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Genetic variability in strawberry (*Fragaria x ananassa* Duch.) cultivars assessed by morphological traits and EST-SSR markers of *Rubus ellipticus*

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Strawberry (*Fragaria x ananassa* Duch.) being an important commercial member of Rosaceae family, accounts for high nutritive value in terms of antioxidative and antibacterial properties. Owing to its unique therapeutic values, present study was done with the objective of characterizing 20 *Fragaria x ananassa* cultivars from Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, India based on their morphological descriptors and expressed sequence tags - simple sequence repeat (EST-SSR) markers of *Rubus ellipticus* L. (Smith). Broad phenotypic variability among the *Fragaria x ananassa* cultivars was detected using morphological descriptors. A set of 68 *R. ellipticus* EST-SSR primers were used for cross transferability analysis in strawberry cultivars for polymorphic marker identification and genetic diversity analysis. Out of 68 EST-SSRs, 51 (75%) showed amplification among the strawberry cultivars. The average (PIC) values of all the polymorphic loci for 20 strawberry cultivars were 0.34. Highest polymorphism information content (PIC) value (0.68) was obtained with contig 218, followed by contig 714 (0.65) and contig 408 (0.63), indicating that contig 218 has the most regions of diversity as amplified with these SSR and was found most suitable for cross transferability. The unweighted pair group method with arithmetic mean (UPGMA) was designed showing two separate groups for 20 cultivars of *Fragaria x ananassa*. The present study revealed that *R. ellipticus* microsatellites when used were able to distinguish strawberry cultivars accurately.

Keywords: Strawberry, molecular markers, EST-SSRs, morphological traits, cross transferability

Introduction

The family Rosaceae comprises of approximately 3000 species which includes fruit crops, berries and ornamental plants. The Rosaceae family is further divided into three sub-families as, Rosoideae, Spiraeoideae and Dryadoideae. The sub-family Rosoideae includes cultivated berries of the genera *Rubus* (raspberry and blackberry) and *Fragaria* (strawberry). Strawberry and raspberry are rich in antioxidants and have high commercial value due to their appearance, taste and aroma. Strawberry and raspberry accounts for the most consumed berries worldwide and their production and consumption has shown a hike by 50% from 2010-2020[1].

In recent years, increased demand of strawberry and raspberry warrants their enhanced production worldwide. This necessitates the development of high yielding and improved quality varieties. However, the rate of success of breeding these fruit crops is slow due to heterozygosity and polyploidy. Therefore, molecular markers here play a crucial role in pacing up the speed and accuracy of germplasm assessment at any stage of plant's growth. Considering breeding in *Fragaria* and *Rubus* it is very complicated due to polyploidy and the heterozygous nature of the germplasm. Therefore, molecular markers here play a crucial role in pacing up the speed and accuracy of germplasm assessment at any stage of plant's growth.

Among molecular markers, simple sequence repeat (SSR) or microsatellite markers consisting of tandem repeats of di-, tri-, tetra-, penta- and hexa-nucleotide are better due to their abundance and robust nature [2-3]. Due to their high polymorphism, co-dominant inheritance and abundance in genome, they play an important role in identifying genomic regions associated with horticulturally desirable traits. SSR markers for *Rubus ellipticus* were first developed in 2020[4]. SSR transferability generally depends on genetic distance between individual species and is more successful if done within closely related species and genera.

The *Fragaria* and *Rubus*, both share the same basic number of chromosome (x = 7) (belonging to same

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subfamily Rosoideae) and have close phylogenetic relationships in terms of their nuclear and chloroplast DNA markers. These facts indicate the collinearity between *Fragaria* and *Rubus* genome[5]. Transferability is very crucial in these species because it helps in molecular characterization of wild species and germplasm that in turn accelerate the breeding of new cultivars by saving time and cost. Keeping the usefulness of EST-SSR markers for transferability analysis and its need to characterize the strawberry germplasm, the present study aimed at precise assessment of genetic diversity among various cultivars of strawberry.

