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Rediscovering Medicinal Plants' Potential with OMICS: Microsatellite Survey in Expressed Sequence Tags of Eleven Traditional Plants with Potent Antidiabetic Properties

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Abstract

Herbal medicines and traditionally used medicinal plants present an untapped potential for novel molecular target discovery using systems science and OMICS biotechnology driven strategies. Since up to 40% of the world's poor people have no access to government health services, traditional and folk medicines are often the only therapeutics available to them. In this vein, North East (NE) India is recognized for its rich bioresources. As part of the Indo-Burma hotspot, it is regarded as an epicenter of biodiversity for several plants having myriad traditional uses, including medicinal use. However, the improvement of these valuable bioresources through molecular breeding strategies, for example, using genic microsatellites or Simple Sequence Repeats (SSRs) or Expressed Sequence Tags (ESTs)-derived SSRs has not been fully utilized in large scale to date. In this study, we identified a total of 47,700 microsatellites from 109,609 ESTs of 11 medicinal plants (pineapple, papaya, noyontara, bitter orange, bermuda brass, ratalu, barbados nut, mango, mulberry, lotus, and guduchi) having proven antidiabetic properties. A total of 58,159 primer pairs were designed for the non-redundant 8060 SSR-positive ESTs and putative functions were assigned to 4483 unique contigs. Among the identified microsatellites, excluding mononucleotide repeats, di-/trinucleotides are predominant, among which repeat motifs of AG/CT and AAG/CTT were most abundant. Similarity search of SSR containing ESTs and antidiabetic gene sequences revealed 11 microsatellites linked to antidiabetic genes in five plants. GO term enrichment analysis revealed a total of 80 enriched GO terms widely distributed in 53 biological processes, 17 molecular functions, and 10 cellular components associated with the 11 markers. The present study therefore provides concrete insights into the frequency and distribution of SSRs in important medicinal resources. The microsatellite markers reported here markedly add to the genetic stock for cross transferability in these plants and the literature on biomarkers and novel drug discovery for common chronic diseases such as diabetes.

Introduction

Nature has produced a significant number of medicinal plants and herbs that contain numerous active ingredients and complex molecules that have yet to be scientifically identified and analyzed. Medicinal plants have long been considered as a healthy source of life for all human beings. In developing countries, including India, over 80% of the population depends directly on plants for their medicinal and nutritional requirements (WHO, 2002). In fact, as up to 40% of the world's poor people have no access to government health services, under such circumstances traditional and folk medicine is the only medicine available to them.

The northeastern (NE) region of India is blessed with a wide range of physiographic and ecoclimatic conditions, and most importantly is the geographical gateway for much of India's endemic flora. In addition, the region represents a vital component of the Indo-Myanmar biodiversity hotspot, among the 25 global biodiversity hotspots recognized to date across the globe. Many precious medicinal plants are closely associated socially as well as culturally. In addition, these plants are widely used for nutritional purpose by the local inhabitants and indigenous communities of this region. Many plants with potential medicinal value also used for various domestic purposes in daily lives are not listed under the medicinal plant category. An extensive literature survey revealed 11 plants having proven antidiabetic properties that

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are largely distributed across the geographical niche of NE India. The plants include: *Ananas comosus* (pineapple), *Carica papaya* (papaya), *Catharanthus roseus* (noyontara), *Citrus aurantium* (bitter orange), *Cynodon dactylon* (bermuda grass), *Dioscorea alata* (ratalu), *Jatropha curcas* (barbados nut), *Mangifera indica* (mango), *Morus indica* (mulberry), *Nelumbo nucifera* (lotus), and *Tinospora cordifolia* (guduchi) (Aderibigbe et al., 1999; Xie et al., 2005; Huralikuppi et al., 2006; Jarald et al., 2008; Sharma et al., 2008; Mishra et al., 2010; Rasineni et al., 2010; Kumar et al., 2010; Maithili et al., 2011; Juarez-Rojop et al., 2012; Sangeetha et al., 2013). Although much progress have been made in recent years in many medicinal plants, the study of genetic potential and improvement through molecular breeding has not been attempted to date for the above mentioned plants.

The ability to investigate DNA sequences directly became available to population biologists only during the late 1970s. Molecular markers have been demonstrated as potential tools to detect genetic diversity and to aid the management of plant genetic resources (Ford-Lloyd et al., 1997; Virk et al., 2000; Song et al., 2003). Currently, a number of DNA-based techniques are widely used for analyzing the genetic diversity in natural populations. These include (i) restriction fragment length polymorphism (RFLP; Botstein et al., 1980), (ii) polymerase chain reaction (PCR; Mullis and Faloona, 1987), and its derivatives, termed as random amplified polymorphic DNA (RAPD; Williams et al., 1990); AP-PCR (Welsh and McClelland, 1990), and (iii) a hybrid of both the above techniques named amplification fragment length polymorphism (AFLP) (Vos et al., 1995). The microsatellite markers are the most powerful derivation of PCR technology that are being widely used in marker assisted breeding programmes. Abundantly dispersed in genome (i.e., both coding and noncoding regions of DNA sequences), SSRs (simple sequence repeats or microsatellites) are short repeat motifs (Toth et al., 2000; Katti et al., 2001; Gupta et al., 2007) that show a high level of length polymorphism due to insertion or deletion mutations in one or more repeats (Tautz and Renz, 1984). As compared to other DNA based markers, SSRs are more convenient, simple, stable, multiallelic, reproducible, and polymorphic, which make them the markers of choice in plant genetics and breeding.

Expressed sequence tags (ESTs) represent short, unedited, randomly selected single-pass sequence reads derived from cDNA libraries and serve as the main source for *in silico* identification of microsatellites. Because of the utility, speed with which ESTs are generated, and the low cost associated with the second generation sequencing technologies, ESTs became point of attention to many scientists. Importantly, most of the projects result in hundreds or thousands of ESTs that are released to the public domain for unrestricted use by the scientific community across the globe. ESTs are highly error prone and require several computational methods for pre-processing, clustering, assembly, and annotation to yield biological information.

Microsatellites developed from ESTs, popularly known as EST-SSRs or genic SSRs, which correspond to functional molecular markers, can be obtained from database searches and other *in silico* methodologies. With the advent of high-throughput next generation sequencing technologies in recent years, focus on functional genomics revolutionized in generation ESTs in large scale from model and nonmodel organisms, including important medicinal plants. In this scenario, evolving high throughput bioinformatics tools have complemented in mining microsatellites from large scale ESTs in a time and cost effective manner (Varshney et al., 2002). Because of the above mentioned advantages, genic SSRs have been identified and extensively studied in most of the model plants and major crop species, such as Arabidopsis, tobacco, rice, maize, wheat, poplar, pineapple, peach, and others (Varshney et al., 2005, Victoria et al., 2011, Duran et al., 2013).

