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Assessment of Genetic Diversity using EST Derived SSR Markers in Pomegranate (*Punica granatum* L.) Germplasms

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Authors' contributions

This work was carried out in collaboration among all authors. Authors HNZ and JSP conceptualized. Authors VNB performed methodology experiments. Authors VNB, HNZ and AM did data analysis and prepared the draft of the manuscript. Authors VK, PCP and SDS. supervised the study. Authors AM, KNP and NVS wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Pomegranate (*Punica granatum* L.) is an economically important perennial crop, with high nutritional, medicinal and ornamental importance. Total 2417 dbEST sequences were downloaded from NCBI and after pre-processing, 1152 sequences were assembled by CAP3. On evaluating by MISA tool, 175 sequences were found to be possessing SSR motifs and further subjected to BlastX hit, 125 sequences were annotated with gene ontology numbers. The distribution of SSR motifs highlighted di-nucleotide repeats as the highest (59.8%) followed by tri-nucleotide (37.7%). Total 124 successful primer pairs were designed and on the basis of unigene function, randomly 32 primer pairs were selected for validation on 46 germplasm. Eight EST-SSR primers were found to be polymorphic which generated 433 scorable bands with average of 54 bands per primer. The PIC and heterozygosity ranged from 0.178 to 0.555 and 0.198 to 0.625 respectively. Thirteen out of 20 gSSR primers were found to be polymorphic which generated 627 scorable bands with average of 48 bands per primer. The PIC and heterozygosity ranged from 0.175 to 0.374 and 0.194 to 0.498 respectively. Clustering pattern of pooled data of EST-SSR and gSSR showed major two clusters A and B having similarity coefficient of 0.37 to 1.00. The results of cluster analysis revealed grouping patterns based on geological distributions and derivation relationships. Thus, the developed EST derived SSR markers based on functional annotation of sequences could be very useful for various research areas in pomegranate, such as identification of the economically important pomegranate cultivars, study evolutionary origin analysis, genetic linkage map construction and marker-assisted selection for breeding.

Keywords: Pomegranate; EST-SSR; diversity; PIC; pomegranate; perennial crop.

1. INTRODUCTION

"Pomegranate (*Punica granatum* L., 2n=16) (Moriguchi *et al*., 1987) is an economically important ancient perennial crop, with high nutritional, medicinal and ornamental importance which belongs to the Lythraceae family. The pomegranate was expanded to Afghanistan, India, China, and Pakistan from the semi-tropics of Persia" (Nafees *et al.*, 2016). "Iran has the richest collection of pomegranate and rank first in pomegranate production in the World. Besides being used as a fresh and processed fruit, pomegranate has extensively been considered as a medicinal plant in the Middle East. Research has shown that pomegranate fruit comprise many bioactive phytochemicals such as sterols, terpenoids, alkaloids fatty acids, organic acids, and flavonols that are useful for the treatment of high blood pressure, diabetes, and cancer" (Sarkhosh *et al.,* 2012). "Moreover, pomegranate has robust flexibility to extreme climates and meagre soils. Therefore, pomegranate could be introduced and cultivated all over the world. Diverse ecological conditions make it display of high diversity of pomological traits, such as fruit colour, seed hardness, sweetness, bacidity, etc" (Luo *et al.,* 2018). Due to its high potential for human health benefit, pomegranate has achieved the title of a "super-food". Pomegranate has a long history of medicinal uses as a herbal cure for cancer, diarrhoea, diabetes, blood pressure, leprosy, dysentery, haemorrhages, bronchitis, dyspepsia, and inflammation (Soni and Kanwar, 2016).

"Due to the long history of pomegranate cultivation in Iran, synonyms or obvious similarities in appearance can be observed between cultivars from different regions. Thus, the precise determination of, and discrimination between cultivars is essential for future pomegranate breeding and commercialisation of promising cultivars. An identification of pomegranate genotypes cultivated in one province of Iran, based on morphological characteristics of the fruit, has been performed. However, morphological characteristics often do not result in a clear discrimination between cultivars due to ambiguous descriptions or phenotypic modifications caused by the environment. Hence, the application of molecular markers for more precise identification of, and discrimination between pomegranate genotypes and cultivars is essential" (Zamani *et al.,* 2007).

"To date, a wide range of DNA marker systems have been deployed in pomegranate. Among these, SSRs become marker of choice because it requires only a small amount of DNA, easily detectable by PCR (Polymerase chain Reaction), high reproducibility, multi-allelic, co-dominant nature, abundant and amenable to high throughput analysis, which provides more

information per unit assay as compared to other marker systems" (Kalia *et al*., 2011). "In pomegranate, SSRs have been employed extensively to study genetic diversity and to understand population structure and association analysis" (Curro *et al.,* 2010; Pirseyedi *et al.,* 2010 and Singh *et al.,* 2015). However, linkage mapping and QTL (quantitative trait loci) analysis based on SSR markers are currently lacking in pomegranate. A possible reason may be limited DNA polymorphism demonstrated in pomegranate by the currently available SSR markers. The length of the repeat motif is of paramount importance while surveying genetic polymorphisms with SSR markers. Alternative to overcome this problem is to develop expressed sequence tag (EST) derived microsatellite/ SSR markers. ESTs are particularly attractive for marker development since they represent coding regions of the genome and are also being developed at an extremely fast pace for many genomes (Kumpatla and Mukhopadhyay, 2005). EST-SSRs, demonstrated some valuable advantages such as they can be rapidly found by electronic sorting and have greater transferability between species than genomic SSR, since genic regions are more conserved among related species (Shirasawa *et al*., 2011 and Chen *et al*., 2015). Furthermore, EST-SSRs usually present in gene-rich regions and can be used as anchor markers for comparative mapping and genetic evolutionary studies (Zhou *et al*., 2014). **"**EST sequencing is a cost-effective method for obtaining sequence information of transcribed genes and the generated information can reveal tissue-specific transcripts or gene expression patterns during development" (Zhang *et al.,* 2017). Therefore, the present study was conducted with the aim to explore new microsatellite loci from public EST database using bioinformatics tools and to assess their potential for genetic diversity analysis in pomegranate germplasm.

2. MATERIALS AND METHODS

2.1 Plant Materials and DNA Extraction

Total forty-six germplasm of pomegranate used for validation of developed molecular markers were procured from Horticulture Farm, S. D. Agricultural University, Sardarkrushinagar (Table S1). Genomic DNA was extracted from the leaves of each genotype by protocol described by Doyle and Doyle (1990). Integrity of DNA was determined by 0.8 % agarose gel electrophoresis and quantified by UV-spectrophotometer. The DNA was diluted to a working concentration of 50 ng/µL.

2.2 Data Mining and Processing of EST Sequences of Pomegranate

A total of 2,417 EST sequences (Accession numbers: JG771192.1 to JZ971827.1) of pomegranate were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov) and further sequences were cleaned for low-complexity regions, poly-A and poly-T tracts and sequence ends rich in undetermined bases and low-quality sequences shorter than 100 bp. These cleaned EST sequences were assembled by employing CAP3 program to reduce sequence redundancy.