Material and Method

Phenotypic: Morphological Characterization of Fruits

The conducted site of the experiment was semi-temperate agro-climatic region at Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India. Healthy and uniform runners of twenty strawberry cultivars i.e. Torrey, Fern, Red Coat, Pride, Addie, Dana, Etna, Tioga, Chandler, Selva, Majestic, Dilpasand, Katrain Sweet, Sweet Charlie, Belrubi, Gorella, Brighton, Douglas, Shasta and Confectura which were grown on raised beds with spacing 45 × 30 cm, in three replications. The breadth and length of berries were evaluated in cm with Vernier calliper and mean was calculated. The weight was recorded for representative fruits of each cultivar from each plant and average weight per berry was noted. The yield per plot was calculated by multiplying the average number of fruits harvested per plants with mean weight. The randomly selected berries from all the cultivars in each replication were used to calculate total soluble solids with refratometer (0-32 ˚Brix range)[6].

Plant Material and DNA Extraction

A total of 20 cultivars of strawberry planted in the field of Department of Fruit Science, Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, were selected to find out the cross species transferability. The list of cultivars used in present study is mentioned in Table 1. Young and healthy leaves were collected in replicates, then immediately put in silica gel to dry until DNA extraction. Genomic DNA was extracted using the modified cetyltrimethyl ammonium bromide (CTAB) method[7-8]. The quality and quantity of genomic DNA was determined by Nano Drop 1000 spectrophotometer and also using uncut lamda DNA standard on 1.0% agarose gel electrophoresis[9].

Cross Amplification of *Rubus ellipticus* EST-SSR Markers in Strawberry

Cross-species amplification of strawberry was performed using unigenes set and 68 EST-SSR primers of *R. ellipticus* which were obtained from an earlier study on *R. ellipticus* (Sharma *et al*, 2021). The polymerase chain reactions (PCR) were performed in 20 µL reaction volume containing 1.0 µL (50 ng) of template DNA, 1.6 µL of 25 mM dNTPs, 0.6 µL (10 µM) of each primer solution, 0.1 µL of *Taq* polymerase, 2.0 µL of 10 X PCR *Taq* buffer and 14.1 µL of sterilized ddH_2O. The PCR amplification protocol was standardized in a touchdown thermal cycler of ProFlex^{TM} thermal cycler (Applied Biosystem, Inc.). The PCR reagents (dNTPs, *Taq* enzyme and buffer) were purchased from Genaxy Scientific Pvt Ltd and primers were synthesized from Eurofins genomics (GeNei^{Tm}). The PCR cycling parameters were used i.e denaturation at 95^{o}C for 5 min followed by 35 cycles of three steps; 1 min of denaturing at 94^{o}C, annealing according to Tm of primer for 1 min, extension at 72^{o}C, and final extension for 10 min at 72^{o}C. Touchdown of 2^{o}C for the first 20 cycles was carried out[10]. The amplified products were mixed thoroughly with 6X loading dye and resolved on 3.5% agarose gel which was run at constant voltage at the rate of 5V/cm under submerged condition of TAE (tris base: glacial acetic acid: EDTA) buffer for two hours. DNA bands were visualized on UV transilluminator and photographs were taken on gel documentation system (Syngene, Cambridge, UK).

Data Analysis

Qualitative and quantitative analysis of morphological data was carried out with R software. Nbclust program of R software was used for the cluster formation between cultivars. The distance between groups was estimated with Euclidian distance matrix. The combined analysis i.e. morphological and molecular data was also carried out by using Euclidian distance matrix. For molecular analysis of EST-SSRs, number of polymorphic bands was scored by each primer as 1 and 0 for presence and absence of band, respectively. The banding pattern in each band of a specific microsatellite marker was recorded for each genotype and was assigned a letter. The analysis of data was carried out with NTSYS-pc (Numerical Taxonomy System, Applied Biostatistics, Inc., New York, USA) version 2.1 software package (Rohlf, 2000). The unweighted pair group method

