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***Research Paper***

# Assessment of Genetic Diversity in *Rubusellipticus* (Smith) Using Molecular Markers

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Genetic relationship among 21 *Rubusellipticus* accessions, collected from different sites in Himachal Pradesh and Uttrakhand, was evaluated by using 20 Expressed sequence tag-Simple sequence repeats and 35 Inter simple sequence repeats. The ESR-SSRs were designed by extracting seven EST sequences from *Rubusellipticus* and 3184 sequences from other species of Rubus, such as; *Rubusulmifolius* and *Rubusideaus* obtained from national center for biotechnology information. The ESTs containing simple sequence repeat motifs were extracted using SSR Identification Tool and 20 primers were designed with primer 3 software. These EST-SSR primers generated 40 polymorphic bands, whereas ISSRs generated 173 polymorphic bands. Polymorphic information content (PIC) for ISSRs was higher (0.886) than that of EST-SSRs (0.667). Effective multiplex ratio and marker index values for EST-SSRs were 1.09 and 0.62 and, for ISSRs, these values were 8.24 and 5.83, respectively, which were used to develop dendrograms. With EST-SSR two cluster 'A' contained 19 collections, whereas cluster 'B' contained two collections with maximum similarity of 67%. For ISSRs, 21 collections were divided into two clusters, i.e., cluster 'C', which contained six collections and cluster 'D' that contained 15 collections with maximum similarity of 56%. Thus EST-SSRs and ISSRs used in the present study revealed a high level of polymorphism in the 21 collections of *Rubusellipticus*, revealing their efficiency for diversity analysis studies.

**Keywords: *Rubusellipticus*; Molecular Marker; Primer Designing; Germplasm Collection; Genetic Diversity**

## Introduction

*Rubus,* known as 'Yellow Himalayan Raspberry', is one of the most diverse genera in the plant kingdom, with more than 500 species. It is medicinally an important wild fruit because it is a source of anthocyanins, catechins, flavonols, flavones, ascorbic acid and tannins (Jenning, 1988; Saklani *et al*., 2012). However, breeding in *Rubusellipticus* is time-consuming because of the highly heterozygous nature of this perennial fruit crop. To promote the cultivation of this important wild fruit, accurate identification of germplasm is a pre-requisite. Recent advances in molecular biology have provided us with novel tools to establish evolutionary and genetic relationships among plants of research interest, which was a cumbersome job earlier. Genetic diversity analysis can be done by observing variations in DNA sequence using molecular markers with high accuracy and throughput which is often not visible at the phenotypic level. Genetic diversity studied via molecular markers can be efficiently used to identify the taxonomic and phylogenetic relationships among cultivars for pedigree analysis and linkage mapping (William *et al*., 1990). One of the important molecular-marker technique is use of simple sequence repeats (SSRs) or microsatellites. Microsatellites are tandemly repeated DNA sequences and variation in the number of repeat motifs is revealed by polymerase chain reaction (PCR) using oligonucleotide primers specific

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to the unique DNA sequences flanking the SSR sequence. The SSR markers are being frequently used for genetic diversity studies across different species and populations (Powell *et al*., 1996). The SSR markers have been successfully developed by mining (Dossett *et al*., 2009) expressed sequence tag (EST) databases in several crops. These markers are derived from the most conserved regions of genome, so they are well suited for application in phylogenetic analysis and comparative genome mapping (Wen *et al*., 2010).Inter-simple sequence repeat (ISSR) are other molecular markers used for genetic mapping and population studies because of their abundance and high degree of polymorphism between individuals of a population of closely related genotypes (Singh *et al*., 2011). The SSRs and ISSRs have become quite useful in various types of molecular genetic studies, including assessment of genetic diversity (Amsellem *et al*., 2001; Ashley *et al*., 2003; Vaidya *et al*., 2012; Vaidya *et al*., 2015; Kaur *et al*., 2015), fingerprinting (Rongwen *et al*., 1995), marker-assisted selection (Fazio *et al*., 2003) and genetic linkage mapping (Bushkara *et al*., 2015). Keeping the usefulness of molecular markers for diversity analysis and need to characterized the *Rubus* germplasm, present study is aimed at precise assessment of genetic diversity among various collections of *Rubusellipticus.*

## Materials and Methods

### *Source of Plant Material*

Leaves of *Rubusellipticus* (Table 1) were taken from 21 collections maintained in the experimental fields of Department of Fruit Science, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan. The leaves were stored at –80ºCuntil needed for further use.

### *DNA Extraction*

Cetyltrimethylammonium bromide (CTAB) method was used to isolate DNA (Doyle *et al*., 1987). A2g sample of fresh leaves was ground in liquid nitrogen

**Table 1: List of *Rubusellipticus* collections used in the study**

| S.No. | Accessions | Collection site" HP | Altitude | Latitude | Longitude |
|---|---|---|---|---|---|
| 1. | Badhu-2 | Badhu | 1813 | 31º29'535" | 77º00580" |
| 2. | Kumarhatti-1 | Kumarhatti | 1590 | 30º53'431" | 77º03'127" |
| 3. | Kaithlighat-3 | Kaithlighat | 1698 | 31º01'220" | 77º06'170" |
| 4. | Khadiyana | Khadiyana | 1328 | 30º56'356" | 77º01'589" |
| 5. | Kharkog-2 | Kharkog | 1760 | 31º27'277" | 76º94'373" |
| 6. | Kukarigalu-2 | Kukarigalu | 1764 | 31º31'518" | 76º59'648" |
| 7. | Kandaghat-8 | Kandaghat | 1415 | 30º58'154" | 77º06'334" |
| 8. | Hiranagar-2 | Hiranagar | 1805 | 31º07'119" | 77º06'104" |
| 9. | Kharkog-1 | Kharkog | 1760 | 31º27'277" | 76º94'373" |
| 10. | Shoghi – 5 | Shoghi | 1804 | 31º02'519" | 77º07'631" |
| 11. | Kandaghat-1 | Kandaghat | 1415 | 30º58'154" | 77º06'334" |
| 12. | Baurgaon-1 | Baurgaon | 1228 | 21º76'790" | 78º87'180" |
| 13. | Hiranagar-1 | Hiranagar | 1805 | 31º07'119" | 77º06'104" |
| 14. | Bhim-boot-1 | Bhim-boot | 1330 | 30º56'276" | 77º01'641" |
| 15. | Nagangi | Nagangi | 1760 | 31º27'277" | 76º94'373" |
| 16. | Deothi -1 | Deothi | 1390 | 30º56' 447" | 77º02'300" |
| 17. | Badashahithul-1 | Badashahithul | 5108 | 34º14' 472" | 74º61'670" |
| 18. | Sanarali-2 | Sanarali | 1650 | 31º20' 265" | 77º11'130" |
| 19. | Guldi-1 | Guldi | 1478 | 30º19' 260" | 78º04'81" |
| 20. | Sarali-3 | Sarali | 1610 | 32º17' 549" | 76º06'986" |
| 21. | Majhgaon | Majhgaon | 1482 | 30º53' 115'' | 77º07' 081" |