The traditional methods of developing simple sequence repeat (SSR) markers are usually time consuming, and cost- and labor-intensive. SSR markers can be rapidly and cheaply identified through computational methods from various public domains. Not only is the *in silico* approach time- and cost effective, but also it allows for the discovery of SSRs from ESTs that represent only the coding region in the genome (Scott et al., 2000; Kantety et al., 2002; Varshney et al., 2002). Recent trends in marker development and studies in plants are more towards gene-specific markers rather than random DNA markers, and microsatellite markers are of great importance in identification of genes (Zhao et al., 2012). Additionally, bioinformatics tools also supplement existing approaches by automating the task of SSR identification from available DNA sequences.

The neglected and the under-utilized status of these locally important crops indicate a risk of disappearance of important plant material without knowing their exact genetic background. One of the important factors restricting their large-scale production and development of better varieties is that very little information is available about their genetic diversity, inter- and intraspecific variability, and genetic relationship among these species. And as such, availability of informative marker is also very scanty. Therefore, attempts to analyze possible untapped genetic diversity and development of marker become extremely essential for breeding and crop improvement. SSR marker reported in the present study shall act as a resource bank for analyzing genetic diversity in various accessions, and the SSR marker linked to the anti-diabetic gene shall act as a benchmark for further improvement of the crop using marker assisted breeding program.

Materials and Methods

Data source and EST assembly

Sequence data were collected from the public domain 'dbEST' at NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov) (Boguski et al., 1993). EST sequences were collected for 11 plants: 5941 for *Ananas comosus*, 77393 for *Carica papaya*, 20168 for *Catharanthus roseus*, 14584 for *Citrus aurantium*, 20497 for *Cynodon dactylon*, 44134 for *Dioscorea alata*, 46862 for *Jatropha curcas*, 1665 for *Mangifera indica*, 4526 for *Morus indica*, 2207 for *Nelumbo nucifera,* and 5498 for *Tinospora cordifolia*. For the pre-processing and assembly steps, an in-house software pipeline ESMP (Sarmah et al., 2012) and EGassembler (Masoudi-Nejad et al., 2006) were used. The updated vector sequences were downloaded from the 'UniVec' database (ftp://ftp.ncbi.nih.gov/pub/UniVec) of NCBI and checked against the downloaded ESTs. The sequences were cleaned to remove the vector contamination using cross_match with min-match and min-score value of 20. The

poly-A trimming was done with the Trimest tool from EMBOSS. The value for minimum length and mismatch in case of Trimest was used as 4 and 1 respectively.

The high quality sequences obtained after pre-processing were assembled using CAP3, which resulted in contigs and singlets. The parameters used in assembly using CAP3 (Huang and Madan, 1999) are listed in Supplementary Material (supplementary material is available online at www.liebertpub.com. Raw data are available from the authors). The assembly in case of *Carica papaya* was done with the help of EGassembler due to the sequence size limitation in CAP3. EGassembler uses CAP3 in an iterative process so that it takes sequence size of more than 50000 bp. In this study the stand-alone processing option was selected in EGassembler, where we allotted 8 CPUs for the analysis.

SSR detection and primer designing

Microsatellite repeats were obtained using MISA (MIcroSAtellite identification tool). MISA is a freely available Perl script, which was downloaded from the Web (http://pgrc.ipk-gatersleben.de/misa/misa.html). Along with ''misa.pl'' another file *viz*., ''misa.ini,'' which contains the search parameters, was also downloaded. The following search parameters were employed in the detection of SSR in MISA: the maximum difference between two SSRs that interrupts to form a compound microsatellite was 100 and the minimum length parameter for the repeated units (unit size/minimum number of repeats): at least ten mononucleotides (1/10); at least six dinucleotides (2/6); at least five trinucleotides (3/5); and five tetranucleotides (4/5), pentanucleotides (5/5), and hexanucleotides (6/5). Primer pairs were designed for the SSRs containing singlets and contigs with the help of primer3 tool (Rozen and Skaletsky, 2000). Perl scripts available on the web (http://pgrc.ipk-gatersleben.de/misa/primer3.html) enabled us simultaneously to use Primer3 for designing primers only for SSR-ESTs and sorting out the results of primer pairs for further studies. For designing primer pairs to flank the EST-SSRs, primer-specificity was optimized for primers greater than 10 bp on either side of the identified SSR and maximum product size of 100–280 bp. The following parameters were employed to design primer pairs in Primer3: optimal size of primer was set to 18 bp with maximum up to 27, melting temperature of 55°C with a minimum of 50°C and maximum of 70°C, and a maximum GC content of 65%.

Functional annotation and GO term analysis

The EST contig sequences containing microsatellites were assigned to putative functions with the help of BLASTx. The nonredundant (nr) protein sequences were downloaded from ftp://ftp.ncbi.nlm.nih.gov/blast/db and configured on the local server with the following command: 'formatdb −p T −i nr.fasta −n nr_db' where 'nrq.fasta' represents the nonredundant protein sequences from nr database of NCBI. SSR containing contigs were given as input to run BLASTx with the following command: 'blastx −query <input fasta sequence> -db nr_db −outfmt 6 −out <result file name>', where all the other parameters were kept as default. InterProScan at EBI is an important tool which was used for analyzing the SSR-contigs to get the functional domain markers (FDMs) (Quevillon et al., 2005). GO terms were assigned to the SSR-contigs using QuickGo (http://www.ebi.ac.uk/QuickGO) at EBI. Custom in-house perl scripts (as listed in Supplementary Material) were used for preparation of the input files for all the above tools.

Prediction of amino acid content for SSR loci

The amino acid content for SSR loci was detected in all the SSR-EST sequences of 11 species using MEGA v. 5.2 and the mean value calculated for each amino acid was extensively studied incorporating Boxplot analysis in R.

Analysis of antidiabetic SSR markers

Antidiabetic compound information in several plants was available in a previous report (Perez et al., 1998). The gene sequences involved with diabetes were curated manually from GenBank of NCBI with reference to the antidiabetic compounds. Here, we have employed locally installed BLASTn program using the SSR containing EST sequences and the gene responsible for antidiabetic compounds. The antidiabetic genes were formatted to create a BLAST database, and the SSR-ESTs were used as query sequences. From the BLAST results, the SSR-EST sequences having significant matches with the genes responsible for antidiabetic compounds were collected and subjected to Blast2go V.2.7.0 to map gene ontology (GO) terms and enrichment analysis. Two datasets were prepared as test and reference for the SSR-ESTs having BLAST matches and the antidiabetic gene sequences, respectively. The enrichment analysis (Fisher's Exact Test) was performed between the two datasets at $p < 1.5$. The GO terms were reduced to more specific terms using the respective option in Blast2GO. An interactive network graph was constructed for the enriched GO terms into three different functional categories [i.e., biological process (BP), molecular function (MF), and cellular component (CC)] using Gephi (https://gephi.org/). The overall workflow of present work is summarized in Figure 1.