Table 1— List of strawberry cultivars used in the study

Sr. no.	Name of cultivars	Species	Maintained location	State	Altitude (m above sea lavel)	Latitude	Longitude	Season	Developer	Release	Pedigree	Notes
1	Torrey	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	California Agriculture Experiment Station	1961	Lassen x Cal 42.8-16	Fruits are large in size, firm flesh and capped easily.
2	Fern	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	S-pring	University of California	1973	Tufts x Cal 69.63-103	Day neutral, asexually propagated runner.
3	Red coat	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Canadian Department of Agriculture, Ottawa	1957	Sparkle x Valentine	Red bright, very firm and good fruit size throughout the harvest season. Resistant to powdery mildew.
4	Pride	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Spain	1996	Sel. 4–43 × 'Vilanova	Day neutral, asexually propagated runner
5	Addie	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Italy	1982	Senga Pantagruella' × MDUS 3816	Day neutral, asexually propagated runner.
6	Dana	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Europe and Nor America	-	unknown	Large fruited, high yielded.
7	Etna	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Europe and Nor America	-	unknown	Large fruited, high yielded.
8	Tioga	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	California Agriculture Experiment Station	1964	Fresno x Torrey	Fruit having short harvesting season, easily capped and get separated without calyx.
9	Chandler	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	University of California	1983	Douglas x Cal 72.361-105	Well suited for southern planting. A Californian variety that is adaptable to the eastern U.S. susceptible to anthracnose disease.
10	Selva	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early September	Belgium	1987	Cal 70.3-117 x Cal 71.98-605	Having greater average fruit.
11	Majestic	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	-	-	Unknown	Everbearer.
12	Dilpasand	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Imported variety in India	-	Unknown	Best suitable for hilly area.
13	Katrain Sweet	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Mid season	Imported variety in India from America	-	Unknown	Best suitable under summer.
14	Sweet Charlie	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early Season	University of Florida, Gulf Coast Research and Education Center		FL 80-456 x Pajaro	Resistant to crown rot, most fruit rot, two spotted spider mites, powdery mildew. Susceptible to leaf blight.
15	Belrubi	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early Season	Imported variety in India	1933	Unknown	Conical to wedge shaped, large sized fruits.
16	Gorella	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early September	Netherland	1969	Juspa x US-3763	Fruits are large, crimson red conical, well exposed upright foliage with green tip.
17	Brighton	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early Season	-	-	Unknown	Day-neutral june bearing strawberries
18	Douglas	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early September	USA	1979	('Tioga' × 'Sequoia') × 'Tufts'	Large fruited, high yielded.
19	Shasta	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early Season	University of California	1945	Unknown	Due to poor ancestor not consider for further analysis.
20	Confectura	*Fragaria* × *ananassa*	Nauni, Solan	Himachal Pradesh	1760	31˚ 27' 277"	76˚ 94' 373''	Early Season	Imported variety in India	-	Unknown	Large fruited, high yielded.

arithmetic average (UPGMA) was used to construct a dendrogram by using similarity matrix[11]. The estimation of the discriminatory power of a locus was provided by polymorphism information content (PIC) by taking into account not only the number of alleles that are expressed but also the relative frequencies of those alleles. The PIC value was calculated using software Power Maker version 3.25. The PIC value was calculated by using given formula:

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

pi frequency of the $_i$th allele[12-13].

Results and Discussion

The knowledge regarding the genetic diversity, relatedness with and between strawberries cultivars are of paramount importance to ensure the long-term success of strawberry improvement programs. Populations with maximum genetic diversity are useful source for broadening the genetic base in any breeding program. Molecular markers in this direction approved as an efficient and powerful tools to illustrate genetic diversity, determining percentage and revealing phylogenetic relationships among various strawberry species.

Morphological Characterization of Fruits

Wide range of diversity was reflected for various morphological characters found within the genus. Table 2 depicts morphological variability among 20 cultivars of strawberry. The cultivars of strawberries showed significant variation among themselves with respect to total soluble solids (TSS) ranging from minimum in 'Red coat' (8.0) to maximum in 'Selva' (12.3). The variation observed in TSS is due to varietal differences as evident from earlier reports[14-15]. The highest total sugar (8.7) reducing sugar (4.9) and non reducing sugar (3.4) was observed in 'Sweet Charlie' followed by 'Chandler' and 'Dilpasand' (Table 2) whereas the lowest total sugar (6.0), reducing sugars (3.3) and non-reducing sugars (2.4) was observed in 'Etna'. In the previous study in strawberry, high sugar content, reducing sugar and non-reducing sugar was observed in cultivars 'Chandler', Torrey and Selva whereas low sugar content, reducing sugar and non-reducing sugar were observed in cultivars 'Etna' and 'Dana'. The differences in fruit sugar are mainly due to the growing conditions and climatic variation[16].