and incubated for 30 min at 65ºC in extraction buffer (10% CTAB; 1M TrisHCl pH 8.0; 4M NaCl; 0.5M EDTA; 2% PVP; 0.2% β-mercaptoethanol). Nucleic acids were purified with chloroform/isoamyl alcohol (24:1) and phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with isopropanol by using a centrifuge and dissolved in the TrisHCl-EDTA buffer. The DNA concentration was determined spectrophotometrically at 260 nm. Before conducting further analyses, the DNA was diluted to10 ng/µl.

***Search for dbEST-SSRs Through Datamining***

The EST sequences of *Rubusellipticus* were screened *in silico* from EST database available on NCBI website (www.ncbi.nih.gov/nucest). The obtained sequences were converted into Fast adaptive shrinkage thresholding algorithm (FASTA) format. The SSR Identification Tool (SSRIT) (Temnykh *et al*., 2001) was used to identify EST sequences containing SSR motifs. Software PRIMER3 was used to design SSR primers (Rozen *et al*., 2000). The designed primers were then custom-synthesized.

***Amplification and Electrophoresis***

A set of 35 ISSR and 20 EST-SSR markers were used to analyze 21 *Rubusellipticus* collections. The PCR protocol was standardized for carrying out amplification using ISSR and SSR primersin 20 µl of reaction mixture which consisted of 1X Taq buffer A (with 75mM $MgCl_2$), 1mM dNTP, 10 picomoles primer (forward and reverse in case of SSRs), 1U Taq DNA polymerase, 50ng DNA.

To carry out PCR amplification with ISSR primer, the following protocol was used: Initial denaturation at 94ºC for 2min, followed by 40 cycles repeat of denaturation at 94ºC for 10sec, annealing according to primer Tm for 30sec, extension at 72ºC for 65sec. followed by final extension at 72ºC for 10 min (Sharma *et al*., 2016). In the case of EST-SSR, thermal profile used was as follows: initial denaturation at 95ºC for 5min, followed by 40 cycles repeat of denaturation at 94ºC for 1min, annealing according to primer Tm for 1min, extension at 72ºC for 2min and final extension at 72ºC for 5min (Vaidya *et al*., 2015).

The amplified DNA was mixed thoroughly with 6X loading dye and then electrophoresed in 1.2% agarose gel for ISSRs and 2.0% agarose gel for EST-SSRs. The gel was run at constant voltage at the rate of 5V/cm under submerged conditions for about two hours. Ethidium bromide at the rate of 0.5 µg/ml was incorporated in the gel. Stock solution of ethidium bromide @ 10 mg/ml was kept ready before hand. DNA profiles visualized on UV Transilluminator and photographs were taken on Gel Documentation System (Syngene, Cambridge, UK).

***Effective Multiplex Ratio (E)***

Calculated for a marker system, effective multiplex ratio denotes the number of polymorphic loci in the germplasm (Varshney *et al*., 2007). The following formula was used to compute E:

$$E = n\beta$$

where. β is the fraction of polymorphic markers and is estimated after considering the polymorphic loci (np) and non-polymorphic loci (nnp) as β = np/(np + nnp) and n is the multiplex ratio, measured as the average number of DNA fragments amplified/ detected per genotype using a marker system.

***Marker Index (MI)***

The utility of a given marker system is a balance between the level of polymorphism detected and the extent to which an assay can identify multiple polymorphisms. Marker index provides a convenient estimate of any marker system utility (Varshney *et al*., 2007) and was estimated as follows:

$$MI = PIC \times E$$

where, PIC = polymorphic information content and E = effective multiplex ratio, as defined above.

***Data Analysis***

Gel images for the two marker systems were transformed into a binary matrix. Software NTSYSpc ver. 2.02h (Rohlf, 2000) was used to study the genetic diversity among *R. ellipticus* collections by separately analyzing ISSR and EST-SSR data. NTSYSpc included the construction of similarity matrices using the Jaccard's coefficient (Jaccard, 1908) based on SIMQUAL function and a dendogram was constructed by using the unweighted pair group method, deploying the arithmetic means (UPGMA) (Sneath *et al*., 1973) based on SAHN (Sequential Agglomerative Hierarchical Nesting) function of NTSYSpc. However, dissimilarity matrices were

constructed by using UPGMA function of DARwin5. The polymorphism information content (PIC) values, which provide an estimate of the discriminative power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of those alleles in the genotypes, were calculated using the below-given formula:

$$PIC = 1-\Sigma pi^2,$$

where, *pi* is the frequency of the *i*th allele (Smith *et al*., 1997).

