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RESEARCH ARTICLE

Large-scale discovery of Simple Sequence Repeat markers in snake gourd (*Trichosanthes cucumerina* L) and their cross-species validation

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Abstract

Snake gourd (*Trichosanthes cucumerina* L) is a medicinally important vegetable crop belonging to the Cucurbitaceae family. India, being the center of origin of snake gourd, is endowed with a rich genetic diversity in this crop, hence offering a tremendous scope for genetic improvement. The lack of sufficient molecular markers in the crop is, however, a big limitation that curtails the endeavors undertaken for crop improvement. In this study, a total of 18,604 SSR (Simple Sequence Repeat) markers were developed in snake gourd (TcSSR) through low coverage sequencing. A total of 1.5 gigabases (Gb) of sequencing data was assembled *de novo* and thereafter used for genome-wide SSR mining. The markers were validated by mapping onto the published whole genome sequence (WGS) of snake gourd. More than 77% of the SSR markers were mapped on the genome, which confirms the accuracy of the sequencing data. A few markers were wet lab validated through polymerase chain reaction (PCR) amplification across three different snake gourd genotypes (*T. cucumerina*) and analysed for cross-species transferability. A total of 40 out of 56 markers tested were identified to be transferable across the five different *Trichosanthes* species (other than *T. cucumerina*), which reflected a high degree of genetic relatedness among the species. To the best of our knowledge, this is the first report on the large-scale discovery of SSR markers in snake gourd. The markers identified in this study would serve as valuable genomic resources for germplasm characterization in snake gourd and facilitate trait improvement efforts in various breeding programs.

Keywords: Snake gourd, Simple sequence repeats, Next generation sequencing, Genomics, Cross-species transferability.

Introduction

Snake gourd (*Trichosanthes cucumerina* L.), popularly called viper gourd, snake tomato or long tomato, is a vegetable crop belonging to the family Cucurbitaceae. It has a chromosome number of 22 (2n=2x=22) (Clark 1879). It is widely cultivated in many Asian countries, where the fruit of the plant is consumed (Lertphadungkit et al. 2021). The crop originated in India or the Indo-Malayan region in tropical Asia (Clark 1879). The plant has a high degree of pharmacological and therapeutical significance, due to the presence of a wide variety of medicinally important phytoconstituents like flavonoids, carotenoids, phenolic acids, etc (Islam et al. 2020; Kumar and Pandit 2022). The myriad health benefits that this crop and other such neglected and underutilized crops offer make them prospective crops for combating global hunger and malnutrition, a deliverable of the UN SDG2 (United Nations Sustainable Development Goal).

Despite its nutritional importance, not much progress has taken place to enhance its productivity (Rana and Pandit 2011). Compared to the other commercial cucurbits, the yield of snake gourd is low and varietal improvement

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Fig. 1. The snake gourd plant with fruit shown

remains laggard (Sivan et al. 2016). This is primarily due to the lack of sufficient genomic resources in the crop. The DNA-based molecular markers available in snake gourd include a few expressed sequence tag – simple sequence repeat (EST-SSRs) and expressed sequence tag – single nucleotide polymorphism (EST-SNPs) (Sivan et al. 2016), randomly amplified polymorphic DNA (RAPD) (Rashid et al. 2016), and SSRs derived from a different but related species (Adeyemo et al. 2020). There is, however, no report available with regard to the large-scale discovery of SSR markers. The latter are the preferred molecular markers for genotyping purposes for various reasons. To name a few, these are co-dominant, multi-allelic, reproducible and highly amenable to automation (Yadav et al. 2013; Mishra et al. 2023; Gaikwad et al. 2025).

With the recent advances in next-generation sequencing (NGS) technologies, sequence information can be derived for most of the organisms, including the large and complex eukaryotes. Keeping this in view, the present study was undertaken to develop novel SSR markers in snake gourd, which would immensely facilitate various crop improvement endeavors.

Materials and methods

Plant sample and DNA preparation

The genomic DNA was isolated from leaves of a single plant of the snake gourd variety, Kaumudi, using Qiagen DNeasy Plant Mini Kit for sequencing (Fig. 1).