2.3 Functional Annotation of Unigenes

The assembled contigs and singletons were subjected to functional annotation using Blast2GO, online tool for functional annotation of (novel) sequences (Conesa et al. 2005). Blastx of contigs was carried out by comparing against the NCBI non-redundant protein sequence data-base (nr) using BLASTX with minimum e-value cut-off 10^{-6}. The sequences resulting from BlastX were mapped and annotated using mapping and annotation function of Blast2GO set at default parameters. The functional categories of these unique sequences were further analyzed according to GO (Gene Ontology) terms based on InterPro GO slim provided by InterPro with Blast2GO tool. All assigned GO terms were used to generate combined GO graph of cellular component, Molecular function, and biological processes using online tool WEGO (Ye et al. 2006; http://wego.genomics.org.cn/cgi-bin/wego/).

2.4 Selection of EST-SSRs and Function Prediction

The EST sequences were scanned using perl script MIcroSAtellite (MISA) program (Thiel et al. 2003; http://pgrc. ipk-atersleben.de/misa/website) to identify SSRs with the parameters: (i) unit size/ minimum number of repeats: (2–6) (3–5) (4–5) (5–5) (6–5) and (ii) maximal number of bases interrupting 2 SSRs in a compound microsatellite=100.

2.5 Primer Designing

The SSR containing contigs and singletons (Unigenes) were used to develop EST-SSR

primer pairs with the BatchPrimer3 online program (You et al. 2008; http://probes.pw.usda.gov/cgi-bin/batchp rimer3/ batchprimer3) with following the standard criteria: PCR product size- 100–250; primer length- 18–27 bp; Tm- 57–63 °C; GC content 40–60 %, maximum Tm difference between forward and reverse primer-1.5 °C. The designed primers were further checked for desired characteristics like hairpin structure, primer dimer using online Scitool, Oligoanalyzer, Integrated DNA Technologies available at http://www.idtdna.com/pages/scitools. The newly developed SSR primers were named with prefix PG-ES (Pomegranate EST-SSRs) and synthesized from Eurofins Genomics, India.

2.6 Polymorphism Survey of Genomic SSR and Newly Designed EST- SSR Markers

Twenty genomic SSR primers were used for initial screening of 46 pomegranate genotypes. The sequence of these primers were selected from literature of pomegranate (Curro *et al.,* 2010; Pirseyedi *et al.,* 2010; Hasnaoui *et al.,* 2012). Newly designed 32 EST-SSR primers out of 124 primers were tested for amplification on all 46 genotypes as mentioned above. The PCR mixture (10 µL) contained 5 µL of 2X PCR master mix, 1 µL of genomic DNA (50 ng), 0.8 µL primers (10p moles of each forward and reverse primers), and 3.2 µL of nuclease free water. For genomic SSR PCR thermal profile performed for 94℃ for 4 min of initial denaturation, followed by 35 cycles of 94℃ for 30 s, 55-45 for 45 s, and 72℃ for 1 min, followed by final extension for 7 min at 72℃. And for EST-SSR touchdown PCR thermal profile was performed for 94 ℃ for 10 min of initial denaturation, followed by first 11 cycles of 94 ℃ for 30 s, 62 ℃ to 52℃ for 30 s and 72 ℃ for 2 min, with 1 ℃ decrement in annealing temperature per cycle, then 24 cycles of 94 ℃ for 30 s with constant annealing temperature of 57 ℃ for 30 s and 72 ℃ for 1 min followed by a final extension for 7 min at 72 ℃. Amplified PCR product of gSSR markers were separated on 3% agarose gel and PCR product of EST-SSRs were separated on 3.5% agarose gel (0.5 µg/ml Et Br) along with 100 bp DNA ladder in 1X TBE buffer at constant power 120 V for about 2.5–3 h. The gels were visualized and documented by gel documentation system. The amplified bands were scored as presence (1) or absence (0). Polymorphism information content (PIC) and heterozygosity (H) was calculated using GeneCalc software (Binkowski and Miks, 2018). The data was entered into binary matrix and subsequently analyzed using NTSYSpc version 2.02 (Rohlf, 1994) for phylogenetic tree/dendrogram construction.

3. RESULTS

3.1 Identification, Characterization and Frequency of EST-SSRs

A total of 2,417 EST sequences (1,702,115 bases) of pomegranate (*Punica granatum* L.) were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov) and further sequences were filter out the low quality and low complexity sequences by using EGassembler (Masoudi-Nejad *et al.,* 2006). Assembly generated 1,152 (847,590 bases) unigenes containing 254 (223,034 bases) contigs and 898 (657,752 bases) singletons with N50 length (781 bp), Maximum contig length (2875 bp) and Average length (710 bp). The statistics expended for assembly were number of bases utilized for assembly, the maximum contig length, N50 contig length were used to assess assembly (Table 1).

A total number of 1152 (8,47,590 bases) unigenes sequence were evaluated for the presence of SSR motifs by MIcroSAtellite identification tool (MISA). The SSR developed were competently called EST-SSR. Using the criteria of motif repeats, a total of 199 SSRs from the 175 (15.2%) non-redundant ESTs were identified by Perl script of MIcroSAtellite (MISA). From 175 non-redundant ESTs, an attempt has been made to develop a total of 124 novel EST-SSR markers that are not available in public database. Among the 199 SSRs, 153 (76.88%) had simple repeat motifs, 22 ESTs contained more than one SSR, while 14 were compound types (Table 2) with an average frequency of one SSR per 4.25 Kb. The frequency of SSRs varies with respect to the parameters of SSR identification and the sequenced data under study. Furthermore, the variation in SSR finding parameters also contributes to the frequency of SSR in study.

EST-SSRs were variously classified depending upon their size, type of repeat unit, motif length. Depending upon the number of nucleotides per repeat unit, SSR's were classified as di-, tri-, tetra-, penta- or hexa-nucleotides (Table 3). The distribution of SSR motifs in different repeat type classes highlighted di-nucleotide repeats as the

highest 119 followed by tri-nucleotide 75, tetra-nucleotide 3 and penta-nucleotide and hexa-nucleotide repeats were found to be 1, respectably (Table 3). The di-nucleotide repeat class AG/CT (66.4%) was found most abundant followed by AT/AT and AC/GT (Table 4). The tri-nucleotide code AAG/CTT was followed by AGG/CCT, CCG/CGG, and ATC/ATG.

3.2 Functional Annotation of Unigenes

The total numbers of assembled sequences harbouring SSRs were subjected to functional annotation using Blast2GO. Out of the total 175 sequences subjected to BlastX with non-redundant database, 125 sequences were annotated. The annotated sequences were also analysed for blast hit with other species in the Nr database (Fig. S1). In case of blast hits distribution, the annotated sequences showed the maximum similarity with *Punica granatum* (338) followed by *Eucalyptus grandis* (125), *Rhodamnia aegentea* (89), *Syzygium oleosum* (78) *Juglans regia* (77), *Gossypium barbadense* (71), *Hevea brasiliensis* (66), *Gossypium tomentosum* (59), *Gossypium hirsutum* (59), *Gossypium darwinii* (57) and *Gossypium mustelinum* (57). The top blast hit distribution of pomegranate sequences was attributed to *Punica granatum* (138) followed by *Ensete ventricosum* (2), *Hevea brasiliensis* (1), *Oryza sativa* japonica group (1) and *Gossypium barbadense* (1) (Fig. S2).