All cultivars of strawberry produced white color flower with hermaphrodite flowering type. Maximum plant height was observed in 'Chandler' (24.9 m) and 'Sweet Charlie' (22.9 m) whereas minimum plant height in 'Red coat' (15.5 m). Maximum number of leaves varied from 31.3 in 'Chandler' to 15 in 'Red coat'. Different cultivars of strawberry showed significant variation in berry weight and yield per plot. The maximum berry weight was obtained in 'Gorella' (12.3 gm) followed by 'Belrubi' (11.5) and 'Chandler' (10.7) whereas minimum 4.6 in 'Majestic' (Table 2). Kumar *et al.*[17] cultivated nine cultivars of strawberry at Krishi Vigyan Kendra; Pulwama, Jammu and Kashmir observed that 'Tioga' (12.24 gm) produced maximum berry weight followed by 'Chandler' (12.11 gm) whereas minimum was observed in 'Selva' (8.71 gm).

The strawberry cultivars also showed significant difference in terms of number of fruits per plant, yield and yield per plot. Cultivar 'Chandler' produced large number of fruits per plant i.e. `32.0' as compared to `13.0' in Tioga. Maximum yield per plot of berries was observed in 'Gorilla' (2.7 kg/plot) and 'Chandler' (2.7 kg/plot) whereas minimum was observed in 'Fern' (0.64 kg/plot). It was also noted that 'Tioga' (2.26 kg) and 'Chandler' (2.19 kg) produced marketable maximum yield whereas minimum yield

Table 2 — Morphological and biochemical analysis of strawberry cultivars

Sr. No	Name of cultivars	Plant height (m)	Number of leaves	flower type	Flower color	Duration of flowering	Number of fruits/plant	Average berry (g)	Yield (g)	Yield/plot (g)	TSS (°B)	Reducing sugar	Non-reducing sugar	TS
1	Torrey	19.4	15.7	Harmaphordite	White	40.3	15.7	7.5	58.3	932.8	10.4	3.7	4.0	8.1
2	Fern	22.9	18	Harmaphordite	White	42	16	7.5	40.1	640.8	8.2	4.2	2.9	7.5
3	Red coat	15.5	15	Harmaphordite	White	42.7	25.3	5.4	68.4	1094.4	8.0	4.1	2.9	7.4
4	Pride	18.6	17.3	Harmaphordite	White	50.7	28	6.3	87.9	1406.72	10.9	4.7	3.4	8.5
5	Addie	19.6	16.7	Harmaphordite	White	42.7	27	6.6	89.1	1425.6	10.3	4.5	3.2	8.1
6	Dana	17.5	22.7	Harmaphordite	White	48.7	16	5.3	42.5	679.7	9.5	3.6	2.5	6.4
7	Etna	24.2	18	Harmaphordite	White	51	28	7.9	110.5	1767.4	9.3	3.3	2.4	6.0
8	Tioga	22.7	25.3	Harmaphordite	White	51.3	12.7	8.6	54.5	872	9.5	3.6	2.6	6.5
9	Chandler	24.9	31.3	Harmaphordite	White	54.3	32	10.7	170.7	2731.5	11.1	4.7	3.3	8.4
10	Selva	26	22	Harmaphordite	White	51.3	23	9.0	102.9	1646.9	12.3	4.0	3.3	7.7
11	Majestic	19.1	19	Harmaphordite	White	50	16	4.6	36.3	581.12	9.3	3.4	3.1	6.9
12	Dilpasand	21.1	22.7	Harmaphordite	White	51.7	19	7.5	70.7	1130.9	11.0	4.7	3.3	8.4
13	Katrain Sweet	22.7	20	Harmaphordite	White	52	16	6.8	54.2	866.6	10.0	3.7	2.6	6.6
14	Sweet Charlie	22.9	21.7	Harmaphordite	White	53	21.3	6.0	63.7	1018.7	11.3	4.9	3.4	8.7
15	Belrubi	19.3	23	Harmaphordite	White	42.7	22.7	11.5	130.5	2088	10.5	3.7	2.7	6.7
16	Gorella	18.7	24	Harmaphordite	White	49.7	28	12.3	171.4	2741.8	9.2	3.7	2.6	6.6
17	Brighton	20.3	21	Harmaphordite	White	48	22	9.4	103	1647.4	10.0	4.2	2.9	7.5
18	Douglas	22.3	21	Harmaphordite	White	44.3	28.7	8.6	123.2	1970.3	9.6	3.6	2.9	6.9
19	Shasta	22.5	20.3	Harmaphordite	White	51.3	15.3	7.7	59.2	947.2	9.3	3.4	3.1	6.9
20	Confectura	20.3	21	Harmaphordite	White	49	18.7	6.3	58.3	932	9.1	3.8	2.7	6.8
		0.0548	1.939			2.068	1.172	1.706	4.925	1.111	0.972	2.006	5.025	1.200

