**

High quality genomic DNA was isolated from the leaves of selected *G. morella* genotypes using modified CTAB method (Ravishankar et al., 2000). Total genomic DNA was sequenced using next generation sequencing Illumina HiSeq2000 platform at M/s Genotypic Pvt. Ltd, Bengaluru facility. Raw data obtained was processed for quality control. High quality data were used for assembly into contigs. *De novo* assembly of reads into contigs was performed using SOAPdenovo2-src-r240 software (Luo et al., 2012).

***Survey, identification and design primers for genomic SSR markers***

All assembled scaffolds were screened for the presence of SSRs using MISA software (http://pgrc.ipk-gatersleban.de/misa). MISA files were transferred to Microsoft Excel where SSRs were classified into mono-, di-, tri-, tetra-, penta- and hexa-nucleotide and compound repeats. Primer pairs flanking the repeats were designed using Primer 3 software (http://wwwgenome.wi.mit.edu/genomesoftware/other/Primer3.html) (Koressaar & Remm, 2007).

***PCR standardization and Genotyping***

Genomic DNA of thirty *G. morella* genotypes was adjusted to a final concentration of 25 ng/µl separately. A total of 50 SSR primers was selected randomly and was synthesized with M13 tail. These M13 tailed primers were first screened for amplification using pooled total genomic DNA from five randomly selected genotypes. Fluorescence based M13 tailing PCR method of Schuelke (2000) was used to amplify the microsatellites in a quick, accurate and efficient manner. PCR was carried out in the 20 µl reaction volume containing 2 µl of 10X reaction buffer, 2.0 µl of 1 mM dNTPs, 0.9 µl (5 pmol) of forward, 0.9 µl reverse primers (5 pmol), labeled M13 probe 1.2 µl (5 pmol), 5.0 µl (50-75 ng) of template genomic DNA, 0.8 µl (2 U) of Taq DNA polymerase and 7.2 µl 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 sec., 55°C for 30 sec., 72°C for 1 min and a final extension at 72°C for 5 min. The primers were employed for amplification of thirty genotypes and two related species *Garcinia indica* and *Garcinia gummigutta*. These PCR products were separated on the automatic 96 capillary automated DNA Sequencer (Applied Biosystems, ABI 3730 DNA Analyzer) at Eurofins facility, Bengaluru.

**Genetic analysis of SSR markers**

The raw data generated were analyzed and compiled using Peak Scanner V1.0 software (Applied Biosystems, USA) for detecting the allele size in bp**.** The results obtained were used for genetic analysis using Cervus 3.0 software (Kalinowski et al., 2007). The number of alleles, observed heterozygosity (Ho), expected heterozygosity (He) and Polymorphic information content (PIC) were estimated. The probability of identity (PI) was calculated using IDENTITY 3.0 software (Wagner & Sefc, 1999).

## RESULTS AND DISCUSSION

**Sequence analysis, assembly, SSR identification and primer design**

The NGS technology platform IlluminaHiseq 2000 was used for sequencing genomic DNA from *G. morella*. The sequencing run yielded 311687120075 bases from 118.7 millinon reads. Low quality reads were filtered out. Finally, around 11254 Mbp paired end reads were obtained. Using SOAPdenovo2-src-r240 (Luo et al., 2012) software, assembly optimization was done. Assembly with Kmer-63 was selected, as it is having the optimal reading for N50. This has resulted in 16,13,263 contigs with total assembly size 632, 848,976 bases. The weighted mean assembly size in scaffold (N50) was 426 bp. The total assembled size of the contigs was 632.8Mbp (Table 1).