Results

In the present study, ESTs of 11 important plants having antidiabetic property were screened for identification of genic microsatellites and functional characterization. Initially the raw sequences obtained from public domains were preprocessed and subsequently subjected to sequence assembly programme CAP3. The number of SSRs obtained from assembled (i.e., contigs and siglets) sequences of all the eleven plants and related information has been summarized in Table 1.

SSR detection and primer designing

The SSR survey detected a total of 47,700 numbers of SSRs in 109,609 numbers of ESTs from 11 plants. The density of SSRs in total was found to be 1 SSR per 1.54 kb, and 25.38 % of the total ESTs contain these SSRs. The SSR density ranged from 1 SSR/5.3 kb in *Mangifera indica* to 1 SSR/0.8 kb in *Cynodon dactylon*. A total of 15,013 numbers of SSRs were found in the compound formation. The highest numbers of compound microsatellites (cSSRs) were present in *Carica papaya* (i.e., 8979) and the lowest in *Mangifera indica* (i.e., 10) as shown in Table 1.

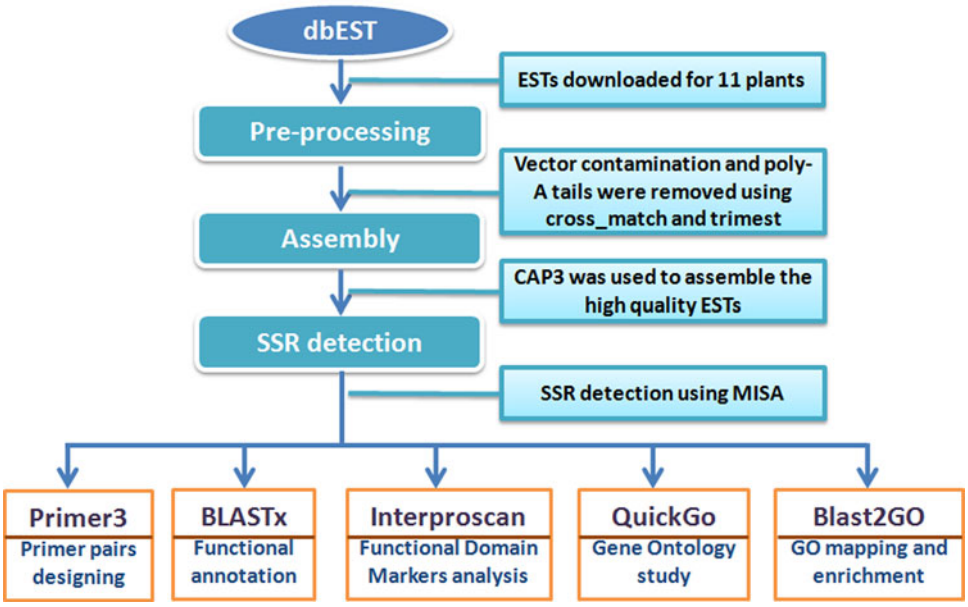


FIG. 1. Work flowchart for the EST-SSR mining. The description of total in silico analysis (i.e., SSR detection, functional annotation, gene ontology study, and GO enrichment analysis).

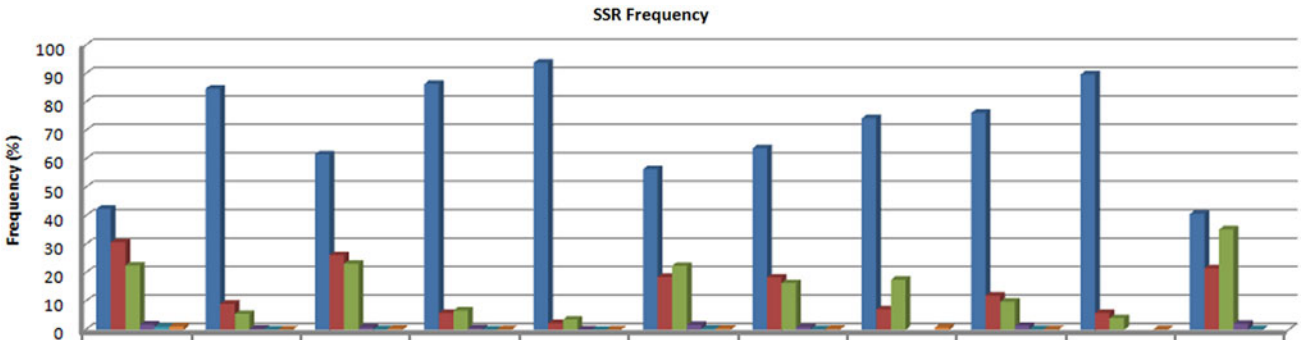
The frequency of SSRs across the EST sequences was analyzed very carefully. Generally, mononucleotides were found to be the abundant repeat type. Apart from mononucleotide repeats (MNR), dinucleotide repeats (DNR) and trinucleotide repeats (TNR) were also present abundantly. The density of DNRs was found to be 30.8% and 2.3% in *Ananas comosus* and *Cynodon dactylon* respectively and TNRs was 35.3% and 3.6% in *Tinospora cordifolia* and *Cynodon dactylon,* respectively. The hexanucleotide repeats were completely absent in *Tinospora cordifolia* and the tetra- and pentanucleotide repeats were completely absent in both *Mangifera indica* and *Nelumbo nucifera* (Fig. 2).

The most frequent classified repeat types were A/T in mononucleotides, AG/CT in dinucleotides, and AAG/CTT in trinucleotides. The exceptions were found in *Dioscorea alata* in case of dinucleotides, where AT/AT was the abundant type, and in case of trinucleotides, AGC/CTG and CCG/CGG were the exceptions found in *Citrus aurantium* and *Cynodon dactylon,* respectively. The frequency of A/T was found to be 60.9% and 99.7% in *Citrus aurantium* and *Dioscorea alata,* respectively, the frequency of AG/CT was 50% and 88.9% in *Citrus aurantium* and *Mangifera indica,* respectively, and the frequency of AAG/CTT was found to be 19.9% and 55.6% in *Ananas comosus* and *Tinospora cordifolia,* respectively (Table 2). A boxplot plotted using R and Bioconductor describes the distribution of the unit repeat type SSRs across the 11 plant species (Fig. 3).

