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Isolation and characterization of microsatellite markers from *Garcinia indica* and cross species amplification

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Abstract: *Garcinia indica* popularly known as ‘Kokum’ or Murugalu”, is a medium sized evergreen tree found in western-ghats of India. This tree species is highly exploited to produce anti-obesity drugs and culinary purposes. Its population is threatened by over exploitation and loss of habitat. Development of microsatellite markers would help in understanding genetic structure and further to develop appropriate conservation strategies. In this study, using next generation sequencing platform Illumina Hiseq 2000, we have sequenced partial genome of *G. indica* and identified 3725 microsatellites. Forty-eight microsatellite markers were analyzed using 30 accessions. Polymorphism information content (PIC) values ranged from 0.718 to 0.968 with a mean value of 0.922. Allele per locus ranged from 3 to 33 per locus. Probability of identity values ranged from 0.00329 to 0.30489. Cross species amplification SSR primers in the related species, showed a moderate transferability from 12.5 % (for *G. morella*) to 18.7% (for *G. gummigutta*)

Keywords: Cross-species amplification *Garcinia indica* , Microsatellite markers and Next-generation sequencing (NGS).

Garcinia indica Choisy (Thouars; Family Clusiaceae), is a perennial tree. *G. indica* is commonly known as a Brindonia Tallow tree or ‘Kokum Butter’ tree in English. Kokum has many uses in cuisines and an important ingredient in locally prepared medicines. The seeds are a rich source of Kokum butter, which is nutritive, demulcent, agent for smoothening, softening and used for cosmetic, confectionery, culinary purposes. Raw fruits, young leaves and bark are also used as medications against several disorders. The fruit rind is a rich source of Hydroxy Citric Acid (HCA) that prevents accumulation of fat in the human body cells.

Therefore, *G. indica* has become the natural source for production of anti-obesity drugs. (Baliga *et al.*, 2011). *Garcinia* species are endemic and distributed in tropical rain forests of the Western Ghats. Perceiving the threat of over exploitation, FRLHT (Foundation for Revitalization of Local Health Traditions) and IUCN (International Union for Conservation of Nature) have recognized this species as 'Vulnerable' and 'Threatened' category respectively (Hareesh and Vasudeva, 2010). A few studies examined diversity in this species using general DNA markers like RAPD and ISSR markers (Thatte *et al.* 2012; Palkar and Sellappan, 2019). However, so far there are no efforts to develop species specific, highly reproducible microsatellite markers or SSR markers in this species. Keeping this in view, an attempt has been made to develop microsatellite or SSR markers using next generation sequencing technology. The development of molecular markers would help in studying its diversity, analyzing the genetics of traits, and further help in evolving conservation strategies and improvement.

The plant material was obtained from the germplasm collection of the College of Forestry, Sirsi (University of Agricultural Sciences, Dharwad), Karnataka state, India. Total genomic DNA was isolated from the leaves of *G. indica* genotypes using modified CTAB method (Ravishankar *et al.*, 2000). Genomic DNA was sequenced using Illumina HiSeq2000 platform at M/ s Genotypic Pvt. Ltd, Bengaluru facility following manufactures instructions. High quality sequence data was used for assembly into contigs. De novo assembly of reads into contigs was performed using SOAPdenovo2-src-r240 software (Luo *et al.*, 2012). This has resulted in 92125 contigs. The total assembled size of the contigs is approximately 25.6 Mbp. An SSR survey of genomic sequences using MISA software ([http:// pgrc. ipk- gatersleben.de/misa](http://pgrc.ipk-gatersleben.de/misa)), showed that 3590 contigs contained at least one microsatellite (Ravishankar *et al.* 2015). A total of 3725 microsatellite was identified. A total of 1374 microsatellites (ESM1) primers were designed using Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>; Untergrasser *et al.*, 2012). From these, randomly 50 loci were selected for initial screening. Finally, 48 SSR primers were selected for genetic analysis based on clear amplification of PCR products. We employed Thirty genotypes of *Garcinia indica* for assessing polymorphism at each locus. The fluorescence based M13 tailed PCR method of Schuelke (2000) was followed to amplify the microsatellites in a quick, accurate and efficient manner. PCR was carried out in the 20 μ l reaction volume containing 2 μ l of 10X reaction buffer, 2.0 μ l of 1 mM dNTPs, 0.9 μ l (5 pmol) of forward, 0.9 μ l reverse primers (5 pmol), labeled M13 probe 1.2 μ l (5 pmol), 5.0 μ l (50-75 ng) of template genomic DNA, 0.8 μ l (2 U) of Taq DNA polymerase and 7.2 μ 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 30sec., 55°C for 30 Sec., 72°C for 1 min and a final extension at 72°C for 5 min. Amplified products were separated on 96 capillary Automated DNA Sequencer (Applied Biosystems, ABI 3730 DNA Analyzer) at M/S Eurofin facility, Bengaluru.