Table 1. Summery of EST sequences and assembly using CAP3 assemblers

Parameters/ Assemblers	EST sequences	Unigenes (CAP3)
Number of sequences	2,417	1,152
Number of bases	1,702,115	847,590
N50 length (bp)	720	781
Maximum length (bp)	1,096	2,875
Average length (bp)	686	710
Number of contigs	-	254
Singletons	-	898
N50 contig length (bp)	-	890
Maximum contig length (bp)	-	2,875
Average contig length (bp)	-	802

Table 2. Statistics of SSRs identified in pomegranate using MISA

SSR mining/ Features	Total
Total number of assembled sequences examined	1,152
Examined sequences size (bases)	8,47,590
Total number of identified SSRs	199
Number of SSR containing sequences	175 (15.2%)
Number of sequences containing more than one SSR	22
Number of SSRs present in compound formation	14
Relative abundance of SSRs (considering 8,47,590 bases)	One per 4.25 kb

Table 3. Distribution and frequencies of SSR repeat types with repeat numbers in pomegranate

Motif length	Repeats number								
	5	6	7	8	9	10	>10	Total	%
Di-nucleotide	-	30	27	14	14	8	26	119	59.8
Tri-nucleotide	46	17	6	5	-	-	1	75	37.7
Tetra-nucleotide	2	1	-	-	-	-	-	3	1.5
Penta-nucleotide	1	-	-	-	-	-	-	1	0.5
Hexa-nucleotide	1	-	-	-	-	-	-	1	0.5
Total	50	48	33	19	14	8	26	199	-
%	25.3	24.2	16.7	9.6	7.1	4	13.1	-	-

Table 4. Frequencies of different repeat motifs of di- and tri-nucleotide repeats in pomegranate EST-SSRs

Repeat motif	Repeats number								
	5	6	7	8	9	10	>10	Total	%
AC/GT	-	-	15	1	-	-	-	16	13.4
AG/CT	-	24	8	11	10	8	18	79	66.4
AT/AT	-	6	4	2	4	-	8	24	20.2
Total	-	30	27	14	14	8	26	119	-
AAC/GTT	-	-	-	1	-	-	-	1	1.3
AAG/CTT	16	4	1	1	-	-	1	23	30.7
AAT/ATT	3	2	-	-	-	-	-	5	6.7
ACC/GGT	4	2	-	1	-	-	-	7	9.3
ACG/CGT	1	-	-	-	-	-	-	1	1.3
AGC/CTG	4	1	-	-	-	-	-	5	6.7
AGG/CCT	7	5	4	-	-	-	-	16	21.3
ATC/ATG	3	2	1	2	-	-	-	8	10.7
CCG/CGG	8	1	-	-	-	-	-	9	12.0
Total	46	17	6	5	-	-	1	75	

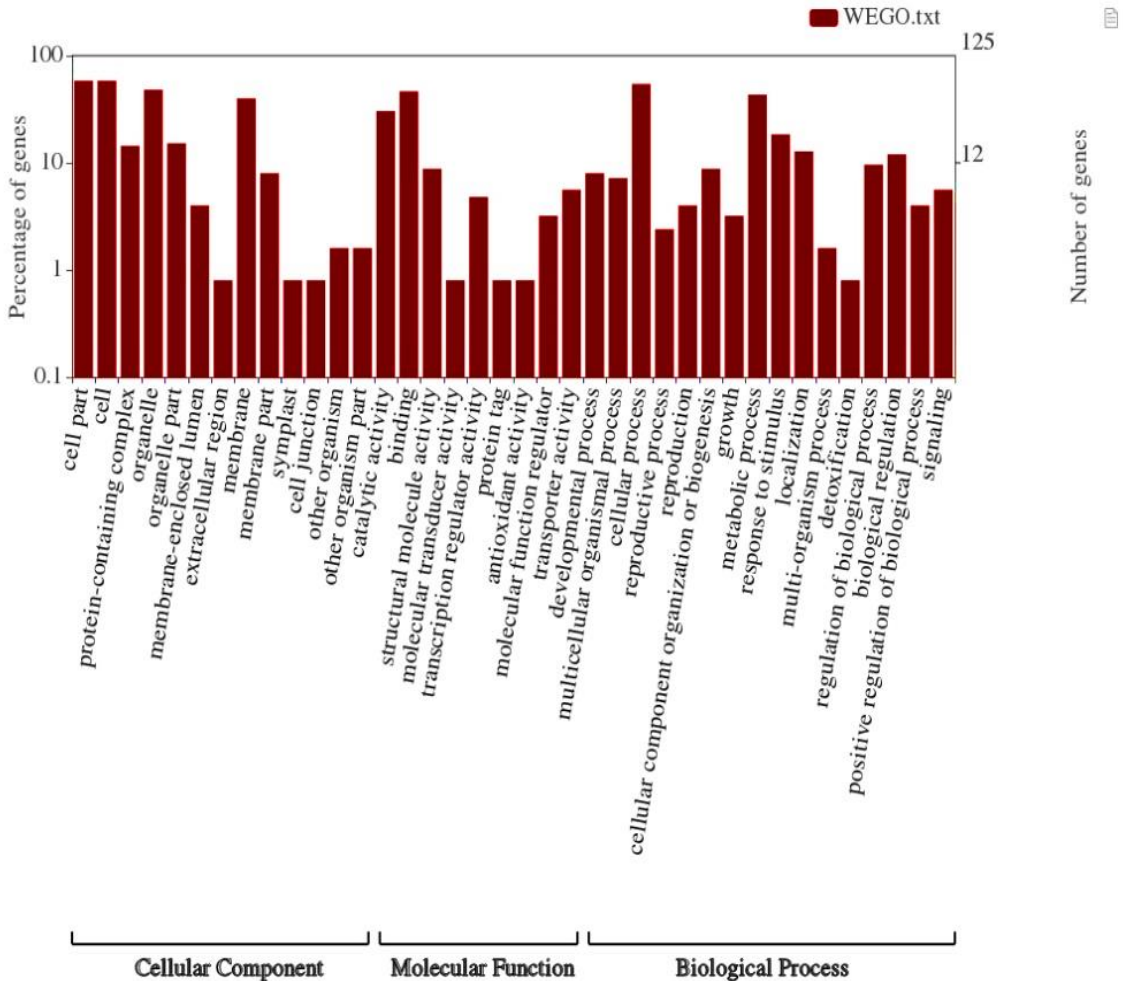

Fig. 1. Distribution of most abundant Gene Ontology (GO) terms assigned to 125 annotated SSR containing sequences

Table 5. Details of EST-SSR primers showing polymorphism among 46 pomegranate germplasm

Primer	Function	No. of loci	No. of alleles	Product size (bp)	PIC value	Hetero-zygosity
PG-ES-1	Protein translation factor SUI1 homolog 2	36	2	293-312	0.375	0.500
PG-ES-6	Glycine-rich cell wall structural protein 2-like	45	3	145-153	0.398	0.506
PG-ES-12	Transcription repressor MYB4-like isoform X1	89	4	155-185	0.538	0.616
PG-ES-13	Zinc finger A20 and AN1 domain-containing stress-associated protein 8	45	2	156-175	0.353	0.458
PG-ES-14	Fasciclin-like arabinogalactan protein 18	49	3	213-237	0.460	0.513
PG-ES-23	Homeobox-leucine zipper protein HOX11	34	2	293-312	0.186	0.208
PG-ES-24	FCS-Like Zinc finger 15-like	90	3	142-172	0.555	0.625
PG-ES-29	Zinc finger A20 and AN1 domain-containing stress-associated protein 8	45	2	200-211	0.178	0.198

Table 6. Details of SSR primers showing polymorphism among 46 pomegranate germplasm