Primer pairs were not possible to design for all the SSR containing ESTs with the optimum parameter values. However, a total of 57957 numbers of primer pairs were designed from 16724 unique ESTs (as listed in Supplementary Table S1). Highest percentage (83.4%) was found in *Tinospora cordifolia* out of all the SSR-ESTs and the lowest was found in case of *Mangifera indica* with a percentage of only 6.3. The output of primer3 tool was analyzed, which provided the percentage density of number of primer pairs designed *viz.*, the number of primer pairs with respect to 100 of unique SSR-ESTs for which primer designing was possible. The

Table 1. Statistical Summary of the SSRs Identified from 11 Medicinal Plants of North-East India with Potent Antidiabetic Properties

Plant common name	*Plant botanical name*	*No. of sequences examined*	*No. of SSRs*	*SSR frequency*	*No. of SSR-ESTs*	*No. of cSSRs*
Pineapple	*Ananas comosus*	3647	1199	1 SSR/2.4 kb	898	182
Papaya	*Carica papaya*	31554	19662	1 SSR/1.3 kb	8536	8979
Noyontara	*Catharanthus roseus*	8559	1753	1 SSR/2.7 kb	1401	229
Bitter orange	*Citrus aurantium*	11934	5924	1 SSR/1.6 kb	3926	1149
Bermuda brass	*Cynodon dactylon*	11953	9310	1 SSR/0.8 kb	5508	3046
Ratalu	*Dioscorea alata*	22583	4711	1 SSR/2.5 kb	3608	730
Barbados nut	*Jatropha curcas*	11334	2783	1 SSR/2.6 kb	2125	410
Mango	*Mangifera indica*	1241	125	1 SSR/5.3 kb	100	10
Mulberry	*Morus indica*	2455	434	1 SSR/2.2 kb	389	26
Lotus	*Nelumbo nucifera*	1634	1008	1 SSR/0.9 kb	707	155
Guduchi	*Tinospora cordifolia*	2715	791	1 SSR/2.6 kb	616	97



	Ananas comosus	Carica papaya	Catharanthus roseus	Citrus aurantium	Cynodon dactylon	Dioscorea alata	Jatropha curcas	Mangifera indica	Morus indica	Nelumbo nucifera	Tinospora cordifolia
mono	42.5	84.8	61.7	86.5	93.9	56.5	63.8	74.4	76.3	89.8	40.8
di	30.8	9.1	26.2	5.9	2.3	18.6	18.4	7.2	12	5.9	21.6
tri	22.6	5.6	23.2	6.8	3.6	22.5	16.4	17.6	9.9	4.1	35.3
tetra	1.8	0.3	0.8	0.5	0.13	1.7	0.9		1.4		2.1
penta	1.1	0.07	0.2	0.1	0.03	0.3	0.2		0.2		0.2
hexa	1.2	0.1	0.4	0.2	0.04	0.4	0.3	0.8	0.2	0.2	

FIG. 2. Frequency of the individual repeat types in the SSRs obtained from MISA analysis.

percentage ranged from 317.1% to 416.5% in *Catharanthus roseus* and *Morus indica,* respectively (Fig. 4).

Functional annotation

BLASTx identified significant matches for a total of 3736 SSR containing contigs in 11 plant species (as listed in Supplementary Table S2). The average range percentage (%) of sequence identity was 79.3% to 100%. Highest numbers of matches (1472) were found in case of *Cynodon_dactylon,* whereas in *Mangifera indica,* the least number of matches (i.e., 7) was found. The InterProScan results can be summarized with the obtained 21,568 numbers of functional domain markers (FDMs) for a total of 4728 unique contigs as listed in Supplementary Table S3. The gene ontology (GO) term annotation provides a common terminology for the functional description of transcripts comprised of three sub-ontologies: biological process (BP), molecular function (MF), and cellular component (CC). The GO term annotation revealed a total of 9634, 4740, and 18611 numbers of biological processes, cellular components, and molecular functions (Fig. 5). Several important processes related to oxidation, reduction, metabolic, biosynthesis, transport, translation etc. were assigned to the SSR containing contigs. In case of cellular components, the intracellular, membrane, ribosomal, and nuclear components were abundant, and so many others were found for all the SSR-contigs analyzed. Enzyme activity was found to be the most frequent with 44% of the total molecular functions detected. In addition, the other molecular functions were mostly related to binding functions such as ion binding and protein binding.

Analysis of antidiabetic SSR markers

A total of 134 gene sequences involved in the synthesis of antidiabetic compounds were collected manually from available literature and GenBank. The BLASTn between SSR containing ESTs and antidiabetic genes identified a total of 11 unique SSR-ESTs in 5 out of all 11 plants examined, which matched significantly with 30 antidiabetic gene sequences. Figure 6 illustrates the positions of SSR markers, antidiabetic gene similarity region (the redundant regions were avoided in the image). *Citrus aurantium, Carica papaya, Cynodon dactylon, Dioscorea alata, and Jatropha curcas* were found to

TABLE 2. SUMMARY OF THE EACH CLASSIFIED SSR MOTIF TYPES WITH THEIR FREQUENCY IN 11 MEDICINAL PLANTS WITH POTENT ANTIDIABETIC PROPERTIES

Plant name	*Mononucleotides*	*Mononucleotide frequency*	*Dinucleotides*	*Dinucleotide frequency*	*Trinucleotides*	*Trinucleotide frequency*
Ananas comosus	A/T	98.2	AG/CT	84.6	AAG/CTT	19.9
Carica papaya	A/T	70.4	AG/CT	57.4	AAG/CTT	51.5
Catharanthus roseus	A/T	95.5	AG/CT	63.6	AAG/CTT	36.2
Citrus aurantium	A/T	60.9	AG/CT	50	AGC/CTG	24.2
Cynodon dactylon	A/T	88.9	AG/CT	60.2	CCG/CGG	27.3
Dioscorea alata	A/T	99.7	AT/AT	55.9	AAG/CTT	23
Jatropha curcas	A/T	88.7	AG/CT	79.4	AAG/CTT	39.8
Mangifera indica	A/T	93.5	AG/CT	88.9	AAG/CTT	45.5
Morus indica	A/T	98.8	AG/CT	62.2	AAG/CTT	37.2
Nelumbo nucifera	A/T	63.5	AG/CT	84.7	AAG/CTT	39.1
Tinospora cordifolia	A/T	99.4	AG/CT	67.3	AAG/CTT	55.6