The raw data generated was analyzed and compiled using Peak Scanner V1.0 software (Applied Biosystems, USA) for estimating the allele size in bp. The allele size data was used for genetic analysis using Cervus 3.0 software (Kalinowski et al., 2007). We have calculated observed heterozygosity, expected heterozygosity, polymorphic information content (PIC). The probability of identity (PI) was calculated using IDENTITY1.0 software (<http://www.uni-graz.at/~sefck/>; Wagner and Sefc, 1999). Genetic analysis of 48 SSR loci, showed PIC values ranging from 0.718 to 0.968 with a mean value of 0.922. The mean values of observed and expected heterozygosity are 0.2813 (Table 1) and 0.933 respectively (Table 1 and 2). The allele per locus ranged from 13 to 41 with a mean of 16.395. The probability of identity (PI) values ranged from 0.00329 to 0.304896 with a mean of 0.03506. The total probability of identity is 8.132729×10^{-80} . In cross species amplification, out of 48 SSR primers, 6 amplified in *G. morella*, accounting 12.5 per cent transferability and 9 amplified in *G. gummigutta* accounting 18.8 percent transferability (ESM2). This relatively low cross-species transferability compared to what has been observed in *G. gummigutta* species (Ravishankar et al., 2017).

This is the first report of SSR markers for *Garcinia indica*, where 3725 microsatellites were identified and primers were designed for 1374 microsatellites. The genetic analysis showed that the majority of the SSR primers developed have high PIC values indicating high heterozygosity in the species. The low probability of identity values of many SSR loci is useful for molecular characterization. Finally, the SSR developed will be useful in studying genetic diversity, mapping and fingerprinting of *Garcinia indica* and related species.

Table 1
Genetic analysis of microsatellite markers developed for *Garcinia indica*