Primer Name	Total No. of loci	Total No. of alleles	Product size (bp)	PIC	Heterozygosity
PG-SSR-1	44	2	159-200	0.373	0.496
PG-SSR-2	43	2	125-144	0.351	0.454
PG-SSR-5	46	2	170-205	0.373	0.496
PG-SSR-6	46	2	261-287	0.343	0.440
PG-SSR-8	46	2	321-343	0.265	0.315
PG-SSR-9	45	2	170-177	0.360	0.470
PG-SSR-10	46	2	105-115	0.323	0.406
PG-SSR-12	46	2	187-215	0.363	0.476
PG-SSR-14	43	2	174-209	0.235	0.273
PG-SSR-15	46	2	163-178	0.175	0.194
PG-SSR-16	85	2	177-193	0.374	0.498
PG-SSR-18	46	2	236-364	0.357	0.466
PG-SSR-20	45	3	243-278	0.332	0.336

The functionally annotated SSR harboured sequences were categorized into the cellular components, molecular functions and biological processes as presented in Fig. 1. In cellular component ontology, the maximum number of sequences were associated with cellular anatomical entity, GO:0110165 (99) followed by protein containing complex, GO:0032991 (13). The maximum number of sequence in molecular function were attributed to nucleotide binding, GO:0005488 (54) followed by catalytic activity GO:0003824 (36), structural molecule activity GO:0005198 (11), molecular function regulator GO:0098772 (10), transporter activity GO:0005215(7), molecular transducer activity GO:0060089(4), antioxidant activity GO:0016209(1) and protein tag GO:0031386. In biological function, the maximum number of sequences were represented by cellular process, GO:0009987 (71) followed by metabolic process GO:0008152, response to stimulus GO:0050896 (23), biological regulation GO:0065007 (16), localization GO:0051179 (16), regulation of biological process GO:0050789 (13), developmental process GO:0032502 (10), multicellular organismal process GO:0032501 (9), signalling GO:0023052 (7), positive regulation of biological process GO:0048518 (5), reproduction GO:0000003 (5), growth GO:0040007 (4), reproductive process

GO:0022414 (3), detoxification GO:0098754 (1), interspecies interaction between organisms GO:0044419 (1), negative regulation of biological process GO:0048519 (1) and multi-organism process GO:0051707 (1). The maximum number of annotated sequences were attributed to transferase (31%) and hydrolase (31%) followed by oxidoreductase (15%), translocase (11%), ligase (4%), isomerase (4%) and lyase (4%) (Fig. S3).