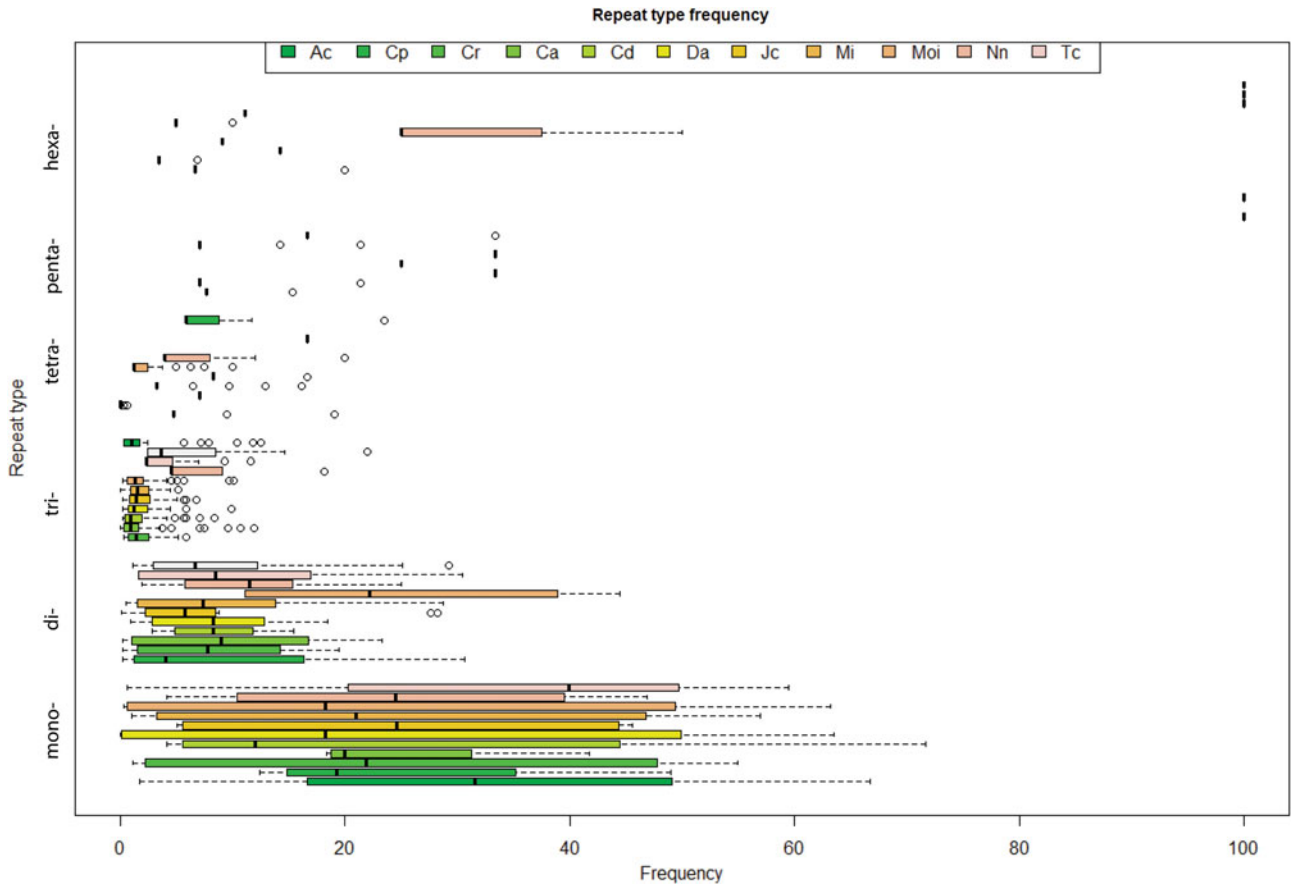


FIG. 3. Box plot to illustrate distribution of the unit repeat type SSRs across the 11 plant species.

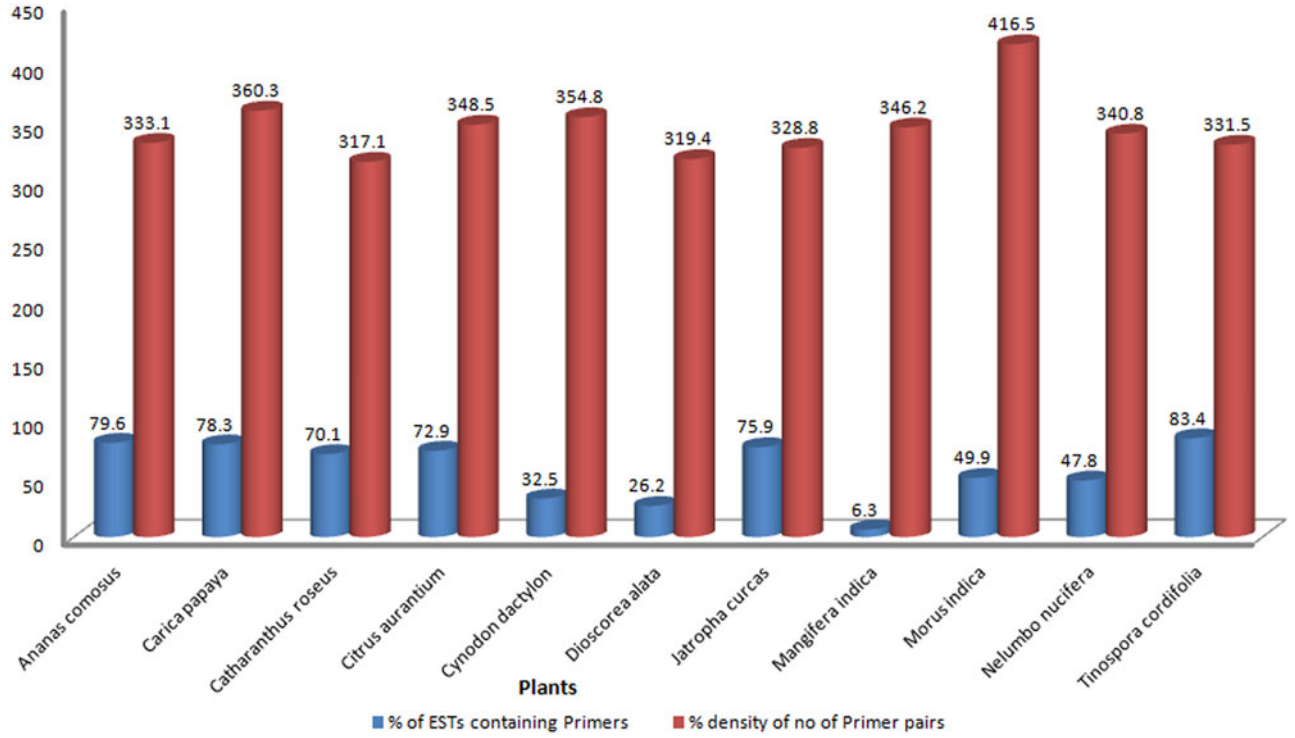


FIG. 4. Primer statistics of SSR containing ESTs.