Locus	Forward Sequence 5'→3'	Reverse Sequence 5'→3'	Repeat Type	Number of Allele (k)	Allele size range (bp)	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Polymorphic Information Content (PIC)	Probability of Identity (PI)
GI_KVRa577	TTTGGCGAGGGTGTGGTGA	ACACGTGTAGGCTGACCAAC	(GT) ⁶	20	140-230	0.345	0.924	0.902	0.012828
GI_KVRa614	TGTGAGTTGTTGGCATGGTGA	GGAGGGTGAGCAATACAGCTCA	(TG) ²²	26	197-290	0.185	0.962	0.941	0.005254
GI_KVRa615	TGTGAGGGGTGAGGTGAGGCT	ACAAACGCATCCCCACTCTCGG	(AT) ⁶	27	283-379	0.259	0.953	0.933	0.006829
GI_KVRa651	TGGGTGGCAAAATTTGGGAGGAAA	TGCCGCCAAGGAGAGAGGAAA	(AC) ⁸	24	185-277	0.2	0.971	0.95	0.006622
GI_KVRa723	TGCACAGGAGGGTACAGACT	ACAACGAGGCTTCCAACAGGA	(AC) ¹⁰	21	412-488	0.143	0.926	0.904	0.011916
GI_KVRa747	TGACAGATCGACAGGCTAGACTCGAA	TGCCCCCGTCTATGTATCAGTC	(AT) ⁶	25	432-531	0.192	0.962	0.941	0.006535
GI_KVRa748	TGAATGCCGAGAGCAATTGTGCC	TCACATCACAAGGCTTGTCTCAACA	(TA) ⁶	33	140-214	0.519	0.979	0.96	0.003290
GI_KVRa834	GTGCACATGTCCCATAAAGATGGA	ACCTACCCCTCCATAACATGCTT	(AT) ⁶	16	105-180	0.133	0.853	0.828	0.036897
GI_KVRa861	GGCCCATGGCTCTCTCATACAA	TGGGAAGGACAATTAAAGTCGGA	(TA) ⁶	15	103-185	0.138	0.721	0.695	0.087401
GI_KVRa862	GGCAGATGTGTCTACACCGCAC	TGTGGACAGGTAGGTACAGGT	(AT) ⁷	9	233-294	0.143	0.855	0.819	0.037316
GI_KVRa961	CCACACACAAATGCCACAAATCCA	TGTGCGTGTGTGTTGACAGGT	(CA) ⁶	14	99-124	0.286	0.847	0.816	0.036213
GI_KVRb069	AGACATCCGTACCGGGCTCAT	TGCCATTGTATGTGTTGGCGG	(CA) ⁷	10	99-125	0.214	0.873	0.841	0.029837
GI_KVRb130	ACCCGCATTCAACAATGCACATACA	GTGGCGCTATTGGGAAATGAGTACA	(CA) ⁷	8	233-341	0.000	0.86	0.823	0.033681
GI_KVRb131	ACCCCTAACGGTGGGTTCTGTC	TGAGGGTCTTGAGTTCTCCCT	(AT) ⁶	13	99-190	0.148	0.905	0.879	0.017689
GI_KVRb132	ACCCCTAACGGTGGGTTCTGTC	TGGCCTTCGGTTGAGTTGTCC	(AT) ⁶	10	117-157	0.429	0.774	0.733	0.067668
GI_KVRb174	ACACCGTAAGGTGGTGAGAAGGA	ACACACAGAGTACCCATATACGCACA	(TG) ⁷	12	101-148	0.25	0.783	0.749	0.054954
GI_KVRb175	ACACCGTAAGGTGGTGAGAAGGA	ACACAGAGTACCTCACATACGCACA	(TG) ⁷	18	100-165	0.517	0.915	0.891	0.016365
GI_KVRb176	ACACCGATCCCATCCGACCT	ACACCAACACGCTCCCTTCCT	(TA) ⁷	24	453-524	0.276	0.945	0.925	0.008223
GI_KVRb200	AACACCATCAACATCAACACACGA	TGGAAGGTGTTGAGGTGCGCCA	(CA) ⁶	22	430-514	0.32	0.957	0.934	0.009077
GI_KVRb201	AACGGCTAGCTTTTCACTGACTGT	TGGTAAGTCGATTGTTGGGCTTCG	(TA) ⁶	17	116-179	0.16	0.913	0.887	0.017850
GI_KVRa975	CACCCATACACAACCAATTCCC	GGTGTATGTGCTGGATAAATGAAGGT	(CA) ⁶	23	201-285	0.103	0.938	0.918	0.009212
GI_KVRa976	CACATCTTACATGTACAAGTCCAC	CTGACCGGCTAAACATCAAGTTCCA	(TA) ⁷	20	316-397	0.083	0.926	0.901	0.016775
GI_KVRa977	CACATAAGGAACAACAAGGCTCA	GGCGGAGGCGGTACAATTGTGTT	(AT) ⁷	24	99-171	0.433	0.856	0.835	0.031996
GI_KVRa978	CAATCTCATCTAGACAACCTGCACA	AGTTGATCCAGGATTGGCGAGGGT	(AC) ⁶	20	99-148	0.414	0.933	0.912	0.011202
GI_KVRa979	CAAGGCTGCTCGGACGTGCAAT	ATCCCAACGGCTCGAGCAAGAA	(CT) ⁶	23	428-582	0.286	0.905	0.883	0.015518
GI_KVRa980	CAACATGCTTCAACAAGCACATACAA	TGCTACTAOCCTAGGAGACATGCATCA	(TG) ¹¹	21	112-198	0.444	0.942	0.92	0.009296
GI_KVRa981	CAACAAGGGCATTTCATGCACACA	TTGGGGAGGAACCAAGCAAGT	(AT) ⁶	24	313-399	0.633	0.955	0.936	0.006817
GI_KVRb047	AGCGAGGACAAGGGAAGGACG	TGGCGGATATGTGTCTGGCG	(TA) ⁷	19	323-365	0.36	0.911	0.885	0.018187

Table 1
Contd....