per plot was recorded in 'Selva' (1.08 kg) (Kumar *et al*, 2012). From this study, it was observed that yield/ plot is influenced with various environmental factors like temperature, photoperiod and light intensities. Pedigree analysis of strawberry cultivars revealed crosses between different parents. None of the cultivar has similar parents. The present investigation showed that among 20 cultivars Chandler, Sweet Charlie, Red coat, Selva, Gorella and Belrubi were the best in physico-chemical and yield attributes at Himachal Pradesh conditions.

Cross Transferability Polymorphism of *Rubus ellipticus* EST-SSRs

Analysis of variability through PCR based molecular markers is the cheapest and most rapid method of characterizing the relationships among different genotypes. Among various PCR based molecular markers, EST-SSRs are mostly used marker systems for genetic diversity analysis[18]. The previous transcriptomic study on *R. ellipticus* showed that majority of best matches were obtained with *Fragaria vesca* followed by *Prunus persica* (Sharma *et al*, 2021). So, the development and transferability of SSR markers from EST sequences saves both, time and cost and hence, present study made with aim to minimize labour and time consumption. Inter Proscan analysis of indicate that p-loop containing nucleoside triphosphate hydrolase domain and WD40 repeats are abundantly present in the leaves of *R. ellipticus* (supplementary Fig. 1 & 2). These unigenes were successfully blast hits were assigned GO term in which ATP binding are abundantly in molecular function followed by oxidation-reduction process in biological process and integral component of membrane in cellular components.

Till now very few reports are available on the transferability of EST based SSR markers to strawberry. In the present study, a set of 68 EST-SSR primer pairs were selected to carry out polymorphism study in 20 cultivars of strawberry (Table 3). Out of 68 markers, 51 (75%) produced clear amplicon of expected size and 17 failed to amplify DNA product. Out of the successful markers, 48 (94%) showed polymorphism in 20 cultivars of strawberry. In the previous study on *R. ellipticus* 61 out of 68 produced clear amplicon whereas seven were unable to amplify (Sharma *et al*, 2021). High polymorphism level of *R. ellipticus* primers in strawberry revealed that this marker system is suitable for genetic diversity and cross transferability study.

A total of 341 fragments were generated from 68 EST-SSR primer combinations which are distributed between 100 and 1000 bp of which 121 (36%) were polymorphic. The previous study on *R. ellipticus* showed 584 polymorphic bands out of 646 revealing 90.3% polymorphism (Sharma *et al*., 2021). The number of polymorphic fragments ranged from one to 42. The average number of amplified fragments per informative primer was 6.69. In *R. ellipticus,* average number of amplified fragments per informative primer was 10.61 (Sharma *et al*., 2020). The mean genetic distance estimated by the Jaccard coefficient among all cultivars was ranging from 0.46 to 0.94 with an average of 0.70. The mean genetic distance estimated with Jaccard coeffiecient in *R. ellipticus* was ranged from 0.24 to 0.69 with an average of 0.47. The genetic distance was greatest (0.46) between two groups i.e 'Tioga' and 'Confectura', and between 'Tioga' and 'Chandler' and smallest (0.94) between 'Shasta' and 'Confectura'. PIC values reveal allelic diversity and frequency among cultivars which ranged from 0 to 0.68 with an average to 0.34. The highest PIC value (0.68) was obtained with contig 218, followed by contig 714 (0.65) and contig 408 (0.63), The study reveals that Contig 218 has the most regions of diversity as amplified with these SSR and was found most suitable for cross transferability. However, the markers having lower PIC value were less powerful for diversity analysis as compared to those with higher PIC values. The PIC value obtained in the present study is similar that obtained in *R. ellipticus* i.e 0.67 (Sharma *et al*, 2017). Our results indicate the EST-SSRs markers are suitable for diversity analysis and fingerprinting due to their high PIC values.