For validation of the SSR markers, in addition to Kaumudi, genomic DNA was also isolated from the leaf tissues of 22 different accessions belonging to six different *Trichosanthes* species, namely *T. cucumerina* (2), *T. anguina* (11), *T. nervifolia* (1), *T. cochinchinensis* (5), *T. tubiflora* (1) and *T. dioica* (2) through the standard cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). The quantity and quality of genomic DNA were estimated using Nanodrop and Qubit® dsDNA BR Assay Kit (Life Technologies) on a Qubit® 2.0 Fluorometer (Invitrogen Corporation), following the manufacturer's recommended protocol.

Library preparation

Sequencing libraries were prepared as per the manufacturer's instructions (Lemos et al. 2018). Two different diluted libraries (26pM) were used to generate template positive Ion Sphere™ Particles (ISPs) containing clonally amplified DNA. Emulsion PCR was conducted with the OneTouch™ 2 (OT2) System using the Ion PGM™ Template OT2 400 Kit (Thermo Fisher Scientific, Waltham, MA, USA). The template-positive ISPs were enriched with the Ion One-Touch™ ES (Life Technologies), and the quality of template-positive ISPs was assessed by using the IonSphere™ Quality Control Kit (Life Technologies) on the Qubit® 2.0 Fluorometer, following the recommended protocol.

Sequencing, data analysis and genome assembly

Sequencing was performed using the Ion PGM™ (Personal Genome Machine, LT) on a 318C (1000 Mb throughput). After sequencing, the sequence reads were filtered with the PGM software, removing low-quality sequences. All the filtered reads were analyzed with the Ion Torrent Suite Software version. 4.2.1 Using the plug-in file exporter. The output was obtained as sff, FASTQ, BAM/BAI files. BAM files were visualized with Integrative Genomics Viewer (IGV). The FASTQ files were used for the *de novo* assembly. Two sets of short single-end reads were generated and used and the snake gourd genome was assembled with CLC genomics workbench (CLC Bio, Aarhus, Denmark). The *ab initio* gene prediction was done using AUGUSTUS v 2.5.5 (Stanke et al. 2005). The transcription factors (TFs) were predicted using Plant_TF_v5.0 (Jin et al. 2014).

SSR mining and primer designing

In order to identify the SSRs from the assembled contigs, Krait software v 1.5.1 was used (Du et al. 2018). The search criteria were set to include a minimum repeat number of 6 for dinucleotides (di), 4 for trinucleotides (tri) and tetranucleotides (tetra), and 3 for pentanucleotides (penta) and hexanucleotides (hexa). The remaining parameters were set as the default. The primers flanking the identified SSRs were designed using Primer3 v 2.6.1 (Untergasser et al. 2012). The parameters set were: PCR product length of 100–300 bp, primer length of 18 to 24 bp, annealing temperature of 50 to 60°C and GC content of 40 to 60%.

Validation of SSR markers

The SSR markers were mapped to the snake gourd genome (GCA_036507285.1) (Ma et al. 2020) using SeqKit v 2.8.2 (Shen et al. 2016). For wet lab validation, PCR analysis was done. For this purpose, primer pairs were synthesized for a subset of

156 randomly selected TcSSR markers. Validation was done across 23 accessions belonging to *T. cucumerina* and five other *Trichosanthes* species. The reaction was carried out in a total reaction volume of 10 µL, which consisted of 1X PCR buffer, 2.5 mM $MgCl_2$, 1-µM primer, 0.2 mM of each dNTP, 1U Taq DNA polymerase (NEB) and 15 ng template DNA. For amplification, standard PCR conditions were used, with standardization performed for annealing temperatures.

Result and discussion

Sequencing and de novo assembly

The snake gourd genome was sequenced using Ion Torrent PGM sequencing technology. A total of 5,101,625 reads were obtained. The average read length was 289 bp. A total of 1.5 Gb of sequencing data at a coverage of 1.5X was obtained. The raw reads were filtered to remove bad quality reads and the filtered reads were used for *de novo* assembly. The data were assembled into 282,755 contigs, which capture 171 Mb of the snake gourd genome (Table 1). The sequence assembled constitutes 18% of the WGS of snake gourd, which is reported to be 919.8 Mb (Ma et al. 2020).

The assembled contigs were used for gene prediction and identification of TFs. A total of 7006 protein-coding genes were identified to be conserved amongst the 22,874 protein-coding genes previously predicted for the snake gourd genome (Ma et al. 2020). Amongst the various TF families, the bHLH (basic helix–loop–helix) family of TFs was most abundant (10% of the total TFs), followed by the WRKY and ERF (Ethylene Responsive Factor) families (Fig. 2).