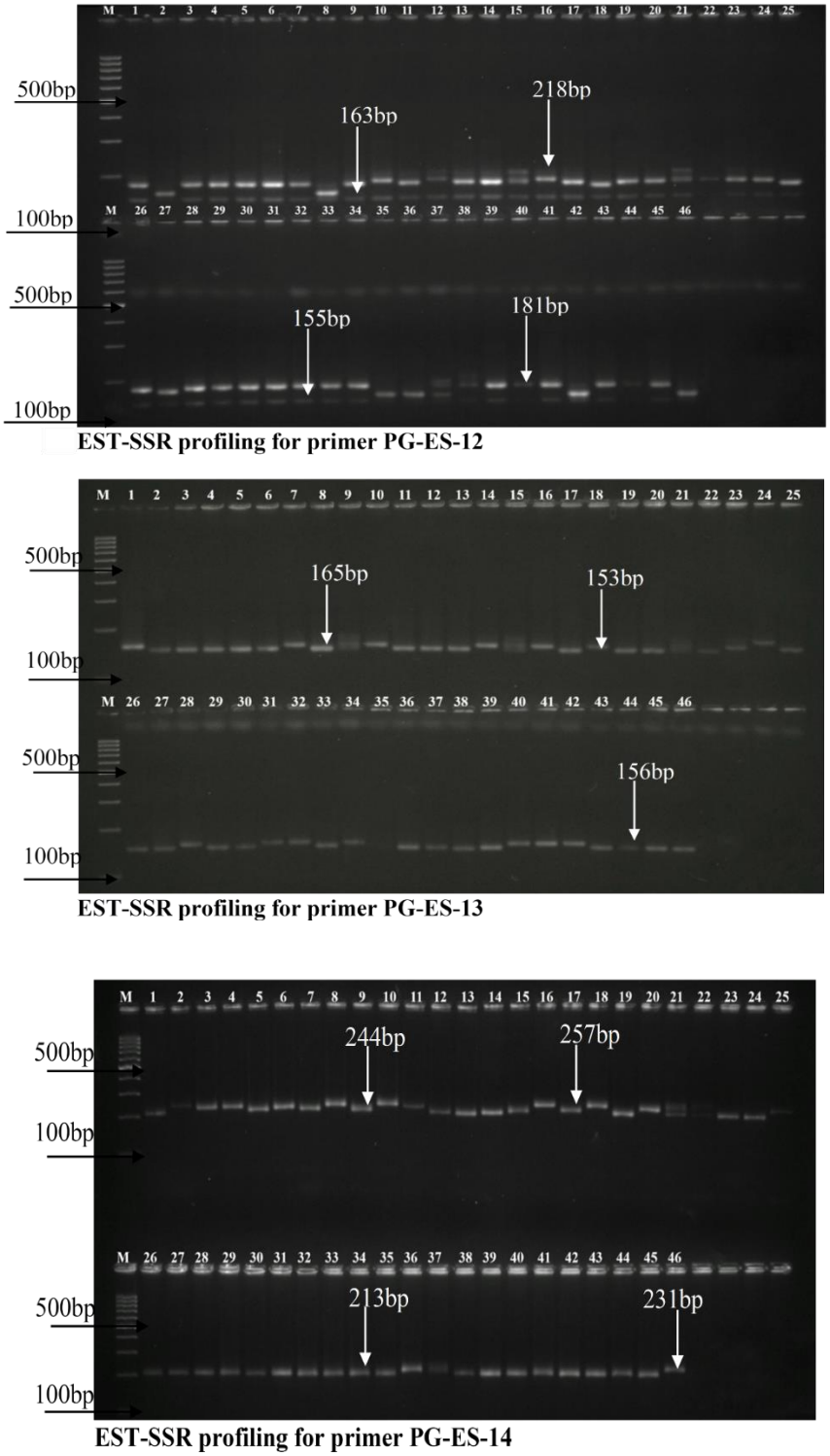

Fig. 2. Electrophoretic profile of PG-ES-12, PG-ES-13 and PG-ES-14 markers

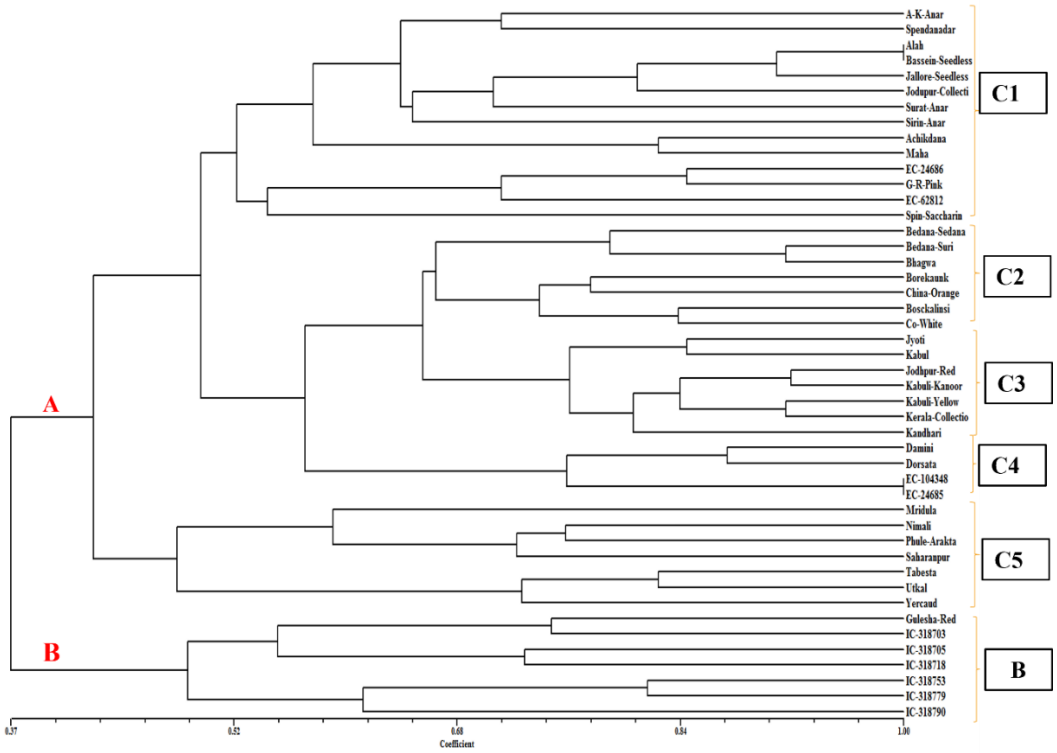

Fig. 3. Dendrogram depicting the genetic relationships among pomegranate germplasm obtained from EST-SSR and SSR markers

3.3 Validation of EST-SSRs and Genomic SSR Markers

The EST-SSR markers were selected on the basis of functionally annotated sequences harboring SSR motifs. Out of the total 1,152 unigenes (harboring 199 SSR motifs) subjected for primer designing, 124 successful primer pairs (70.86%) were designed from 175 sequences with stringent criteria of selection. On the basis of unigene function, randomly 32 primer pairs were selected and are listed in Table S2. A total of 32 EST-SSR markers were validated on 46 germplasm of pomegranate for amplification using touchdown PCR. Out of which only 8 primers were found to be polymorphic and in three primers, non-specific amplification was found showing primer dimers and immense number of null alleles. PCR amplification of genomic DNA of 46 germplasm of pomegranate, using eight EST-SSR primers generated 433 scorable bands with average of 54 bands per primer. The size of the bands ranged from 142 bp to 312 bp. On an average, 2.5 alleles were generated per primer. The highest PIC (0.555) and Heterozygosity (0.625) was exhibited by primer PG-ES-24, while the lowest PIC (0.178) and Heterozygosity (0.198) was exhibited by PG-ES-29. The details of amplification products are given in the Table 5.

Out of 20 primers only 13 primers were found to be polymorphic and other seven primers did not produce any result. PCR amplification of genomic DNA of 46 germplasm of pomegranate, using 13 SSR primers generated 627 scorable bands with average of 48 bands per primer. The size of the bands ranged from 105 bp to 364 bp. On an average, 2 alleles were generated per primer. The SSR primers tested in present investigation produced fragments of different size. The highest PIC (0.374) and Heterozygosity (0.498) was exhibited by primer PG-SSR-16, while the lowest PIC (0.175) and Heterozygosity (0.194) was exhibited by PG-SSR-15. The details of amplification products are given in the Table 6 and SSR primers (PG-ES-12, PG-ES-13 and PG-ES-14) showing polymorphism among 46 pomegranate germplasms are represented in Fig. 2.

The similarity value for all the 46 populations based on combined data ranged from 0.37 to 1.00. The UPGMA tree grouped the population into major two cluster, cluster A and cluster B regardless of their geographical origin while

some accessions from the same region clustered together (Fig. 3).

4. DISCUSSION

In recent years, different kinds of molecular markers have been used widely, including marker-assisted breeding, study of genetic relationships between populations, and screening candidate genes associated with the target traits (Gupta *et al.,* 2004). The simple sequence repeats (SSRs) are increasingly important due to their high polymorphism and convenient techniques. However, EST-SSRs are superior to genomic SSRs for their transcriptional sequence and suitable application in cross-species (Mian *et al.,* 2005). In the present study, we found the average frequency of one SSR per 4.25 Kb, while Ravishankar *et al*., (2015) have estimated the average frequency of one SSR for every 5.56 Kb and Patil *et al.,* (2020) found 527.97 Mb.

We have also analyzed the distribution and frequency of SSR motifs of 2-6 bp and we observed that the SSR frequency decreased with increase in number of repeat units. The distribution of SSR motifs in different repeat type classes highlighted di-nucleotide repeats as the highest 59.8% followed by tri-nucleotide 37.7%, tetra-nucleotide 3% and penta-nucleotide and hexa-nucleotide repeats were found to be 0.5%, respectably. Patil *et al.* (2020) also found the di-nucleotide as the highest repeats and interestingly the also found penta- and hexa-nucleotide repeats in nearly equal proportions but their results were not alike as they found di-nucleotide followed by tetra-, tri-, hexa- and penta-nucleotides. Ravishankar *et al.* (2015) found similar results that number of di-nucleotides (73.33%) was higher than that for tri-nucleotides (12.52%) in pomegranate. The di-nucleotide repeat class AG/CT (66.4%) was found most abundant followed by AT/AT (20.2%) and AC/GT (13.4%). The results obtained were different than earlier studies performed by Patil *et al.* (2020) they reported AT/AT as highest followed by AG/CT. While, Ravishankar *et al.* (2015) also obtained AT/AT as highest followed by GC/CG and TG/GT motifs. The tri-nucleotide code AAG/CTT was followed by AGG/CCT (21.3%), CCG/CGG (12.0%), and ATC/ATG (10.7%). The results obtained were distinguishable than earlier studies in pomegranate in which Patil *et al.* (2020) found AAT/ATT as highest followed by AAG/CTT. The reason behind this difference can be attributed because the parameters of SSR motif identification also have a role in the distribution of SSR, difference in the number of a particular motif directly reflects on its contribution to the total SSR identified in different studies. The reason behind this difference can be attributed because the parameters of SSR motif identification also have a role in the distribution of SSR, difference in the number of a particular motif directly reflects on its contribution to the total SSR identified in different studies. This abundance is dependent on factors like SSR search criteria, size of the dataset, database mining tools and the EST sequence redundancy (Varshney et al. 2005[a,b]; wang et al. 2014).

We successfully validated a set of 52 (32 EST-SSR and 20 SSR) primers out of which 21 (8 EST-SSR and 13 SSR) could reveal polymorphism among 46 pomegranate genotypes. The allele size (bp) obtained across pomegranate genotypes were similar to the expected sizes of the products for each locus.