## Results and Discussion

The important objective of crop improvement programs is the identification of variability among the available genotypes. Diversity analysis at the molecular level using PCR based markers is the cheapest and the most rapid method of characterizing the relationships among different genotypes. Among various DNA based markers ISSRs and EST-SSRs widely used markers systems (Ganopoulos *et al*., 2011). Development of SSR markers from EST databases represents a potentially valuable resource for evolutionary studies because it saves both cost and time since no laborious and time consuming efforts are needed. The EST sequences of *R. ellipticus*/ *Rubus* species were obtained from the NCBI website (www.ncbi.nlm.nih.gov/nucest) in FASTA format. The SSR Identification Tool (SSRIT) was used to screen SSR motifs in the EST sequences with maximum motif length of six and minimum number of three repeats was taken as parameters.

A total of 946 EST sequences of *Rubus* containing SSR motifs with trinucleotide or above repeats were detected with the help of SSRIT. Table 2 reveals that 745, 125, 31 and 45 repeats were recorded to be tri-, tetra-, penta- and hexa-nucleotide repeats, respectively. Rest of the sequences ofESTs contained either mono- or dinucleotide repeat motifs. The EST sequences containing only trinucleotide repeats or above were then selected for further studies (Table 3). Only seven EST sequences of *R. ellipticus* were obtained from dbEST but none of the sequences contained SSRs. So ESTs of *R. idaeus* and *R. ulmifolius* were used for primer designing. Primers were designed by using online interface of PRIMER 3 software.

All of the EST-SSR and ISSR primers were found to be polymorphic with 40 and 173 polymorphic scorable bands respectively (Tables 4 and 5). Total amplified fragments with EST-SSRs and ISSRs were 267 and 866 with an average number of polymorphic bands per primer of 2 and 5.3, respectively (Table 6; Table 7). The results obtained were further analyzed following computer software NTSYSpc ver. 2.02h. On the basis of Jaccard's coefficient matrix values of EST-SSRs similarity coefficient ranged from 0 to 0.667 and 0.015 to 0.564 for ISSR. The highest value of similarity (0.667), was between G1 (Badhu-2) and G4 (Khadiana), which were collected from Mandi and Chamba district of Himachal Pradesh respectively. Minimum similarity of zero was obtained between different pairs of collections. The highest value of similarity for ISSR (0.564) was between G9 (Kharkog-1) and G15 (Nagangi), both were collected from Nauni. Minimum similarity (0.171) was obtained between G6 (Kukorigolu-2) and G19 (Guldi-1).

Dendrograms were constructed for EST-SSRs and ISSR which separated 21 collections of *Rubusellipticus* into different groups. In the EST-SSR dendrogram, the 21 *Rubus* collections were separated into two main clusters, '**A**' and '**B**' (Fig. 1). Cluster A was found to contain 19 genotypes, viz., 'Badhu-2','Khadiana', 'Heera nagar-2', 'Kumarhatti-1','Baurgaon-1', 'Deothi -1', 'Kharkog-2', 'HeeraNagar-1', 'Kandaghat-8', 'Kharkog-1', 'Nagangi', 'Saroli-3', 'Badashahithul-1', 'Majhgaon', 'Shoghi-5', 'Guldi-1', 'Sanarali-2', 'Kandaghat-1', and 'Bhim-boot-1' whereas cluster B contained two genotypes, viz., 'Kaithlee ghat-3', and 'Kukorigolu-

**Table 2: Summary of results of SSRIT for EST-SSRs for *Rubus* species**

| Crop species | No. of EST sequences screened | No. of SSR containing sequences | No. of trinucleotide containing sequences | No. of tetranucleotide SSR containing sequences | No. of pentanucleotide SSR containing sequences | No. of hexanucleotide SSR containing sequences |
|---|---|---|---|---|---|---|
| *R. idaeus* | 3191 | 1978 | 745 | 125 | 31 | 45 |

**Table 3: List of SSR sequences detected by SSRIT in ESTs of *Rubus idaeus* and *Rubus ulmifolius***

| S.No. | Sequence Id | Crop species | SSR | SSR size | SSR start position | SSRE end position |
|---|---|---|---|---|---|---|
| 1. | gi\|192387345\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (accaca)$_4$* | 24 | 243 | 266 |
| 2. | gi\|192389107\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (cttcct)$^3$ | 18 | 121 | 138 |
| 3. | gi\|192386981\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (ccggct)$^3$ | 18 | 390 | 407 |
| 4. | gi\|192385718\| | *R.* var. inermis x *R. thyrsiger* | (tcttcc) $^3$ | 18 | 79 | 96 |
| 5. | gi\|164351943\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (gaat)$^3$ | 12 | 638 | 644 |
| 6. | gi\|111181980\| | *R. idaeus* | (attc)$^3$ | 12 | 17 | 28 |
| 7. | gi\|192386937\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (tcctct)$^5$ | 30 | 5 | 34 |
| 8. | gi\|192386249\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (cccctc)$^4$ | 24 | 307 | 330 |
| 9. | gi\|192389147\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (gcacca)$^3$ | 18 | 118 | 135 |
| 10. | gi\|192387399\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (atttga)$^3$ | 18 | 268 | 285 |
| 11. | gi\|111182120\| | *R. idaeus* | (gctctt)$^3$ | 18 | 329 | 346 |
| 12. | gi\|111181972\| | *R. idaeus* | (aggtgt)$^3$ | 18 | 453 | 470 |
| 13. | gi\|192386057\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (taatg)$^3$ | 15 | 616 | 630 |
| 14. | gi\|192389165\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (caaa)$^3$ | 12 | 22 | 33 |
| 15. | gi\|192387233\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (ggag)$^3$ | 12 | 540 | 551 |
| 16. | gi\|111181967\| | *R. idaeus* | (ttct)$^3$ | 12 | 630 | 641 |
| 17. | gi\|192387651\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (cct)$^3$ | 9 | 31 | 39 |
| 18. | gi\|192387597\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (ccg)$^3$ | 9 | 56 | 64 |
| 19. | gi\|192387425\| | *R. ulmifolius* var. inermis x *R. thyrsiger* | (acg)$^3$ | 9 | 26 | 34 |
| 20. | gi\|192387279\| | *R. ulmifolius* var. inermis x *R thyrsiger* | (gac)$^3$ | 9 | 83 | 91 |