SSR identification and validation

A total of 37,388 SSRs were identified from the assembly. Low coverage sequencing has been frequently employed for SSR identification in many plants (Sarzi et al. 2019; Li et al. 2020; Hu et al. 2023). This is a cost-effective and less time-consuming method to derive sequence information for preliminary analysis, such as SSR identification. Low coverage sequencing, ranging from as low as 0.04X to 2.63X, was performed for various hardwood tree species for the purpose of SSR identification (Staton et al. 2015). The PGM (Ion Torrent) platform used in this study has the advantages of being cost-effective and having a short run-time, which makes it a suitable approach for SSR discovery, as previously reported by Egan et al. 2012. Elliott et al. 2013 have demonstrated the efficacy of PGM sequencing for SSR discovery vis-à-vis the FLX pyro-sequencing chemistry and have shown that PGM chemistry could produce a significantly larger number of markers at a comparatively lower cost and within a shorter time span. Lemos et al. 2018 used the PGM sequencing chemistry for sequencing of a South American tree species, *Schinus molle,* at a coverage of 0.3X (estimated genome size of 410 Mbp) for SSR discovery.

Table 1. Details of the low coverage sequencing of the snake gourd genome; bp: base pairs

Parameter	Details
Total reads	5,101,625
Mean read length (bp)	289
Total number of contigs	2,82,755
Minimum length of contig (bp)	200
Maximum length of contig (bp)	25,156
Total length of contigs (bp)	171,576,179

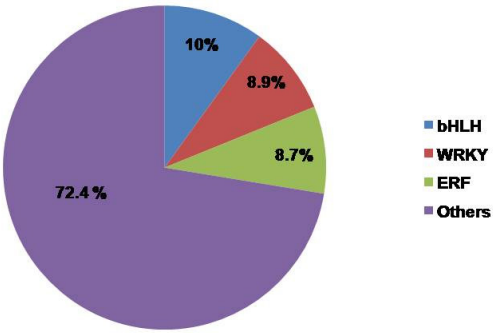

Fig. 2. The relative abundance (in terms of percentage) of various transcription factor (TF) families identified for the snake gourd genome

Amongst the different repeat motifs identified, the trinucleotide repeats were most abundant, followed by dinucleotide repeats. The tetranucleotide repeats were the least abundant (Fig. 3a and Table 2). About 22% of the SSR primers could be designed for the dinucleotide motif regions, while a far greater percentage of SSR primers were designed for the longer motifs. This is in agreement with three different cucurbit species, namely *Cucurbita maxima*, *C. moschata* and *C. pepo,* where it was observed that longer motifs were more suitable for primer design (Zhu et al. 2021).

Out of the 37,388 SSRs identified, flanking primers were designed for 18,604 different loci (Supplementary Table S1 Data not provided). These were mapped onto the published snake gourd genome of 919 Mb (Ma et al. 2020). A total of 14,499 loci were successfully mapped onto the genome (Fig. 3b). This is indicative of the highly accurate identification of SSRs in this study. Highest hits were obtained for the LG01, followed by LG05 (Fig. 3c). The mean marker density was 218.6 SSRs/Mb, which is similar to that reported in other horticultural species like citrus (Singh et al. 2023). In some crops, a very high frequency of SSR occurrence is also reported, even for species with comparable genome sizes for instance, in grapes, the average SSR density was estimated to be 1/Kb, which is significantly higher compared to this study and other reports for horticultural crops. In related species like cucumber (*Cucumis sativus*), SSR density of 551.9 SSRs/Mb was reported (Cavagnaro et al. 2010). Such a significant difference between SSR density, even for species