Investigation of genetic variation in germplasm is key to hastening genetic improvement of plants. To this end, molecular marker technologies counting SSRs have developed as a promising tool to unearth genetic polymorphism in a given set of genotypes/germplasms. In this framework, structure and cluster analyses are effective means for studying genetic relationships related to germplasm resources (Goossens *et al.,* 2002). The UPGMA tree grouped the population into major two cluster, cluster A and cluster B. Cluster A is further divide into five sub-cluster C1, C2, C3, C4 and C5. Subcluster C1 consists of national variety A K Anar from (Turkey), five exotic cultivar Alah (Iran), Sirin Anar and GR Pink from (Russia), Spendanadar (India) and Spin Saccharin from (India, MPKV, Rahuri), two exotic collection EC-24686 and EC-62812 from (India, Rajasthan), five Indian cultivars Bassein Seedless (India, Karnataka), Jallore Seedless and Jodhpur Collection (India, Rajasthan), Surat Anar (India, Gujarat) and Achikdana from (India, Shrinagar) and Maha. C2 consists of exotic cultivar Bedana Sedana from (Afghanistan), China Orange from (China), Bosckalinsi (Tajikistan), two Indian cultivars Bedana Suri (India, Solapur) and Bhagwa (India), exotic cultivar CO-White (India, Tamil Nadu) and Borekaunk. Whereas, subcluster C3 mostly constituted few cultivars from India and few from Afghanistan, Afghan collection Kabul and three exotic breeding line Kabuli Yellow, Kabuli Kanoor and Kandhari were from (Afghanistan), two

Indian cultivars Kerala collection from (India, Kerala) and Jodhpur Red from (India, Rajasthan) and one commercial variety Jyoti from (India, Karnataka)). Similarly, Patil *et al.,* (2020) also found a cluster showing pomegranate germplasm across India and Afghanistan. Subcluster C4 consists of exotic collection Dorsata, EC-104348 and EC-24685 (India, Jallore, NBPGR) and Damini. The subcluster C5 includes exotic commercial variety Nimali from (Shrilanka), exotic cultivar Tabesta from (Iran) three Indian cultivars Mridula from (India, Maharashtra), Yercaud (India, Tamilnadu) and Saharanpur (India), commercial variety Phule Arakta (India, MPKV, Rahuri) and Utkal from (India, Odissa). Whereas the cluster B exclusively contains wild collection genotypes IC-318703, IC-318705, IC-318718, IC-318753 and IC-318779 from (India, Himachal Pradesh) except exotic cultivar Gulesha Red from (Russia). Similar results were shown by Patil *et al.,* (2020) as Gulesha Red lies in a same cluster along with some wild germplasm of pomegranate.

5. CONCLUSION

The newly developed 124 EST derived SSR markers based on functional annotation of sequences have added to the repository of molecular markers for pomegranate and can also be utilized for transferability in other closely related crops having less genetic resources available. Total 21 out of 52 primers (eight out of 32 EST-SSR and 13 out of 20 genomic SSR) showed polymorphic patterns, and revealed genetic relationships among 46 pomegranate germplasm. The results of cluster analysis revealed grouping patterns based on geological distributions and derivation relationships. Thus, the developed EST derived SSR markers based on functional annotation of sequences could be very useful for various research areas in pomegranate, such as identification of the economically important pomegranate cultivars, study evolutionary origin analysis, genetic linkage map construction and marker-assisted selection for breeding.

DECLARATIONS

By submitting this research paper for publication, all authors consent to its dissemination and acknowledge that it has not been previously published nor is it under consideration elsewhere.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

DATA AVAILABILITY STATEMENT

The raw data available as supplementary file.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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SUPPLEMENTARY

Table S1. Details of pomegranate accessions used for molecular characterization

Sr. No.	Accession	Type	Origin/Source
1.	A K Anar	National Variety	Turkey
2.	Achikdana	-	Shreenagar
3.	Alah	Exotic Cultivar	Iran
4.	Bassein Seedless	Cultivar	India(Karnataka)
5.	Bedana Sedana	Exotic Cultivar	Afghanisthan
6.	Bedana Suri	Indian Cultivar	India(Solapur)
7.	Bhagawa	Indian Cultivar	India
8.	Borekaunk	Exotic Cultivar	-
9.	Bosckalinsi	Tajikistan Collection	Tajikistan
10.	China Orange	China Collection	China
11.	Co-White	Exotic Variety	India (Tamil Nadu)
12.	Damini	Variety	
13.	Dorsata	-	NBPGR
14.	EC-104348	Exotic Collection	Jalore, NBPGR
15.	EC-24685	Exotic Collection	Jalore, NBPGR
16.	EC-24686	Exotic Collection	Jalore, NBPGR
17.	EC-62812	Exotic Collection	Jalore, NBPGR
18.	G R Pink	Exotic Cultivar	Russia
19.	Gulesha Red	Exotic Cultivar	Russia
20.	IC-318703	Wild Collection	India (Himachal Pradesh)
21.	IC-318705	Wild Collection	India (Himachal Pradesh)
22.	IC-318718	Wild Collection	India (Himachal Pradesh)
23.	IC-318753	Wild Collection	India (Himachal Pradesh)
24.	IC-318779	Wild Collection	India (Himachal Pradesh)
25.	IC-318790	Wild Collection	India (Himachal Pradesh)
26.	Jallore Seedless	Cultivar	India (Rajasthan)
27.	Jodhpur Collection	Cultivar	India (Rajasthan)
28.	Jodhpur Red	Cultivar	India (Rajasthan)
29.	Jyoti	Commercial Variety	India (Karnataka)
30.	Kabul	Afghan Collection	Afghanistan
31.	Kabuli Kanoor	Exotic Breeding Line	Afghanistan
32.	Kabuli Yellow	Exotic Breeding Line	Afghanistan
33.	Kandhari	Exotic Breeding Line	Afghanistan
34.	Kerala Collection	Indian Cultivar	India (Kerala)
35.	Maha	Variety	-
36.	Mridula	Indian Collection	India (Maharashtra)
37.	Nimali	Exotic Commercial Variety	Shri Lanka
38.	Phule Arakta	Commercial Variety	India (MPKV, Rahuri)
39.	Saharanpur	Indian Cultivar	India
40.	Sirin Anar	Exotic Cultivar	Russia
41.	Spendanadar	Exotic Cultivar	India
42.	Spin Saccharin	Exotic Cultivar	India (MPKV, Rahuri)
43.	Surat Anar	Cultivar	India (Gujarat)
44.	Tabesta	Exotic Cultivar	Iran
45.	Utkal	Variety	India (Odissa)
46.	Yercaud	Cultivar	India (Tamil Nadu)