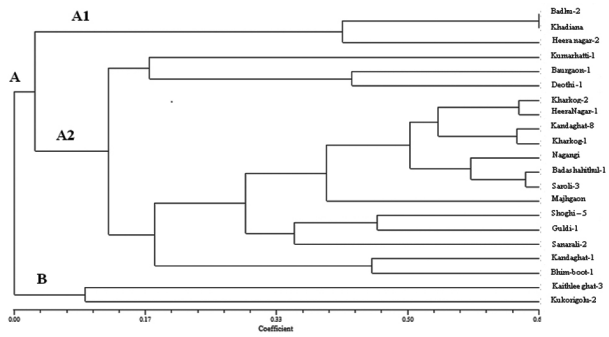


**Fig. 1: Dendrogram of 21 *Rubus* species based on EST-SSR analysis**

2'. Cluster A was further divided into two sub-clusters, i.e., A1 and A2, at a similarity value of 3%. This revealed very less similarity between sub clusters 'A1'and 'A2'because it contained collections from different sites and cluster B ('Kaithlee ghat-3' and, 'Kukorigolu-2') was totally different from rest of 19 accessions. Cluster A1 included 'Badhu-2', 'khadiana' and the remainder 17 accessions belonged to sub-cluster A2. Sub-cluster A2 was further subdivided into 'A21' and 'A22' at 10% similarity. A21 and A22 were further subdivided at 19% similarity. Highest similarity of 67% was found between 'Badhu-2' and 'Khadiana' showing that these two were relatively closely related.

The 21 accessions of *Rubusellipticus* were divided into two main clusters, 'C' and 'D' at 8% similarity using ISSRs (Fig. 2). Cluster C was found to contain six genotypes viz., 'Badhu-2', 'Kukorigolu-

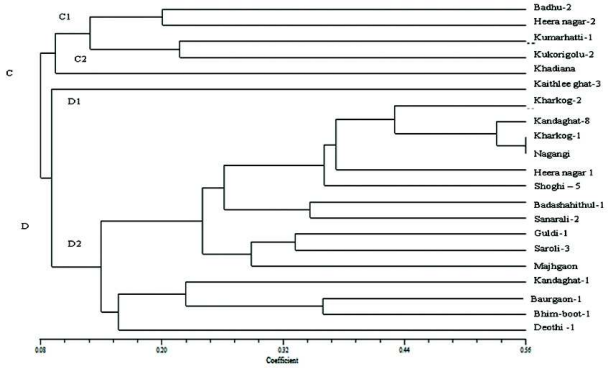


**Fig. 2: Dendrogram of 21 *Rubus* species based on ISSR analysis**

**Table 4: Details of EST-SSR primers of *Rubusidaeus* and *Rubusulmifolius* used for the present study**

| S.No. | Sequence ID | Primer sequence | Tm* (ºC) | GC%** | Length in bp | PIC value | No. of unique bands | Name of the sample | Position of unique band |
|---|---|---|---|---|---|---|---|---|---|
| 1. | gi\|111181967 | **F:**ACGTTCTCCATCGTTTCCTG<br>**R:**AAAACCTCCAGGGTCTGCTT | 60.1160.11 | 5050 | 2020 | 0.55 | 2 | Kharkog<br>2 | 400bp<br>300bp |
| 2 | gi\|111181972 | **F:**CGGAGATCTGAGCAAACACA<br>**R:**GACTCCCAAGGTTAGCACCA | 59.9860.11 | 5055 | 2020 | 0.62 | 0 | - | - |
| 3 | gi\|111181980 | **F:** AGTCCACACACCTCCCAGAC<br>**R:**GGCAGCCTCAAGAAGTGAAG | 60.0160.13 | 6055 | 2020 | 0.593 | 1 | Kandaghat-1 | 400 bp |
| 4 | gi\|111182120 | **F:** GTCGCTCTTGCTCTTGCTCT<br>**R:**ATCCACAAGATCGGAGATGC | 60.0460.04 | 5550 | 2020 | 0.26 | 0 | - | - |
| 5 | gi\|164351943 | **F:** GGCAGCCTCAAGAAGTGAAG<br>**R:** AGTCCACACACCTCCCAGAC | 60.1360.01 | 5560 | 2020 | 0 | 0 | - | - |
| 6 | gi\|192385718 | **F:** CGCACTGTCTCGCTCTACAC<br>**R:** GTAGATCCCGTGCTTGTGGT | 59.8060.00 | 6055 | 2020 | 0 | 0 | - | - |
| 7 | gi\|192386057 | **F:** AGCATGGTTGCACCCTTTAC<br>**R:** GCCAAGGACTGGTCAGAAAG | 60.0059.84 | 5055 | 2020 | 0 | 0 | - | - |
| 8 | gi\|192386249 | **F:** GGTTTGTTCAACACGCACAC<br>**R:** AGGAGGAGGTCGTCGAAGTT | 60.0660.25 | 5055 | 2020 | 0.4 | 0 | - | - |
| 9 | gi\|192386937 | **F:** CCACAACTCCCACAACCTCT<br>**R:** GAGGTAGTCGACGAGGATGC | 60.0059.83 | 5560 | 2020 | 0.196 | 1 | Majhgaon | 480bp |
| 10 | gi\|192386981 | **F**: GGGCTCAGGTGTACTCTCCA<br>**R**: GGAGCCAATGTCAGCTTCTT | 60.2659.43 | 6050 | 2020 | 0.496 | 0 | - | - |
| 11 | gi\|192387233 | **F:** AGGCATCTTCTCCCCAATCT<br>**R:** TGCCGTAGTCCTTGACCTCT | 60.0459.87 | 5055 | 2020 | 0 | 0 | - | - |
| 12 | gi\|192387279 | **F:** GATCTTCGGAACCTCCTTCC<br>**R:** AATGGCCATGAAACGAGAAG | 60.0160.07 | 5545 | 2020 | 0 | 0 | - | - |
| 13 | gi\|192387345 | **F:** GCTACGAGAGCACCGGTAAG<br>**R:** CATCTCTGGTCACGGTTGTG | 60.0460.15 | 6055 | 2020 | 0.49 | 0 | - | - |
| 14 | gi\|192387399 | **F:** TGAAGGAGCTGGAGGAGTGT<br>**R:** GAACCACATACGGACCCAGT | 59.9959.70 | 5555 | 2020 | 0.475 | 0 | - | - |
| 15 | gi\|192387425 | **F:** ACGACGACGAACATCTCCTC<br>**R:** CCAAATGGCTCTTCCTGAAC | 60.2759.67 | 5550 | 2020 | 0.25 | 0 | - | - |
| 16 | gi\|192387597 | **F:** TAGTGTCGCTCATCGTCGTC<br>**R:** AGGAGCCGATTCTCCTTCTC | 60.0259.92 | 5555 | 2020 | 0.488 | 0 | - | - |
| 17 | gi\|192387651 | **F:** CATCTCCGGTTCCGATAGAA<br>**R:** CCGTATAGTGCTGGGTGCTT | 60.0360.15 | 5055 | 2020 | 0.32 | 1 | Kharkog-2 | 400bp |
| 18 | gi\|192389107 | **F:** CACCCTTCCTTCCTCTTCCT<br>**R:** GGTCTGAGGAGGGTTTAGGG | 59.6759.93 | 5560 | 2020 | 0.544 | 0 | - | - |
| 19 | gi\|192389147 | **F:** CTGCAGCCCTTCTCATCTTC<br>**R:** CCCCAAACTTCAACGTGTCT | 60.1060.01 | 5550 | 2020 | 0.42 | 0 | - | - |
| 20 | gi\|192389165 | **F:** AACGGGGTTCTCAGTGTGAC<br>**R:** TTAAGCGCTCACAACGACAC | 60.0160.06 | 5550 | 2020 | 0.44 | 0 | - | - |