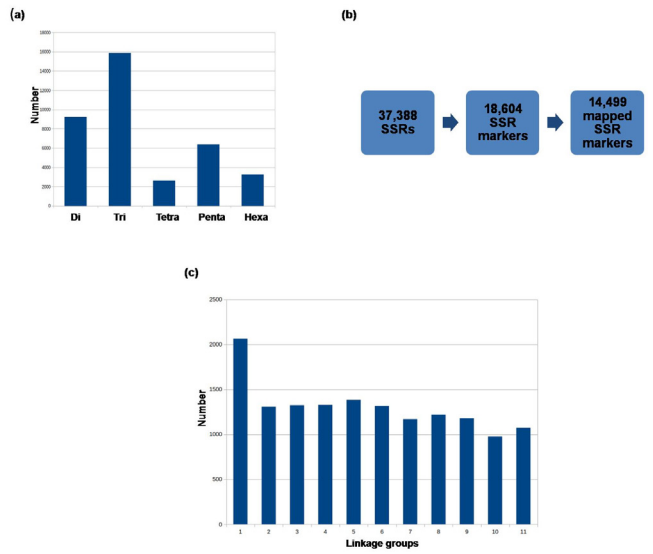

Fig. 3. The SSRs identified for the snake gourd genome. (a) The abundance (in numbers) of different types of repeat motifs for the SSRs was identified. (b) The number of SSRs identified, SSR markers designed and mapped onto the published snake gourd genome (Ma et al. 2020). (c) The number of SSR markers mapped across the 11 different linkage groups of snake gourd

Table 2. Distribution of SSR markers in the snake gourd genome

Parameter	Number
Total no. of SSRs identified	37388
No. of SSRs with Di-repeats	9243
No. of SSRs with Tri-repeats	15865
No. of SSRs with Tetra-repeats	2633
No. of SSRs with Penta-repeats	6393
No. of SSRs with Hexa-repeats	3254

with comparable genome sizes demonstrates the lack of a correlation between genome size and SSR density, an observation previously made for certain cucurbit species (Zhu et al. 2021). The plausible reason for this was posited to be differences in the software used, the parameters adopted for SSR detection and the quality of the genome sequence (Zhu et al. 2021). Another possible reason for the variation in SSR density could be the inter-specie variation in the GC content (percentage of Guanine + Cytosine in a sequence) across the genes as it is known that high GC content will increase the probability of replication slippage, one of the reasons leading to creation of differences in repeats (Zhao et al. 2023). Given that approximately a fifth of the genome was sequenced and captured in the assembly (171Mb/919 Mb), it is predicted that a significantly larger number of SSRs will be present across the snake gourd genome (more than 1 lakh). This is in line with the number of genome-wide SSRs identified for most species with similar genome sizes and repeat content (Gaikwad et al. 2023; Zhao et al. 2023).

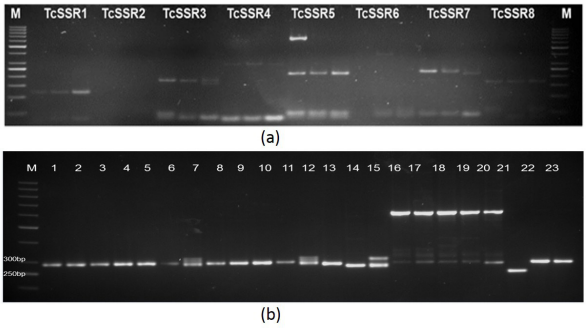

Fig. 4. (a) Representative gel picture depicting PCR amplification profile obtained with a few TcSSRs (eight shown) across three different accessions (one of them being Kaumudi) of snake gourd (*T. cucumerina*). (b) Cross-species amplification profile of TcSSR1 in different *Trichosanthes* species. *T. cucumerina* (lane 1-3), *T. anguina* (lane 4-14), *T. nervifolia* (lane15), *T. cochinchinensis* (lane 16-20), *T. tubiflora* (lane 21) and *T. dioica* (lane 22-23). M is the DNA molecular weight standard 50bp ladder (Thermo Scientific)

A few SSR markers were validated through PCR amplification. Out of the 156 TcSSR markers, 56 produced reproducible banding pattern on PCR analysis using three *T. cucumerina* accessions (Fig. 4a). The cross-species transferability of TcSSR markers was also done using the same set of 56 TcSSR markers (Fig. 4b).

As high as 71% (40/56) primers were transferable across all the five different species examined. Highest transferability of the TcSSRs was identified for the species *T. anguina* (56/56) while least transferability (50/56) was observed for the specie, *T. dioica* (Supplementary Table S2). This observation is in agreement with previous observation where the two species *T. anguina* and *T. cucumerina*, showed higher resemblance to each other. Infact according to one hypothesis, *T. anguina* could have evolved from *T. cucumerina* (Singh and Roy 1979). However, since the SSRs are derived from a relatively smaller proportion of the entire genome, they can be considered to represent diversity existing in only the sequence captured. Inclusion of more SSRs which provide a wider genome coverage might therefore alter the results.