Table S2. Detailed list of 32 EST-SSR primers

Sr. No.	Sequence ID	Function	Primer Name		Sequence (5' to 3')	Tm	Motif length	Product size ~ Bp
1	CONTIG103	protein translation factor SUI1 homolog 2	PG-ES-1	F	CCTACCTAGAGAGAGAGAGAGGC	59.847	(AG)6	297
				R	GGACATACTCCTTTGTTCCAGC	60.004		
2	CONTIG117	gibberellin-regulated protein 1-like	PG-ES-2	F	GACAAAAGACAGGACACTCCATT	59.538	(TTA)5	377
				R	GCTAGGTGCCAGTTATCATCG	59.744		
3	CONTIG171	MADS-box transcription factor 15	PG-ES-3	F	CCTTATGCTTGAGTCCATTTCC	59.966	(CTC)5	239
				R	TATTTTGTGGTGGAAGAAGTGC	59.134		
4	CONTIG247	phosphoprotein ECPP44	PG-ES-4	F	ATCATCGTCTACTCATCGGAGC	60.628	(GAG)5	394
				R	CCTCCTCGTGTTTCTCCTTCTT	61.119		
5	CONTIG54	cold and drought-regulated protein CORA-like	PG-ES-5	F	TCATAGGAGTGATGGAAGTTGG	59.052	(TG)7	220
				R	GGACATAATTCTCGGATTTTCG	59.808		
6	CONTIG96	glycine-rich cell wall structural protein 2-like	PG-ES-6	F	GGAAAGGGCTATTGTTTGACTG	60.001	(AT)13	135
				R	CTAGCAGGGCTCCTCTTATTCA	60.001		
7	CONTIG98	universal stress protein A-like protein	PG-ES-7	F	GGACTCGGAAGAATTAAAAGGG	60.295	(TG)7	331
				R	CCGCATCTGACTGAAAGTGAT	60.27		
8	JG771255.1	agamous-like MADS-box protein AGL1	PG-ES-8	F	AACTTCTGGTGTCTCTTCCACC	59.645	(AG)18	233
				R	CTTCCTTCCGGTAAATCTCTTG	59.26		
9	JG771277.1	CBL-interacting serine/threonine-protein kinase 6	PG-ES-9	F	CACGCTGGAGTACAATCAGTTC	59.803	(GTTG)6	288
				R	TAATTGAGAGACCCACCAGAGC	60.63		
10	JG771376.1	auxin-responsive protein SAUR32	PG-ES-10	F	TCAATCAAGCCAACAGCTAGAA	60.018	(TGG)8	386
				R	CGACTATATCAATCACCGGCTC	60.84		
11	JG771408.1	ribulose-1,5-bisphosphate carboxylase small subunit	PG-ES-11	F	AGTGCATGAAGGTACACACCAC	59.96	(AGCT)5	395
				R	GTATGTGGATATTGACCCCGAC	60.327		
12	JG771444.1	transcription repressor MYB4-like isoform X1	PG-ES-12	F	TCCCGAGAAAGTTGCATATCTA	58.854	(AG)18	191
				R	ATTTTGTCCTCAAGAGCAGTCC	59.757		
13	JG771472.1	zinc finger A20 and AN1 domain-containing stress-associated protein 8	PG-ES-13	F	ATAATAAATCGCATCCCTCCGT	60.86	(TC)13	174
				R	GTGTTCCATTTTCTCGATCCTC	59.947		

Sr. No.	Sequence ID	Function	Primer Name		Sequence (5' to 3')	Tm	Motif length	Product size ~ Bp
14	JG771677.1	Fasciclin-like arabinogalactan protein 18	PG-ES-14	F	AGATCGTAATGTTGTGCTTCCC	60.381	(GAA)6	206
				R	CATCTCCTTTTCTTTCGTCCAC	60.11		
15	JG771772.1	cysteine synthase, chloroplastic/chromoplastic-like	PG-ES-15	F	CGAAGAAGAAAGAATCAGTCGG	60.365	(CT)10	288
				R	CTTTGCAGACCACATAGAAGCC	61.162		
16	JG771931.1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3-B	PG-ES-16	F	ACGGCAAACTAAAGAAGCAAAC	59.834	(TGG)5	335
				R	AGGGAGAGAGAGAGAGATCGGT	59.981		
17	JG771964.1	late embryogenesis abundant protein At5g17165	PG-ES-17	F	CGCTGGTCACACTACTTACTCG	59.995	(AT)12	341
				R	GTTCCGTCCCACTAGCATAGA	59.208		
18	JZ122382.1	FCS-Like Zinc finger 15-like	PG-ES-18	F	GAGTGGTTCTCGCAAGTTCAG	60.043	(TC)8	357
				R	ATAGGCCGTAGCCTTTACATGA	60.01		
19	JZ122495.1	organic cation/carnitine transporter 7	PG-ES-19	F	GCGTTAAATTGTTAGTCTTTGTCCC	61.346	(GTG)6	348
				R	GGCTTTGTTCCTGAGCAGATAG	60.393		
20	JZ122556.1	probable WRKY transcription factor 75	PG-ES-20	F	ATCATCTCCTCCTTTCATGGC	60.424	(CT)6	397
				R	CTTCTGTCCGTACTTCCTCCAG	60.294		
21	JZ122558.1	B-box zinc finger protein 24-like	PG-ES-21	F	GTTCGGAGAGCTAGAGTGGCTA	60.172	(GAT)6	279
				R	CCAATGCGAGATAGAGTAAGGG	60.109		
22	JZ122607.1	E2F-associated phosphoprotein	PG-ES-22	F	CTGCTGCTCAGTTTGCTGTACT	59.886	(TA)16	364
				R	CATCACGGTATTTGGACAACAC	60.154		
23	JZ122718.1	homeobox-leucine zipper protein HOX11	PG-ES-23	F	AAATCTCTCTTGTTGCCCCTC	59.705	(TA)7	301
				R	ATTACCTCTTTGCCTTCCTGCT	60.608		
24	JZ122970.1	FCS-Like Zinc finger 15-like	PG-ES-24	F	TTCCATCCGTCAACTAACCTCT	59.998	(AG)21	142
				R	AACCCACCATCTCTCACTCACT	60.036		
25	JZ123039.1	WAT1-related protein At5g47470	PG-ES-25	F	ATTGCTTGGGCAGTTAGATTTG	60.489	(CTT)5	252
				R	CTGTAAGACGACATTGCTGGAG	59.936		
26	JZ123108.1	fasciclin-like arabinogalactan protein 2	PG-ES-26	F	AAGTGACGTTGAAGACGAAGGT	60.209	(GAA)5	387
				R	GTCTGAGCAATTACACCCGATT	60.381		
27	JZ123458.1	outer envelope pore protein 16-2, chloroplastic	PG-ES-27	F	TGATTCTGCTTCAGTTCGAGAG	59.757	(CTC)6	380
				R	AGAGTTTCTGTTGGTTTCCCCT	60.381		
28	JZ123542.1	ethylene-responsive	PG-ES-28	F	AAGAGTGTAGCGTCATCAGTGG	59.425	(TA)13	282

Sr. No.	Sequence ID	Function	Primer Name		Sequence (5' to 3')	Tm	Motif length	Product size ~ Bp
		transcription factor ERF039-like		R	GACAAGAATCCTAAGCCCTTCA	59.725		
29	JZ123587.1	zinc finger A20 and AN1 domain-containing stress-associated protein 8	PG-ES-29	F	ATAATAAATCGCATCCCTCCGT	60.86	(TC)12	209
				R	GTGTTCCATTTTCTCGATCCTC	59.947		
30	JZ123818.1	calcium-binding protein PBP1-like	PG-ES-30	F	CTCAGAAGCCTGAAGAGGAACT	59.273	(AT)12	321
				R	AGCAGGATACATGGCTCAATTA	58.744		
31	JZ123822.1	50S ribosomal protein L21, chloroplastic	PG-ES-31	F	GCTCGCTCTCTTTCTCTCTCTC	59.258	(GAG)6	275
				R	CTAACGGTGGGTTCTTCTGTTT	59.56		
32	JZ123848.1	glycine-rich RNA-binding protein-like	PG-ES-32	F	GTCACCTTCAGCAACGAGAAGT	60.857	(GCG)5	379
				R	CCCACCACCAGAGAGTAGAGAC	60.17		