*Tm- Annealing temperature

**GC- Guanine and Cytosine content

**Table 5: Details of ISSR primersused for the present study**

| S.No. | Sequence ID | Primer sequence | Tm* (°C) | GC%** | Length in bp | PIC | No. of unique bands | Name of the sample | Position of unique band |
|---|---|---|---|---|---|---|---|---|---|
| 1. | Primer 3E | ACACACACACACACACG | 52.8 | 52.9 | 17 | 0.787 | 2 | Guldi-1 | 200bp |
| | | | | | | | | Deothi-1 | 220bp |
| 2 | Primer 1 | CACACACACACACACAAGG | 56.7 | 52.6 | 19 | 0.799 | 2 | Sanarali-2 | 600bp |
| | | | | | | | | Kharkog-1 | 100bp |
| 3 | UBC-855 | ACACACACACACACACCTT | 55 | 47.4 | 19 | 0.375 | 1 | Badashahithul -1 | 500bp |
| 4 | UBC-850 | GTGTGTGTGTGTGTGTCTC | 57 | 52.6 | 19 | 0.74 | 0 | - | - |
| 5 | UBC-840 | GAGAGAGAGAGAGAGACTC | 57 | 52.6 | 19 | 0.625 | 2 | Badhu-2 | 550bp |
| | | | | | | | | Heera Nagar-1 | 220bp |
| 6 | Primer 2 | TGTGTGTGTGTGTGTGC | 52.8 | 52.9 | 17 | 0.444 | 1 | Kaithlee ghat-3 | 500bp |
| 7 | ISSR 6 | AGAGAGAGAGAGAGAGYG | 55 | 52.8 | 18 | 0.444 | 0 | - | - |
| 8 | ISSR3C | TCTCTCTCTCTCTCTCA | 50.4 | 47.1 | 17 | 0.571 | 1 | Shoghi-5 | 500bp |
| 9 | ISSR 3A | CTCTCTCTCTCTCTCTT | 50.4 | 47.1 | 17 | 0.651 | 0 | - | - |
| 10 | ISSR 3B | CACACACACACACACAT | 50.4 | 47.1 | 17 | 0.692 | 1 | Deothi-1 | 340bp |
| 11 | ISSR8 | CACACACACACACACA | 48 | 50.0 | 16 | 0.765 | 0 | - | - |
| 12 | ISSR I | CACACACACACACACACG | 56 | 55.6 | 18 | 0.64 | 0 | - | - |
| 13 | IISRS 3D | TCTCTCTCTCTCTCTCG | 52.8 | 52.9 | 17 | 0.5 | 0 | - | - |
| 14 | IISRS 3G | GTGGTGGTGGTGGTG | 53.3 | 66.7 | 15 | 0.642 | 3 | Guldi-1 | 900bp |
| | | | | | | | | Nagangi | 700bp |
| | | | | | | | | Bhim-boot-1 | 100bp |
| 15 | IISRS 3H | GACGACGACGACGAC | 53.3 | 66.7 | 15 | 0.804 | 0 | - | - |
| 16 | UBC 829 | TGTGTGTGTGTGTGTGC | 52 | 52.9 | 17 | 0.602 | 0 | - | - |
| 17 | ISSR 3 | GAGAGAGAGAGAGAGAYC | 55 | 52.8 | 18 | 0.773 | 1 | Deothi-1 | 500bp |
| 18 | ISSR 5 | AGAGAGAGAGAGAGAGYC | 55 | 52.8 | 18 | 0.784 | 0 | - | - |
| 19 | ISSR 7 | ACACACACACACACACYC | 55 | 52.8 | 18 | 0.809 | 0 | - | - |
| 20 | UBC 854 | TCTCTCTCTCTCTCTCCAGC | 60 | 55 | 20 | 0.795 | 0 | - | - |
| 21 | ISSRS 3F | GACAGACAGACAGACA | 49.2 | 50 | 16 | 0.856 | 0 | - | - |
| 22 | IISRS 3L | GACAGACAGACAGACA | 49.2 | 50 | 16 | 0.855 | 1 | Kumarhatti-1 | 900bp |
| 23 | IISRS 3M | ACACACACACACACAC | 49.2 | 50 | 16 | 0.792 | 0 | - | - |
| 24 | ISSR 808 | AGAGAGAGAGAGAGAGC | 52 | 52.9 | 17 | 0.815 | 0 | - | - |
| 25 | UBC 880 | GGAGAGGAGAGGAGA | 49 | 60 | 15 | 0.877 | 0 | - | - |
| 26 | IISRS 3O | CACACACACACACACAGC | 56 | 55.6 | 18 | 0.886 | 0 | - | - |
| 27 | UBC 841 | GAGAGAGAGAGAGAGACTC | 57 | 52.6 | 19 | 0.788 | 0 | - | - |
| 28 | UBC 848 | CACACACACACACACAAGG | 57 | 52.6 | 19 | 0.728 | 0 | - | - |
| 29 | ISSR 4 | AGAGAGAGAGAGAGAGYT | 53 | 47.2 | 18 | 0.444 | 0 | - | - |
| 30 | ISSR 2 | CAGAGAGAGAGAGAGAYT | 53 | 47.2 | 18 | 0.875 | 0 | - | - |
| 31 | IISRS 3N | CACACACACACACACATG | 53.7 | 50 | 18 | 0.685 | 0 | - | - |
| 32 | IISRS 3K | GAGAGAGAGAGAGAGATC | 53.7 | 50 | 18 | 0.774 | 0 | - | - |
| 33 | UBC 876 | GATAGATAGACAGACA | 43 | 37.5 | 16 | 0.776 | 0 | - | - |
| 34 | ISSR HB 11 | GTGTGTGTGTGTCC | 44 | 44 | 57.1 | 0.769 | 0 | - | - |
| 35 | ISSR HB 10 | GAGAGAGAGAGACC | 44 | 44 | 72.7 | 0.625 | 1 | Kaithlee ghat-3 | 200bp |