**Table 1 : Sequence analysis of *G. morella* genome**

| Sequence details | 1613263 |
|---|---|
| Total number of contigs | 161326 |
| Total number of examined sequences (bp) | 632848976 |
| Total number of identified SSRs | 121199 |
| Number of SSR containing sequences | 111109 |
| Number of SSR containing more than 1 SSR | 9112 |

An SSR survey of genomic sequences using MISA software (http://pgrc.ipk-gatersleban.de/misa), revealed that 1613263 contigs contained 121199 SSR markers. The 2777 SSRs were present on CDS. Among the identified SSR repeats, the direpeats were the most abundant, accounting for 42.5% of total SSRs, followed by mono-repeats (30.4%), tri-repeats (7.9%), tetra-repeats (2.02%), penta-repeats (0.9%), and hexa-repeats (0.2%) and compound-repeat (16.3%) nucleotide types (Table 2, Fig. 1). Among the

repeat motifs of the di-nucleotide, the AT and TA repeat was the most common, and the predominant motifs of tri-nucleotide AAT and TTA (Table 3). AT-rich repeats were also more common repeats in tetra-, Penta- and hexanucleotide SSRs. Primers were designed for 52901 SSRs. The results of mapping SSR markers to coding sequences showed that 2777 SSR markers mapped on to the coding sequences. Among the repeat types mapped, trinucleotide repeats form predominant SSRs (68.9 %) on coding sequences (Table 3).

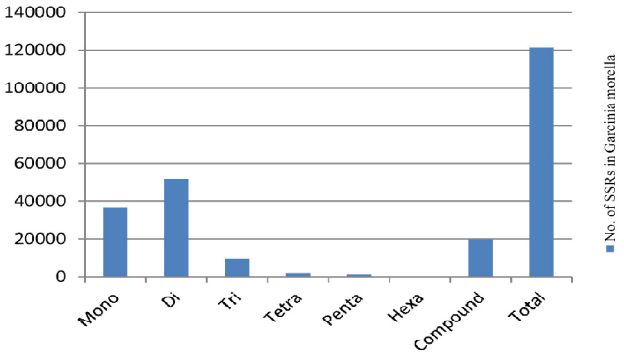


Fig.1 : Distribtuion of SSRs in *Garcinia morella* genome

**Table 2 : Simple sequence repeat types in the *G. morella* contigs sequences**

| Motif length | Number of SSRs | Frequency (%) |
|---|---|---|
| Mononucleotide | 36840 | 30.4 |
| Dinucleotide | 51569 | 42.5 |
| Trinucleotide | 9630 | 7.9 |
| Tetra nucleotide | 1893 | 1.6 |
| Penta nucleotide | 1110 | 0.9 |
| Hexanucleotide | 297 | 0.2 |
| Complex/compound | 19680 | 16.3 |
| Total | 121199 | |

**Table 3 : Distribution of SSR mapped on coding sequences**

| Motif length | Number of SSRs | Frequency (%) |
|---|---|---|
| Mononucleotide | 367 | 13.2 |
| Dinucleotide | 291 | 10.5 |
| Trinucleotide | 1916 | 68.9 |
| Tetra nucleotide | 2 | 0.007 |
| Penta nucleotide | 10 | 0.36 |
| Hexanucleotide | 26 | 0.93 |
| Complex/compound | 165 | 16.3 |
| Total | 2777 | - |

**Genetic analysis and transferability of genomic SSRs**

Genetic analysis of 48 SSR markers, using 30 accessions showed PIC values ranging from 0.067 to 0.939 with a mean value of 0.7547. The mean values of observed and expected heterozygosity were 0.2026 and 0.7852, respectively. The allele per locus ranged from 2 to 24 with a mean of 13. The probability of identity values ranged from 0.0058 to 0.7742 with a mean value of 0.1018 (Supplementary Table 4, and 5). The total probability of identity was 5.666690e-068. In cross species amplification, four out of 48 SSR primers amplified in *G. indica* and 39 amplified in *G. gummigutta* (Table 6).