The dendrogram obtained by UPGMA hierarchical clustering analysis identified the formation of two groups with a clear separation of the cultivars at 61% similarity. Group I consisted of 13 cultivars viz Torrey', 'Selva', 'Red coat', 'Addie', 'Etna', 'Majestic', 'Douglas', 'Dilpasand', 'Katrain Sweet', 'Sweet Charlie', 'Dana', 'Brighton', 'Tioga' whereas group II contained seven cultivars *viz*., 'Fern', 'Pride', 'Belrubi', 'Gorella', 'Chandler', 'Shasta', 'Confectura'. Cluster A was further divided into sub-clusters, i.e. A1 and A2 at 64% similarity. The sub-cluster A1 was found to contained only twelve cultivars *viz* Torrey', 'Selva', 'Red coat', 'Addie', 'Etna', 'Majestic', 'Douglas', 'Dilpasand', 'Katrain Sweet', 'Sweet Charlie', 'Dana' and 'Brighton', whereas sub-cluster A2 contained only one cultivar *viz* 'Tioga'. Cluster B

Table 3 — Details of EST-SSR primers used in present study

Sr. No	Sequence Id	Sequence Id	Primer sequence	Tm* (˚C)	GC%*	Length in bp	Total number of amplified alleles	Size range of amplified fragments (bp)	PIC value
1	Contig11	1-aminocyclopropane-1-carboxylate oxidase	F: TGCTCTTTTGTCATCTTCTCCA	56.5	40.9	22	14	200-700	0.44
			R: ATTTGAACCAACACTTGCACAC	56.5	40.9	22			
2	Contig218	Auxin-induced AUX28	F: AGCCTCTCTTCCTCATCATCAC	60.3	50	22	25	600-750	0.68
			R: ACCCTCCTTCCTTTCTCTAACG	60.3	50	22			
3	Contig397	60S ribosomal L27a-3	F: ACCCACTTTACCAAAATACCCC	58.4	45.5	22	13	100-300	0.42
			R: ACCCGTGGCTGAAAACCTA	56.7	52.6	19			
4	Contig408	Pollen-specific SF21-like	F: GCGACAGACACATAACCATGAC	60.3	50	22	4	300-500	0.63
			R:ACAGGGACAGAAGCAGAGAGAG	62.1	54.5	22			
5	Contig506	Cytochrome c	F: CAATTCACAGCCATGTTCTTGT	56.5	40.9	22	28	400-800	0.58
			R: TTCGTTACTTGTTCGTCTGGAA	56.5	40.9	22			
6	Contig528	Copper transporter	F: TTTGTTGGTTTTGGTGTAGTGG	56.5	40.9	22	7	400-800	0.59
			R: CTCCTTGCTCCAGTAGAACGTC	62.1	54.5	22			
7	Contig530	Negative regulator of sporulation MDS3	F: CCAAAACTCCATCAACCTCTCT	58.4	45.5	22	42	300-600	0.46
			R: AGCTCTACCAATTAAGCAGCCA	58.4	45.5	22			
8	Contig585	Ras-related RABD2c	F: TTGATAGTCTTCCCATCCTGCT	58.4	45.5	22	20	200-600	0
			R: GAGAAACTTGGGCTCATTCTTG	58.4	45.5	22			
9	Contig620	Eukaryotic translation initiation factor 5A-2	F: GGCAGTGAAGATGTCAATACCA	58.4	45.5	22	20	500-800	0
			R: TATAAATACCCGCTTCTCCCAA	56.5	40.9	22			
10	Contig656	FANTASTIC FOUR 1-like	F: CACCCAATTAAAAGGCTCAAAG	56.5	56.5	22	20	300-600	0
			R: TAGGGATGATGAGGAGGAAGAA	58.4	58.4	22			