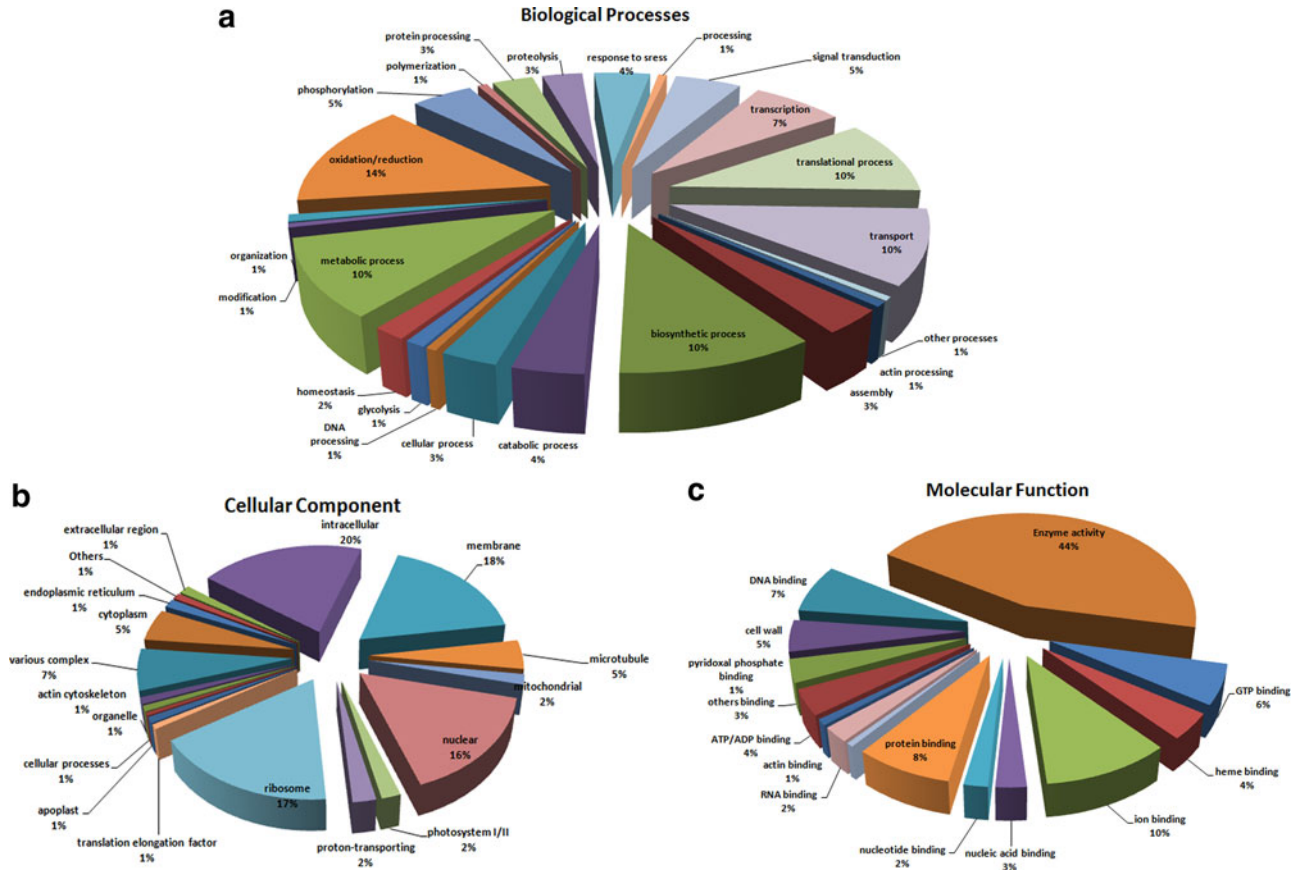


FIG. 5. Statistics of GO annotation of the SSR-contigs; **(a)** biological processes, **(b)** cellular components, and **(c)** molecular functions.

contain 3, 3, 2, 2, and 1 antidiabetic SSR-ESTs, respectively. Blast2GO analysis identified 126, 37, and 30 numbers of GO terms associated with several biological processes, molecular functions, and cellular components, respectively. The statistical significance of GO terms associated with the 11 SSR-ESTs (test datasets) having match with the 30 antidiabetic genes (reference datasets) was well explored with the help of enrichment analysis in Blast2go tool. The enrichment analysis at $p < 1.5$ detected total of 80 specific GO terms distributed in to three sub-ontologies (i.e., 53 biological processes, 17 molecular functions and 10 cellular components respectively).

A modular architecture (Fig. 7) was constructed with the help of Gephi which clearly illustrates the networks between the antidiabetic SSR-ESTs and enriched GO terms associated with them. Both the Contig1455 and Contig6377 of *Cynodon dactylon* and *Carica papaya* were found to be connected with

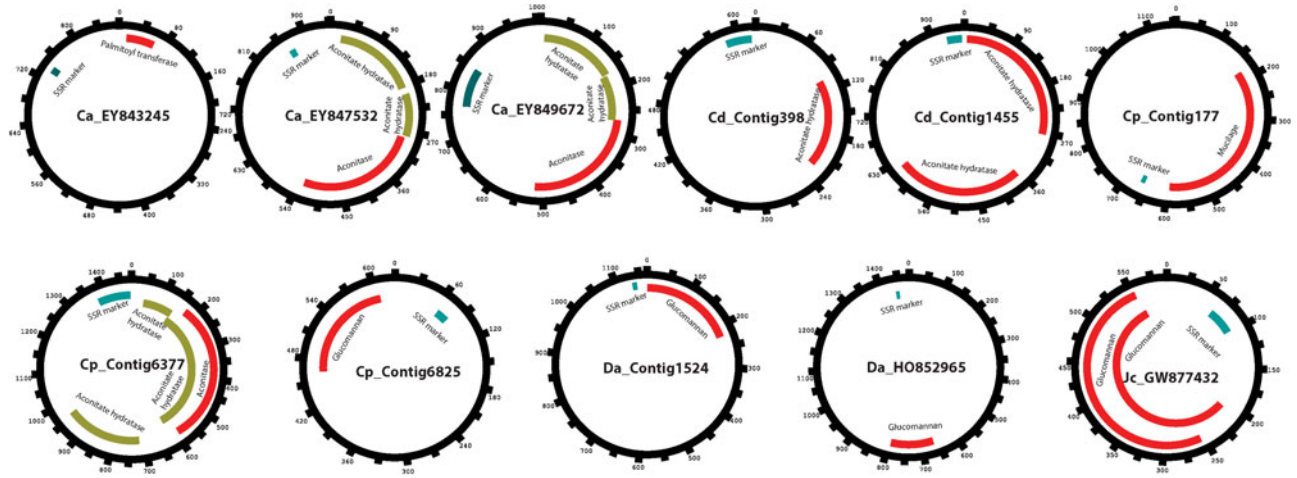


FIG. 6. Figures showing the positions of SSR markers and regions similar to antidiabetic gene for all 11 SSR-ESTs.