GI_KVRb048	AGCGAATGCATCGGTGTAGCGA	ACGATCACCTTGGGGACGCTCA	(AT) ⁶	19	472-527	0.261	0.871	0.846	0.031785
GI_KVRb204	AACCCAGTGAGTGAATGCGAATTGT	TGTTGTTGGCTTATAGCCGAATGTGA	(CA) ⁷	21	102-195	0.107	0.948	0.927	0.007728
GI_KVRb205	AACCCATGAGTGAATGCCAGTTGT	ACTGTGGTTGGCTTATGGCCTGA	(CA) ⁶	21	103-197	0.5	0.919	0.898	0.015233
GI_KVRb206	AACAGGACCGGTGTGCGGTTGA	TCCGCACATGTGTCCACACCAA	(TA) ⁸	21	201-341	0.423	0.909	0.885	0.016389
GI_KVRb207	AACACGTGGCAGACGCTCAAGG	TGGTGAGGTGCGTCCAACACAGGA	(AT) ⁶	8	117-178	0.233	0.793	0.757	0.070882
GI_KVRb208	AACACGCGGAGGACATACTGC	CCAAGCCTCTCTCCATTGTGTC	(TA) ⁶	7	154-171	0.679	0.774	0.72	0.077586
GI_KVRb209	AACACCTGCACGGGTTTGTGG	ACTTTCATCTCGACACGCGG	(TA) ⁷	10	330-413	0.000	0.89	0.86	0.023726
GI_KVRb213	AAAGGACCGGCGAAGAAAGCGG	CCAGCTCAAAACCGATGCCCAA	(AG) ⁶	10	134-250	0.000	0.881	0.85	0.026089
GI_KVRb214	AAAGAGAGTCTCTTAGTGAGGGGG	TGTTGGCTTGGTGTAAACGGCT	(GT) ⁶	6	150-251	0.148	0.792	0.742	0.062789
GI_KVRb219	TGTTGGGAAGTAAAGGAGGGAGCA	TGACCTAGGCATCCATCTCCCT	(TGT) ⁵	7	113-178	0.5	0.785	0.733	0.063197
GI_KVRb220	TGTGGGGATGGCAAATGAGGTGA	TGCCATTGCGTTGGGGCATCT	(CA) ⁵	10	143-173	0.115	0.829	0.788	0.044338
GI_KVRb234	TGGCGTGCAGTTCTTCTCCCA	GGGATCGCATCCAACATTCTTTCCA	(CAA) ⁵	3	173-215	0.154	0.335	0.303	0.304896
GI_KVRb242	TGCAACAACAGGCTCAGGCACA	TGGTGAGGCAACGGGTTGAACA	(CCA) ⁵	15	189-215	0.5	0.907	0.881	0.018089
GI_KVRb243	TAGCGACCGTGCCTGATGTTG	AGGGCTCCTCACCTCTACCTTA	(CAG) ⁵	13	141-171	0.36	0.864	0.83	0.032098
GI_KVRb341	ACAAGCATGCCAAACGTAGCCGA	TGAAGAAGTGCCCAACCCACT	(TGG) ⁵	12	136-170	0.517	0.78	0.741	0.071213
GI_KVRb352	AAGACGGGTGGCGGTGGAGAAA	TGAAGCGAACCTCTCCTCTGA	(TCT) ⁸	13	362-403	0.552	0.866	0.835	0.033609
GI_KVRb357	TGACAATACGTGGGAGATCCGT	TGTTCAAGGCTCAATCCCTTCGTGC	(AATA) ⁷	16	115-191	0.000	0.886	0.861	0.021333
GI_KVRb368	TCCGTGCCAATTCCTTGCAAC	TGACCTGTGCGCTTAGCTACCT	(AAAAT) ⁵	17	249-310	0.192	0.925	0.9	0.014054
GI_KVRb373	AGCTAGGGGGCAACCTGTACCA	TGCTATTGAATTCTGTGTTGGTGTGA	(CAATAC) ⁵	8	151-168	0.481	0.818	0.778	0.048049
GI_KVRa011	TCCGTCCATCCGTTCTGTCGTT	ACCGGATGGGATCCAGCGATGT	(CGTC) ⁶ cggt (CGTC) ⁷	12	100-136	0.172	0.75	0.722	0.074675

Table 2
Summary of Genetic Analysis

	Mean	Range
Polymorphic Information Content (PIC)	0.8416	0.303- 0.96
Observed Heterozygosity (Ho)	0.2813	0.000- 0.679
Expected Heterozygosity(He)	0.8701	0.335- 0.979
Allele per locus	16.395	3- 33
Probability of Identity (PI)	0.03506	0.00329- 0.304896

Total number of Alleles : 787 Total probability of Identity : 8.132729e-080

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