**Table 6: Summary of the amplified products obtained in 21 *Rubusellipticus*collections using EST-SSRprimers**

| | |
|---|---|
| Total number of primers examined | 20 |
| Number of informative primers | 20 |
| Number of polymorphic primers | 20 |
| Total number of scorable bands | 40 |
| Number of polymorphic bands | 40 |
| Number of monomorphic bands | 0 |
| Average number of polymorphic bands per primer | 2 |
| Total number of amplified fragments | 267 |
| Average number of amplified fragments per collection | 12.71 |
| Average number of amplified fragments per informative primer | 13.35 |

**Table 7: Summary of the amplified products obtained in 21 *Rubusellipticus*collections using ISSR primers**

| | |
|---|---|
| Total number of primers examined | 35 |
| Number of informative primers | 35 |
| Number of polymorphic primers | 35 |
| Total number of scorable bands | 173 |
| Number of polymorphic bands | 173 |
| Number of monomorphic bands | 0 |
| Average number of polymorphic bands per primer | 5.3 |
| Total number of amplified fragments | 866 |
| Average number of amplified fragments per species | 41.2 |
| Average number of amplified fragments per informative primer | 24.7 |

2', 'Khadiana', 'Heera nagar-2', 'Kumarhatti-1'and Badashahithul-1, whereas; cluster D contained 15 genotypes viz., 'Kaithlee ghat-3', 'Baurgaon-1', 'Deothi -1', 'Kharkog-2', 'HeeraNagar-1', 'Kandaghat-8', 'Kharkog-1', 'Nagangi', 'Saroli-3','Majhgaon', 'Shoghi- 5', 'Guldi-1', 'Sanarali-2', 'Kandaghat-1', and 'Bhim-boot-1'. This revealed very less similarity between sub-clusters C and D. Clusters C and D were further subdivided into C21, C22 and D21, D22 at 9% similarity. C21 contained four genotypes and C22 contained only one genotype. D21 contained only one genotype and D22 contained 15 genotypes. Highest similarity (56%) was found between 'Kharkog-1' and 'Nagangi' showing that these two were relatively closely related. The dendrograms revealed that high genetic variability existed within Rubus species. From our results, we indicate that the variation in Rubus species was mostly due to individual variation and cross-pollinationed effect between polyploidy Rubus species.

In the present study, all 20 SSR and 35 ISSR primers produced 100% polymorphism (Figs. 3, 4, 5 and 6). In some earlier studies, high level of polymorphism using SSRs has also been found i.e., 100% in blackberry (Dossett *et al*., 2012); 100% in peach (Li *et al*., 2013); 98.36% in *Rubus* species (Thakur, 2013) – all members of Rosaceae family. High ISSR percentage was also supported by same finding in which ISSR primers showed, 89.6% in Chilean strawberry (Carrasco *et al*., 2007); 100% in strawberry (Nunes*et al*., 2013); 97.81% in apple (Vaidya *et al*., 2014). High levels of polymorphism with two marker systems showed that these two markers types were suitable tools for genetic diversity studies.