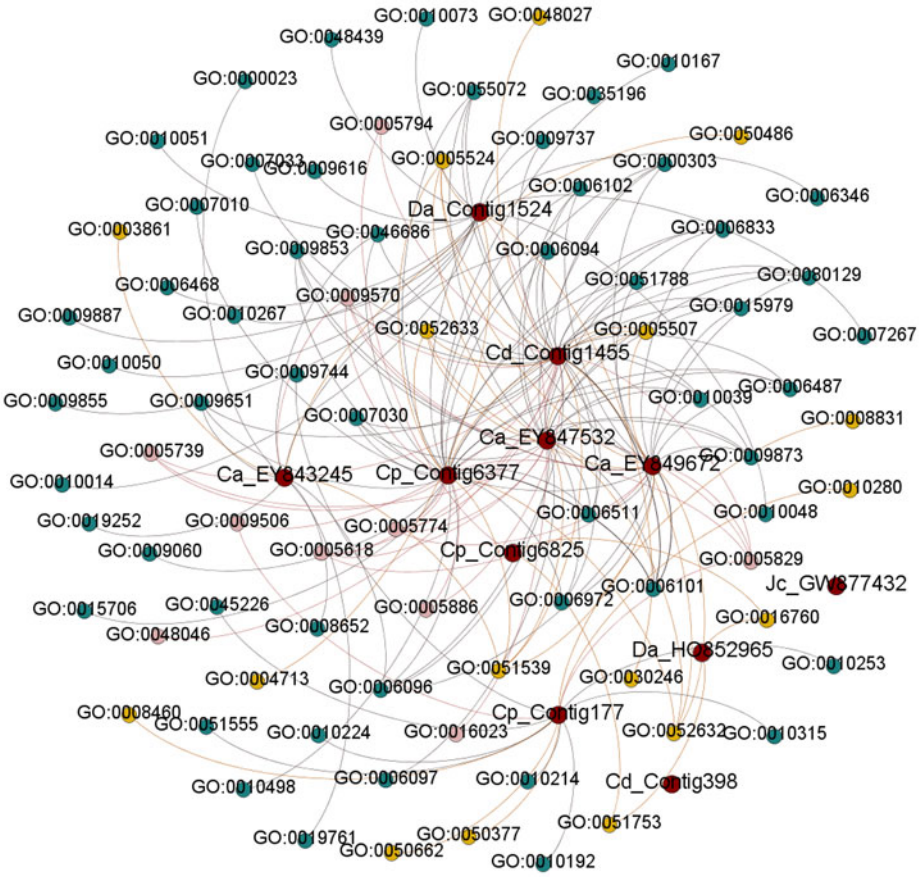


FIG. 7. Modular architecture of the GO terms associated with the anti-diabetic genes. *Red*, *yellow*, *green*, and *blue color* nodes represent the EST-IDs, biological processes, cellular components, and molecular functions, respectively.

37 enriched GO terms, whereas the Contig6825 of *Carica papaya* was connected with least numbers of GO terms (i.e., 3). The Contig398 of *Cynodon dactylon* and GW877432 of *Jatropha curcas* were found to have no association with the enriched GO terms. The most frequent enriched GO terms associated with biological processes were found to be GO: 0006096 and GO: 0006094, and each were mapped to five unique SSR-ESTs. In the case of molecular functions, there were four GO terms (i.e., GO: 0005829, GO: 0009570, GO: 0051539 and GO: 0005524) connected with maximum (i.e., five numbers of unique SSR-ESTs). GO: 0005829 and GO: 0009570 are the two enriched GO terms that belong to cellular component and mapped to five unique SSR-ESTs which is the highest.

Discussion

Over the last 2 decades, molecular techniques have been widely used for the genetic analysis of various crop plants due to their high efficiency (Ford-Lloyd et al., 1997; Virk et al., 2000; Song et al., 2003). The molecular markers, in particular, genic microsatellite (SSR) markers, are the markers of choice due to their high levels of cross-taxon portability, rapid and less expensive development. The applications of SSR markers have been reported in many crops to scrutinize DNA sequence variation(s). The multiallelic nature, reproducibility, high abundance, and extensive genome coverage make microsatellites a powerful tool to get into the genetic makeup of plants (Tautz and Renz, 1984; Gupta et al., 1996; Toth et al., 2000; Katti et al., 2001; Kantety et al., 2002; Varshney et al., 2002). The traditional methods of developing SSR markers are usually time consuming and labor-intensive processes involving genomic library construction, hybridization with the repeated units of nucleotides, and sequencing of the clones. The computational approach for developing SSR markers from ESTs provides a better platform than the conventional approach. Computational biology, along with high throughput bioinformatics tools (both standalone and web-based), have paved the way to screen publicly available EST and GSS data to design EST-SSR markers on a large scale. The publicly available computational tools and large set of EST data available on the web helps researchers to perform data mining rapidly with ease from their local system at a very low cost.

In this study, we present mining of EST sequences of 11 important plants bearing antidiabetic properties, and abundant in the Northeastern region of India, that include pineapple, papaya, noyontara, bitter orange, bermuda brass, ratalu, barbados nut, mango, mulberry, lotus, and guduchi. Though these plants are mainly known for their uses of fruits and flowers, they are also very effective against diabetes, as evident from literature surveys. But as of now, no efforts have

been put into development of efficient molecular markers in these plants, so an attempt was made to derive EST-SSR markers *in silico*. Our *in silico* survey of microsatellite from the ESTs of these plants revealed a total 1199, 19662, 1753, 5924, 9310, 4711, 2783, 125, 434, 1008, and 791 numbers of SSRs markers. The densities of SSRs found to be 1 SSR per 2.4 kb, 1.3 kb, 2.7 kb, 1.6 kb, 0.8 kb, 2.5 kb, 2.6 kb, 5.3 kb, 2.2 kb, 0.9 kb, and 2.6 kb, respectively, for the above plants. Previous studies have shown the SSR densities in several other plants as 1 SSR per 2–10 kb (Morgante et al., 2002; Kantety et al., 2002; Varshney et al., 2002; Kumpatla and Mukhopadhyay, 2005; Poncet et al., 2006; Scaglione et al., 2009), which signifies that SSRs detected are more frequent in our study. A total of 58,159 number of primer pairs were possible to design for all the 11 plants (see Results section).