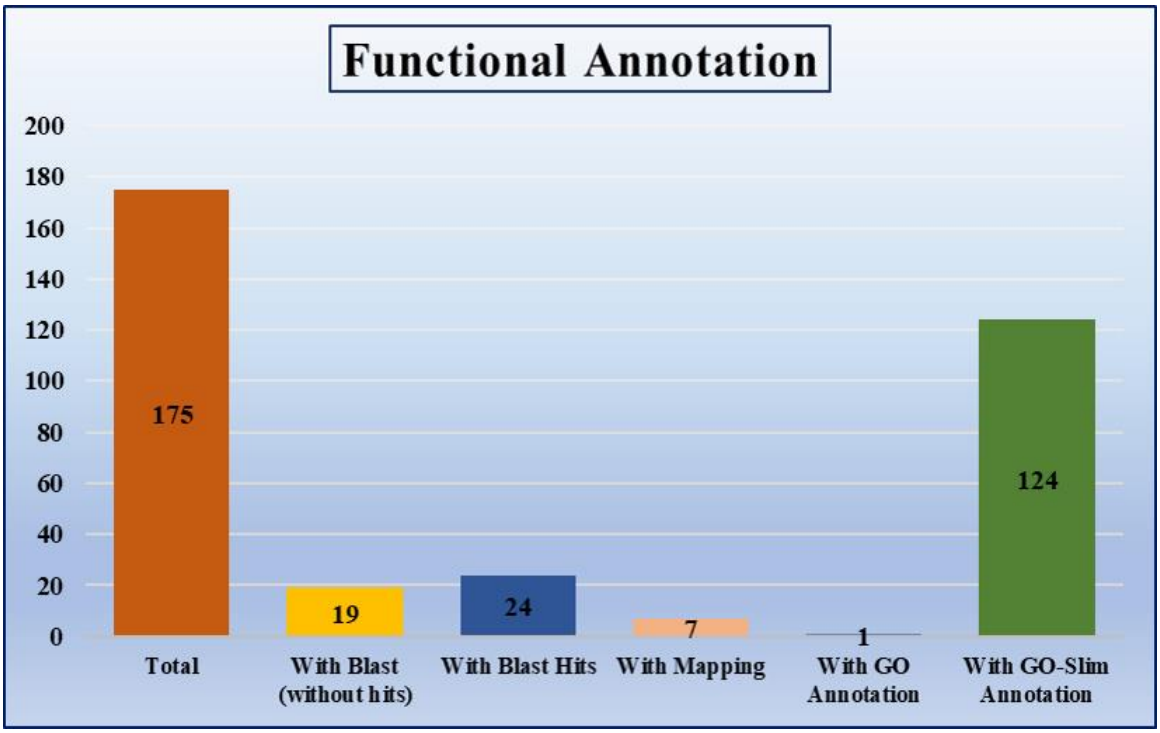

Fig. S1. Blast hit distribution of pomegranate EST-SSR containing sequences

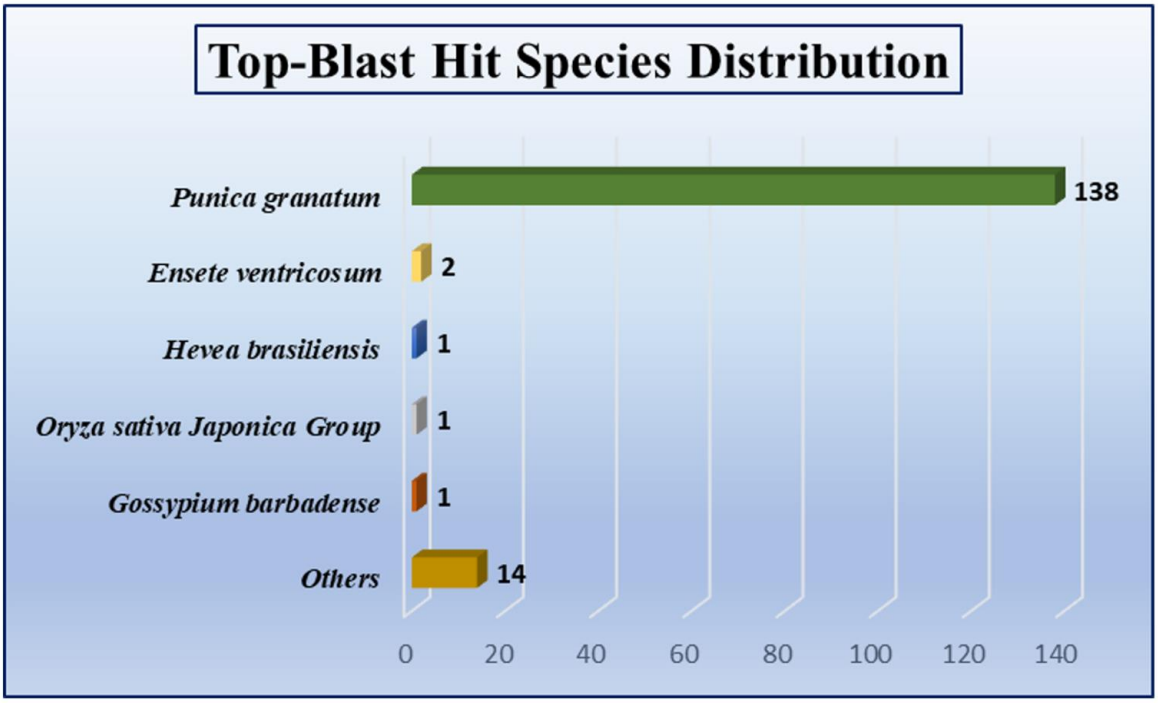

Fig. S2. Top blast hit distribution of pomegranate EST-SSR containing sequences

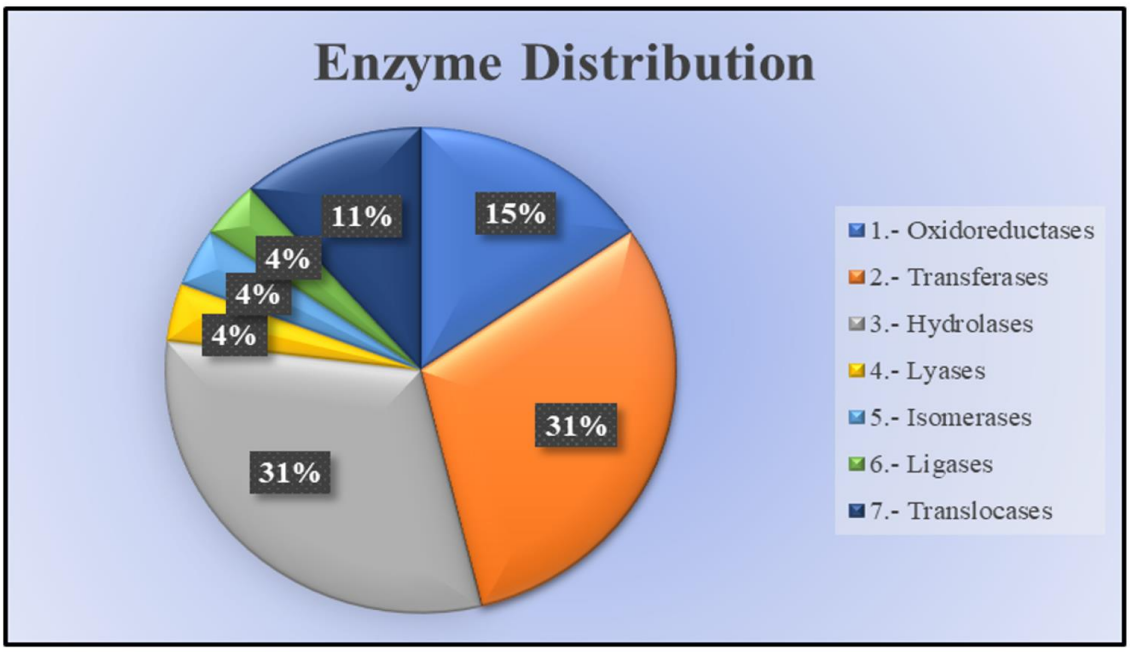

Fig. S3. Enzyme classification of EST-SSRs using KEGG

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