This is the first report describing the large scale discovery of SSR markers in snake gourd. The markers identified would serve as important genomic resources in *Trichosanthes* spp. These markers can be utilized for a variety of purposes like gene mapping or tagging, MAS, germplasm characterization, etc. This would provide the much required momentum to varietal improvement and development in snake gourd.

Supplementary material

Supplementary Table S1 can be obtained from the author and Supplementary Table S2 is provided with the text, and can be accessed at www.isgpb.org.

Author's montributions

Conceptualization of research (ABG); Designing of the experiments (ABG); Contribution of experimental materials (KP); Execution of field/lab experiments and data collection (AK, DG); Analysis of data and interpretation (SY, RK, WM); Preparation of the manuscript (SY, AT, AMCS, GPS, ABG).

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Supplementary Table S2. Cross-species transferability of TcSSR markers identified from *Trichosanthes cucumerina* across five different *Trichosanthes* species. A total of 23 different accessions were used which included 3 accessions of *Trichosanthes cucumerina*, one being Kaumudi. All the 56 TcSSR markers produced amplicons with predicted sizes across all the three accessions of *Trichosanthes cucumerina.* Here, Tc denotes *Trichosanthes cucumerina;* + and – represent presence and absence of amplicon, respectively

S. No.	SSR marker	*T. anguina*	*T. nervifolia*	*T. cochinchinensis*	*T. tubiflora*	*T. dioica*
1	TcSSR3	+	+	-	+	+
2	TcSSR4	+	+	+	+	+
3	TcSSR7	+	+	+	+	+
4	TcSSR9	+	+	+	+	-
5	TcSSR12	+	+	+	+	+
6	TcSSR13	+	+	+	+	+
7	TcSSR20	+	+	+	+	-
8	TcSSR21	+	+	+	+	+
9	TcSSR22	+	+	+	+	+
10	TcSSR23	+	+	+	+	+
11	TcSSR24	+	+	+	+	+
12	TcSSR25	+	+	+	+	+
13	TcSSR27	+	+	+	+	+
14	TcSSR29	+	+	+	+	-
15	TcSSR31	+	+	+	+	+
16	TcSSR33	+	+	+	+	+
17	TcSSR34	+	+	+	+	-
18	TcSSR39	+	+	-	+	+
19	TcSSR40	+	+	+	+	+
20	TcSSR41	+	+	-	+	+
21	TcSSR49	+	+	+	+	+
22	TcSSR50	+	+	+	+	+
23	TcSSR51	+	+	+	+	+
24	TcSSR53	+	+	+	+	+
25	TcSSR54	+	+	+	+	+
26	TcSSR60	+	+	-	-	+
27	TcSSR63	+	-	+	+	+
28	TcSSR72	+	+	+	-	+
29	TcSSR74	+	+	+	+	+
30	TcSSR75	+	-	+	+	+
31	TcSSR84	+	+	+	+	+
32	TcSSR86	+	+	+	+	+
33	TcSSR89	+	+	+	+	+
34	TcSSR102	+	+	+	+	+
35	TcSSR104	+	+	+	+	+
36	TcSSR109	+	+	+	+	+
37	TcSSR111	+	+	+	+	-
38	TcSSR112	+	+	+	+	+

39	TcSSR114	+	+	+	+	+
40	TcSSR116	+	+	+	+	+
41	TcSSR118	+	+	+	+	+
42	TcSSR119	+	+	+	+	+
43	TcSSR123	+	-	-	+	+
44	TcSSR127	+	+	+	+	+
45	TcSSR128	+	+	+	-	+
46	TcSSR129	+	+	+	+	+
47	TcSSR130	+	+	+	-	+
48	TcSSR135	+	+	+	+	+
49	TcSSR136	+	+	+	+	+
50	TcSSR137	+	+	+	-	-
51	TcSSR143	+	+	+	+	+
52	TcSSR145	+	+	+	+	+
53	TcSSR147	+	+	+	+	+
54	TcSSR149	+	+	+	+	+
55	TcSSR154	+	+	+	+	+
56	TcSSR156	+	+	+	+	+