Mononucleotide repeats were the most frequent repeat type in all the plants. Apart from mononucleotide repeats, dinucleotide and trinucleotide-SSR motifs were predominant, whereas tetra-, penta-, and hexanucleotide motifs were detected in much smaller amounts. In *Mangifera indica* and *Nelumbo nucifera*, tetranucleotides and pentanucleotides were completely absent, whereas in *Tinospora cordifolia*, hexanucleotide was completely absent. The dinucleotide repeats (DNRs) are more frequent than the trinucleotide repeats (TNRs) in ESTs of *Ananas comosus*, *Carica papaya*, *Catharanthus roseus*, *Jatropha curcas*, *Morus indica,* and *Nelumbo nucifera,* similar to the previous reports in Actinidia (Fraser et al., 2004), *Camellia* (Sahu et al., 2012), and Picea species (Rungis et al., 2004). But the frequencies of TNRs were found to be more than DNRs in *Citrus aurantium*, *Cynodon dactylon*, *Dioscorea alata*, *Mangifera indica,* and *Tinospora cordifolia,* similar to most previous studies (Cordeiro et al., 2001; Kantety et al., 2002; Varshney et al., 2002; Thiel et al., 2003; Nicot et al., 2004). In most cases, the frequent classified repeat types are found to be A/T in mononucleotides, AG/CT in dinucleotides. The frequently classified repeat types observed in this study perfectly correlate with the earlier studies by Temnykh et al. (2000) and Kantety et al. (2002). In the case of trinucleotides, AAG/CTT is the most frequent classified repeat in nine plants, unlike the previous study by Morgante and co-workers (2002). In *Dioscorea alata,* AT/AT is the most frequent dinucleotide classified repeat type, which is the same as in nonvascular plants, as found for *Physcomitrella patens* (Victoria et al., 2011). AGC/CTG and CCG/CGG are the most frequent trinucleotide classified repeat type in *Citrus aurantium* and *Cynodon dactylon,* similar to *Cymbidium* spp. (Moe et al., 2012).

InterProScan helped functional analysis of translated nucleotides by classifying them into families and predicting domains and important sites. Most importantly, the GO annotations revealed a total of 2619 unique GO terms. Gene ontology analysis revealed that majority of SSR loci were involved in 1332 unique molecular functions, 938 biological processes, and 349 cellular components. Among the discrete biological processes inferred through gene ontology analysis, oxidation/reduction, which was the most abundant biological process, accounted for 14%. Other processes that were more frequently noted include metabolic, translational, transport, biosynthesis, and transcription processes, whereas aminoacylation, peptide processing, and RNA processing were found to be very negligible. Of cellular components, intracellular components had the highest frequency of 20%, followed by membrane at 18%, ribosome at 17%, and nuclear at 16%. The less frequent components were associated with cytoskeleton, chromosomes, and lipid storage bodies. A number of important molecular functions were predicted, among which the most abundant types were enzyme activity with a frequency of 44%. The other frequent functions were associated with ion binding, and protein binding, whereas phospholipid binding, ribosome, and transporter activity weree also detected with less frequency.

Previous studies revealed that the frequency of SSRs detected in a survey greatly depends upon the size of the sequence data, SSR parameter, search criteria and the mining tools used (Varshney et al., 2005). Compound microsatellites (cSSRs) have already been proven to be the most important despite their low density in the nucleotide sequences (Bull et al., 1999). In the present study, the percentage of cSSRs was found to be the highest in *Carica papaya* at 45.7%. In other plants, the percentage of detected cSSRs in the total SSRs were: *Cynodon dactylon* (32.7%), *Citrus aurantium* (19.4%), *Dioscorea alata* (15.5%), *Nelumbo nucifera* (15.4%), *Ananas comosus* (15.2%), *Jatropha curcas* (14.7%), *Catharanthus roseus* (13.1%), *Tinospora cordifolia* (12.3%), *Mangifera indica* (8%) and *Morus indica* (6%).

The frequencies of cSSRs in most of the plants in the present report are better than the previous studies where the frequencies of cSSRs were 11% in human, 4%–25% in seven fully sequenced species (*Maccaca mulatta*, *Mus musculus*, *Rattus norvegicus*, *Ornithorhynchus anatinus*, *Gallus gallus*, *Danio rerio,* and *Drosophila melanogaster*), and 1.75%–2.85% in complete *Escherichia coli* genomes (Weber, 1990; Kofler et al., 2008; Chen et al., 2011). In this study, we identified a total of 7164 cSSRs, where only 6856 unique cSSRs were retained after removal of redundant ones. Out of all the compound microsatellites, 213 were detected more than once. The 6 unique cSSRs: (A)11g(A)12, (A)15g(A)30, (A)22g(A)29, (A)35g(A)29, (A)39g(A)29, and (C)10gg(C)10 were found to have the highest frequency.

The microsatellite markers are the most preferable for studying genetic diversity, linkage map analysis, identification of new genes, and many other important fields. Mainly in plants these markers have shown quite impressive ability to explore the genetic information (Liu et al., 1996; Struss et al., 1998; Ramsay et al., 2000; Ritschel et al., 2004; Varshney et al., 2005; Tang et al., 2006) and to study the cross-transferability across related species (Cordeiro et al., 2001; Morgante et al., 2002; Saha et al., 2006; Wohrmann and Weising, 2011).

In the present study, 11 SSR markers were found to be associated with five unique genes: palmitoyl transferase (carnitine palmitoyltransferase), aconitase, aconitate hydratase, mucilage, and glucomannan (glucomannan 4-beta-mannosyltransferase), which were shown to be the key regulatory elements in curing diabetes. Carnitine palmitoyltransferases 1 and 2 (CPTs; EC: 2.3.1.21) were found to be the key enzymes in the import of long-chain fatty acids into mitochondria and recently have received considerable attention due to their catalytic activity and for the development of novel drugs against diabetes (Rufer et al., 2006). Aconitase is an alternative name for Aconitate hydratase, and both indicate the same enzyme that is associated with diabetes (Boquist et al., 1985). Though the exact connection is still not

fully known, studies have shown that lower aconitase activity is responsible for influencing the rates of the activity of the tricarboxylic acid cycle, thereby impairing mitochondrial function and energy metabolism in diabetic hearts (Lin et al., 2009). The low cost, lack of toxicity, easy availability, soothing action, and nonirritant nature of mucilage makes them preferable for semi-synthetic and synthetic excipients (Malviya et al., 2011). The effect of fenugreek seed mucilage on disaccharide activities has been proven to be beneficial to increase specific activities of intestinal disaccharides significantly during diabetes; it is also found to be better than turmeric (Kumar et al., 2005). A direct association is reported of glycemia with glucomannan, a water-soluble polysaccharide used as a dietary fiber (Vuksan et al., 1999). Although at this point, the exact mechanisms underlying the link of antidiabetic properties and SSR could not be proposed, further studies involving microsatellite linked association with trait studies can shed more light into the microsatellite–gene alliance with medicinal importance in plants.

Conclusions

For the first time, we developed and characterized potential microsatellite markers as well as several efficient gene-based SSR markers for the identification of hypoglycemic agents in 11 traditional plants with potential antidiabetic property. The genic microsatellites developed from our study can be considered as an important repository for future improvement of the crop through marker assisted breeding and also for identification of new ideotypes with antidiabetic property. Moreover, the SSR markers have been proven to be cross transferable across species of the same family. Hence, the EST-SSR markers developed in this study would be useful tools for genetic diversity and conservation studies in several plant species.

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Author Disclosure Statement

The authors declare that there are no competing financial interests.

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