**Table 4 : Summary of genetic analysis**

| Parameter | Mean | Range |
|---|---|---|
| Polymorphic information content (PIC) | 0.7547 | 0.067- 0.939 |
| Observed heterozygosity (Ho) | 0.2026 | 0.000- 0.760 |
| Expected heterozygosity (He) | 0.7852 | 0.07- 0.959 |
| Allele per locus | 13 | 2- 24 |
| Probability of identity (PI) | 0.1018 | 0.0058- 0.7742 |

Total number of alleles is 624; total probability of identity is 5.666690e-068

**Table 5 : Cross species amplification of *G. morella* SSR primers with *G. indica* and *G. gummingutta***

| Locus Name | Species | |
|---|---|---|
| | *G. indica* | *G. gummigutta* |
| GM_KVRw990 | A | NA |
| GM_KVRx003 | A | NA |
| GM_KVRx004 | NA | A |
| GM_KVRx022 | NA | A |
| GM_KVRx038 | NA | A |
| GM_KVRx045 | A | NA |
| GM_KVRx046 | A | NA |
| GM_KVRx047 | NA | A |
| GM_KVRx048 | NA | NA |
| GM_KVRx049 | NA | NA |

| | | |
|---|---|---|
| GM_KVRx075 | NA | NA |
| GM_KVRx076 | NA | NA |
| GM_KVRx077 | NA | NA |
| GM_KVRx090 | NA | A |
| GM_KVRx091 | NA | A |
| GM_KVRx092 | NA | A |
| GM_KVRx093 | NA | A |
| GM_KVRx094 | NA | A |
| GM_KVRx095 | NA | A |
| GM_KVRx133 | NA | A |
| GM_KVRx134 | NA | A |
| GM_KVRx135 | NA | A |
| GM_KVRx432 | NA | A |
| GM_KVRx433 | NA | A |
| GM_KVRx434 | NA | A |
| GM_KVRy205 | NA | A |
| GM_KVRy206 | NA | A |
| GM_KVRy207 | NA | A |
| GM_IIHRb888 | NA | A |
| GM_IIHRb889 | NA | A |
| GM_IIHRg191 | NA | A |
| GM_IIHRg192 | NA | A |
| GM_IIHRt169 | NA | A |
| GM_IIHRx419 | NA | A |
| GM_IIHRx420 | NA | A |
| GM_IIHRx421 | NA | A |
| GM_IIHRx536 | NA | A |
| GM_IIHRx537 | NA | A |
| GM_IIHRz510 | NA | A |
| GM_IIHRz511 | NA | A |
| GM_IIHRz512 | NA | A |
| GM_IIHRz572 | NA | NA |
| GM_MRDa479 | NA | A |
| GM_MRDa828 | NA | A |
| GM_KVRa040 | NA | A |
| GM_KVRa041 | NA | A |
| GM_KVRa042 | NA | A |
| GM_KVRa325 | NA | A |

A: amplified; NA: not amplified

Although *G. morella* holds economic significance as a tree species, studies regarding its genetic diversity are limited. Parthasarathy et al. (2013a) have compared the effectiveness of the inter-simple sequence repeats (ISSR) and RAPD profiling of 12 species of Indian *Garcinia* and showed that combined information of ISSR and RAPD could be a better tool to assess the diversity of Garcinia. Parthasarathy et al. (2013b) have reported that lack of awareness, coupled with habitat destruction, is leading to genetic erosion of *Garcinia morella* and other related species.

At present, the microsatellite markers are most widely used molecular marker for the analysis of diversity, genetic relatedness, mapping, crop breeding programs and population genetics (Varshney et al., 2005; Ravishankar et al., 2015a). SSR markers are highly reproducible, multi-allelic, PCR based, highly polymorphic, easy to use and amenable for automation (Ravishankar et al., 2015b & 2015c). However, the use of microsatellite markers for studying non-model species like wild *G. morella* has been impeded by a lack of available sequence information and genetic studies. Earlier, the detection of genomic SSRs and subsequent conversion to markers was expensive and time-consuming; involving the construction and screening of microsatellite enriched genomic DNA libraries (Glenn & Schable 2005; Ravishankar et al., 2011). 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 next-generation sequencing technologies, such as pyrosequencing, has made this process less complicated and easy (Zalapa et al., 2012; Ravishankar et al, 2018). As a result, a large number of SSR markers can be developed in short span of time and at a lower cost. This approach is especially useful for many tree crops where there are no available SSR markers.