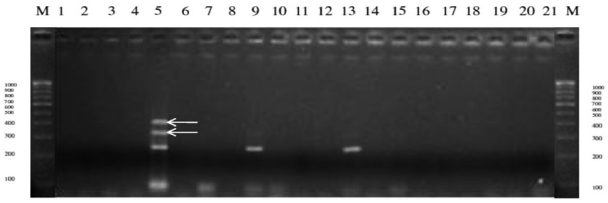


**Fig. 3: Gel image of EST-SSR Primer 111181967**

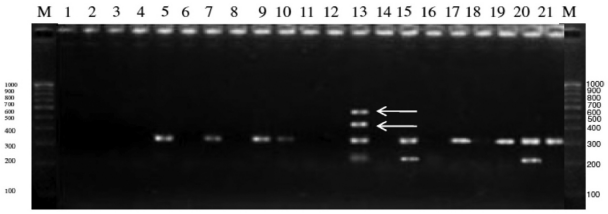


**Fig. 4: Gel image of EST-SSR Primer 111182120**

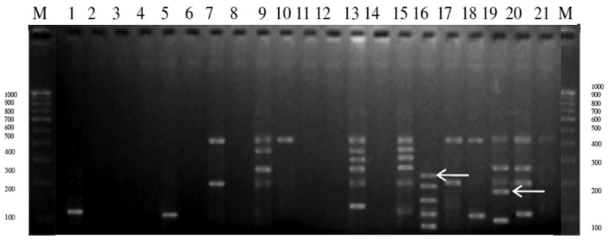


**Fig. 5: Gel image of ISSR Primer 3E**

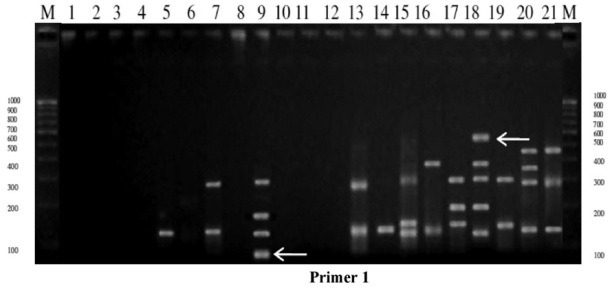


**Fig. 6: Gel image of ISSR Primer 1**

The PIC values, which provide an estimate of the discriminatory power of a primer, were as high as 0.667 for EST-SSRs and 0.886 for ISSRs. With a few exceptions, the markers clearly placed the accessions into different groups, according to their background. High PIC values i.e., 0.76 and 0.593was also reported with EST-SSRs (Lee *et al*., 2016; Jaliseh *et al*., 2015] whereas 0.95 and 0.37 with ISSR (Singh *et al*., 2015; Uzun *et al*., 2016) respectively. Effective multiplex ratio (E) and marker index (MI) values for EST-SSRs were 1.09 and 0.62 and for ISSRs these values were 8.24 and 5.83. These results of high E and MI values were also found in apple (Vaidya *et al*., 2014). The marker index is considered to be an overall measure of the efficiency OF detecting polymorphism. The ISSR primers showed the highest marker index, i.e., 5.834 (Monte *et al*., 2002).Our results indicate that the ISSR marker system suitable for fingerprinting and estimating genetic diversity in breeding populations because it showed high PIC, E. MI values.

The evaluation of genetic background of germplasm is important for the use of genetic resources (Mariac *et al*., 2006; Tani *et al*., 2006). For efficient conservation and management strategies, it is essential to have information regarding genetic differences in *Rubus* species germplasm. Sometimes, uncertain diversity of species and artificial groups of the *Rubus* genuscreate confusion during correct classification of the species at both commercial and scientific levels. So, the use of microsatellite markers and inter simple sequence repeat will have a major impact on genetic analysis such as genetic diversity and evolutionary relationship study among *Rubus* species. The results of this study will hopefully provide information for future breeding programs and would be useful for the conservation and evaluation of genetic resources in various *Rubus* species.

## Conclusion

Results obtained from both the marker systems showed almost overlapping similarity values and cluster formations, and on the basis of cluster analysis, we can state that dendrograms generated by the two marker systems are in congruence with each other. The dendrograms constructed for both the marker systems were grouped collections, to a larger extent is on the basis of their locations. The collections of same place or nearby places have been found to be very near in the dendrograms. The collections from different areas exhibited wide range of diversity. Thus, it was concluded from present study that ISSRs and EST-SSRs are highly useful to find out genetic diversity among *Rubus* collections. Our results indicate that the *Rubusellipticus* germplasm of present study was relatively untapped. This study is an initiative for use of this germplasm in domestication, further in breeding programmes.

## References

Jenning D L (1988) Raspberry and blackberries: their breeding, disease and growth *Academic press London* 230 p

Saklani S, Chandra S, Badoni P P and Dogra S (2012) Antimicrobial activity, nutritional profile and Phytochemical screening of wild edible fruit of *Rubusellipticus. International Journal of Medicinal and Aromatic Plants* **2** 269-274

William J G K, Kubelik A R, Levak K J, Rafaski J A and Tingey S V (1990) DNA polymorphism amplified by arbitrary primer are useful as genetic marker *Nucleic Acid Research* **18** 6531-6535

Powell W, Morgate M, Doyle J J, McNicol J W, Tingey S V and Rafalski A J (1996) Genepool variation in genus glycine subgenus soja revealed by polymorphism nuclear and chloroplast microsatellite *The Genetics Society of America* **144** 793-803

Dossett M, Bassil N, Lewers K S and Finn C E (2009) Transferability of *Rubus* microsatellite markers for use in black raspberry *Horticulture Science* **44** 1092p

Wen M, Wang H, Xia Z, Zou M, Lu C and Wang W (2010)

Development of EST-SSR and genomic-SSR markers to assess genetic diversity in Jatropha Curcas. L *BMC Research Notes* **3** 1-8

Singh D R, Srivastava A K, Srivastava A and Srivastava R C (2011) Genetic diversity among three *Morinda* species using RAPD and ISSR markers *Indian Journal of Biotechnology* **10** 285-293

Amsellem L, Dutech C and Billote N (2001) Isolation and characterization of polymorphic microsatellite loci in *Rubusalcefoleius* Poir (Rosaceae) an invasive weed in La Reunion island *Molecular Ecology Notes* **1** 33-35

Ashley M V, Wilk J A, Styan S M N, Craft K J, Jones K L, Feldheim K A, Lewers K S and Ashman T L (2003) High variability and disomic segregation of microsatellite in the octoploid *Fragaria virginiana* Mill (Rosaceae) *Theoretical and Applied Genetics* **107** 1201-1207

Vaidya E, Kaur R and Bhardwaj S V (2012) Data mining of ESTs to develop dbEST-SSRs for use in a polymorphism study of cauliflower (*Brassica oleracea* var. botrytis) *Journal of Horticultral Science and Biotechnology* **87** 57-63

Vaidya E, Kaur R, Kumar K and Sharma N (2015) Exploitation of Malus ESTs for development of SSR markers after in-silico analysis *Journal of applied botany and food quality* **88** 164-169

Kaur R, Shilpa, Vaidya E and Kumar K (2015) Development, characterization and transferability of peach genic SSRs to some Rosaceaespecies *Advances in Research* **3** 165-180

Rongwen J, Akkaya M S, Bhagwat A A, Levi U and Cregan P B (1995) The use of microsatellite DNA markers for soyabean genotypes identification *Theoretical and Applied Genetics* **90** 43-48

Fazio G, Chung S M and Staub J E (2003) Comparative analysis of response to phenotypic and marker assisted selection for multiple lateral branching in cucumber (*Cucumis sativa* L.) *Theoretical and Applied Genetics* **107** 875-883

Bushakra J M, Bryant D W, Dossett M, Vining K J, VanBuren R, Gilmore B S, Lee J, Mockler T C, FinnC E and Bassil N V (2015) A genetic linkage map of black raspberry (*Rubusoccidentalis*) and the mapping of *Ag4* conferring resistance to the aphid *Amphorophoraagathonica*. *Theoretical and Applied Genetics* **128** 1631-1646

Doyle J J and Doyle J J (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissues *Phytochemical Bulletin* **19** 11-15

Temnykh S, Clerk G, Lukashova A, Lipovich L, Cartinhour S and McCouch S R (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential *Genome Resource* **11** 1441-1452

Rozen S and Skaletsky H J (2000) Primer 3 on the www for general users and for biologist programmers *In*: Krawetz S and Misener S (eds). Bioinformatics methods and protocols: methods in molecular biology, Humana Press, Totowa, NJ 365-386

Sharma N, Kaur R and Vaidya E (2016) Potential of RAPD and ISSR markers for assessing genetic diversity among *Stevia rebaudiana* Bertoniaccessions *Indian Journal of Biotechnology* **15** 95-100

Varshney R K, Chabane K, Hendre P S, Aggarwal R K and Graner A (2007) Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys *Plant Science* **173** 638-649

Rohlf F J (2000) NTSYSpc numerical taxonomy and multivariate analysis version 2.0.h New York: Applied Biostatics Inc. p 25

Jaccard P (1908) Nouvelles Recherches sur la distribution florale *Bulletin de la Societe Vaudoise des Sciences Naturelles* **44** 223-270

Sneath P H A and Sokal R R (1973) NumericalTaxonomy. W H Freeman and Company, San Francisco, California 431p

Smith J S, Chin E C, Shu H, Smith O S, Wall S J, Senior M L, Mitchell S E and Kresovich S and Zeigle J (1997) An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): comparisions with data from RFLPs and pedigree *Theoretical and Applied Genetics* **95** 163-173

Ganopoulos J V, Kazantzis K, Chatzicharisis J, Karayiannis I and Tsaftaris A S (2011) Genetic diversity, structure and fruit trait associations in Greek sweet cherry cultivars using microsatellite based (SSR/ISSR) and morpho-physiological markers *Euphytica* **181** 237-251

Dossett M, Bassil N V, Lewer K S and Finn C E (2012) Genetic diversity in wild and cultivated black raspberry (*Rubusoccidentalis* L.) evaluated by simple sequence repeat markers *Genetic Resources and Crop Evolution* **10** 40-57

Li X W, Meng X Q, Jia H J, Yu M L, Ma R J, Wang L R, Cao K, Shen Z J, Niu L, Tian J B, Chen M J, Xie M, Arus P, Gao Z S and Aranzana M J (2013) Peach genetic resources: diversity, population structure and linkage disequilibrium *BMC Genetics* **14** 1-16

Thakur R (2013) Studies on development of genic-SSRs in raspberry (*Rubusellipticus* Smith.) and their transferability across related species. MSc. Thesis. Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, India 58p

Carrasco B, Garces M, Rojas P, Saud G, Herrera R, Retamales J B and Caligari P D S (2007) The chilean strawberry [*Fragariachiloensis* (L.) Duch.]: genetic diversity and structure *Journal of the American Society for Horticultural Science* **132** 501-506

Nunes C F, Ferreira J L, Generoso A L, Dias M S C, Pasqual M and Cancado G M D A (2013) The genetic diversity of strawberry (*Fragariaananassa* Duch.) hybrids based on ISSR markers *Acta Scientiarum* **35** 443-452

Vaidya E (2014) Studies on QTL analysis for growth characteristics in apple *Malus* x *domestica* Borkh. PhD. Thesis, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, India 136p

Lee K J, Lee G A, Kang H K, Lee J R, Raveendar S, Shin M J, Cho Y H and Ma K H (2016) Genetic Diversity and Population Structure of *Rubus* Accessions Using Simple Sequence Repeat Markers *Plant Breed Biotech* **4** 345-351

Jaliseh S A, Mehregan I, Tarang A and Nejadsattari T (2015) Genetic diversity of Rubus L. (Rosaceae) in the Northern Iran *Bulletin of the georgian national academy of sciences* **9** 387-394

Singh A, Singh N P, Husain R and Sengar R S (2015) DNA fingerprinting based identification and classification of indica rice (*Oryza sativa* L.) to assess genetic diversity via ISSR & RAPD markers *The Ecoscan* **9** 1015-1021

Uzun A, Ozongun S, Gulsen O, Yilmaz K U, Kaymak S and Ercisli S (2016) Determination of genetic relatedness among Turkish apple germplasm based on ISSR markers *Journal of applied botany and food quality* **89** 82-88

Monte C L, Goulao L and Oliveira C (2002) Discrimination of Pear cultivars with RAPD, AFLPTM and ISSR *Acta Hort* **596** 187-191

Mariac C, Luong V, Kapran I, Mamadou A, Sagnard F and Deu M (2006) Diversity of wild and cultivated pearl millet accessions (*Pennisetum glaucum* [L.] R. Br.) in Niger assessed by microsatellite markers *Theor Appl Genet* **114** 49-58

Tani N, Yoshimaru H, Kawahara T, Hoshi Y, Nobushima F and Yasui T (2006) Determination of the genetic structure of remnant Morusboninensis Koidz. trees to establish a conservation program on the Bonin Islands, Japan *BMC Ecol* 6-14.