In the present study high-throughput pyrosequencing Illimina platform Hiseq 2000 was used to develop genomic SSR markers in *G. morella*. The assembly of reads of the long sequences 16,13,263 contigs covering 632 Mb of the genome (Table 1). It was observed that di-repeats were predominant in nature accounting for 42.5%, followed by mono- (30.4%) and tri- (7.9%) repeats (Table 2). The mono, di and tri nucleotide repeats contributed to the major proportion of SSRs (80.8%) and very small share was contributed

by tetra, penta and hexa nucleotide repeats (Table 2). Among the repeat motifs of the di-nucleotide, the AT and TA repeat was the most common, and the predominant motifs of tri-nucleotide AAT and TTA. Among the SSRs mapped to coding sequences, the tri-nucleotide repeats were in very high frequency in *G. morella* (68.9%). This was also observed by Lawson et al. (2006) in both monocots (Rice- 64%) and dicots (Arabidopsis- 65.4%) in coding regions. The abundance of tri-nucleotides in coding regions is hypothesized to be the result of purifying selection which eliminates any SSRs causing frame shift mutations. However, it is unknown if selection is involved in the distribution of SSR types in genomic DNA (Celik et al., 2014). A similar trend was observed in other Garcinia species, *G. indica* (Ravishankar et al., 2021) and *G. gummigutta* (Ravishankar et al., 2017).

It was hypothesised that the dominant presence of a repeat motif with a specific sequence and length in the plant genome arises from the selection pressure exerted on that motif during its evolution. However, the molecular origins and development of microsatellites remain unclear. Replication slippage, considered the most common mutation mechanism, involves the addition or removal of motifs. Other processes, such as duplication events, uneven crossing over, and nucleotide substitution, may also contribute to microsatellite variation. However, these processes cannot explain the species-specific accumulation of certain motif repetitions (Sonah et al., 2011).

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

In this study, 48 out of 50 SSR primers examined, amplified PCR products for *G. morella*. A high rate of successful amplification can be due to high-quality sequence data and the appropriate primer parameters, such as high GC content. In the present study, the genomic SSR markers exhibited a high degree of variation, with an average PIC value of 0.7547. Several of the genomic SSR markers amplified multiple alleles (averaging 13 per loci), as detailed in Supplementary Tables 4, and 5. This pattern could be attributed to the substantial heterozygosity within the species, which resulted in multiple number of alleles. Previous investigations employing RAPD markers revealed a substantial molecular diversity, with the heterogeneity index across species ranging from 0.81 to 0.82 in four specific species: *G. gummigutta, G. indica, G. cowa, and G. xanthochymus* (Parthasarathy et al., 2013b). In the present study, 35 SSR markers (72.9%) had more than 10 alleles per locus, indicating that high heterozygosity and diversity of accessions were used (Supplementary Table 4, and 5). The probability of identity (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 (mean =0.1018). These low PI values confirm their applicability and use in DNA fingerprinting. Thus, these SSR markers can be easily employed for genotyping elite individuals. In cross species amplification using *G. indica* and *G. gummi-gutta* species, these SSR markers showed a relatively high percentage of amplification in *G. gummi-gutta* (81.25%) and low in *G. indica* (8.33%) (Table 6).

The present work describes the pyrosequencing-based approach for identifying and analysing microsatellite markers from *G. morella's* partial genome sequences. The *G. morella* genome sequence was thoroughly examined, and a large number of SSRs were found. The results from this study is an invaluable set of molecular markers that is essential for genetic research, genotyping, and the conservation studies on *Garcinia morella.*

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