11	Contig685	GDSL esterase lipase APG-like	F: AGCAAAGGGTAGCAGTTTTCAG	58.4	58.4	22	27	500-700	0.58
			R: GCAATCCAAAGTCGATAGGAAC	58.4	58.4	22			
12	Contig714	DNA ligase 1-like	F: CCTCTATTTCAATCTCCCTGGAT	58.9	58.9	23	26	400-700	0.65
			R: TCGAGGTAACAGAAGACCAGAA	58.4	58.4	22			
13	Contig925	Znc finger A20 and AN1 domain-containing stress-associated 8-like	F: CTTAGCCTGCTCCTGTTTCATC	60.3	60.3	22	35	500-600	0.47
			R: ACGTCTCTCTCTCTCGTCGTCT	62.1	62.1	22			
14	Contig926	Hypothetical protein PRUPE_ppa014191mg	F: AAGCAACCAGAGATCAGAAGAA	56.5	56.5	22	21	100-600	0.6
			R: TATGCTCACCAACAACTCATCA	56.5	56.5	22			
15	Contig935	Transmembrane ascorbate ferrireductase 1-like	F: ATGGACAAGCAAAATCACACAC	56.5	56.5	22	2	300-800	0
			R: CGTCACTCTCTTATTTGGGGTC	60.3	60.3	22			
16	Contig969	50S ribosomal chloroplastic	F: ACAATCTCTTGGAGGACTCTGC	60.3	50	22	0	0	0
			R: AAAAGGGTCAATGGGAGAAAGT	56.5	40.9	22			
17	Contig1053	Major centromere autoantigen B-like	F: ATGTCGAGTGTTCCTTGTCCTT	58.4	45.5	22	0	0	0
			R: ATGGGATACCTACTTTGGAGCA	58.4	45.5	22			
18	Contig1075	Palmitoyl-acyl carrier chloroplastic-like	F: TATAGATGACAGGTGGGAAGGA	58.4	45.5	22	0	0	0
			R: GGAGTATAGGAGGGAGTGTGGA	62.1	54.5	22			
19	Contig1137	NAC domain-containing 2	F: AGAGGTAGTGCTTGACGAGTTC	60.3	50	22	0	0	0
			R:TTAGCCCAAGAAGAGAGTAGAGTT	59.3	41.7	24			
20	Contig1169	Translation factor SUI1 partial	F: CTTCTTCAATCCCTGGACAGTT	58.4	45.5	22	0	0	0
			R: TTTCCAAACAGCTTCTCTGGT	55.9	42.9	21			
21	Contig1171	Wound-induced 1- partial	F: TCTAAGTAGCTTCAGCCCATCC	60.2	60.3	22	1	400	0
			R: CAAGTTCTCAAAGTCCTCCTCC	59.3	59.3	22			
22	Contig1193	Nuclear transcription factor Y subunit B-3-like	F: ACAGAGTCGGAGACAGACACAA	59.3	60.3	22	1	200	0
			R: AGGCGGGTTTAATGGAGTTTAT	60.2	59.3	22			
23	Contig2039	Hypothetical protein PRUPE_ppa023800mg	F: GTACGATGATGAGGATGAGGTG	60.3	50	22	1	500	0
			R: ATAGATAGCGCGGCAAGTTAAG	58.4	45.5	22			
24	Contig2047	Asterix-like	F: AAACCGGAGTAATCGACTGGTA	58.4	45.5	22	1	300	0
			R: AATTTGGATCGAGACCTGAAAC	56.5	40.9	22			
25	Contig2122	THO complex subunit 4A	F: TAAGAGGCCCAAACTCCATAAG	58.4	45.5	22	1	700	0
			R: GCCGTAGTGGTGAGAAGGTATC	62.1	54.5	22			
26	Contig2303	Transmembrane 45A-like	F: ATAGTGCTCTCCATTTTCGACC	58.4	45.5	22	1	100	0
			R: AGGAGGAAAGAATTGGTGTACG	58.4	45.5	22			
27	Contig2568	Probable sulfate transporter	F: CGATCACATTCGGCTTCTC	56.7	52.6	19	1	900	0
			R: CCAAAAGAGGACTTGAGCTTGT	58.4	45.5	22			
28	Contig2692	PREDICTED: uncharacterized protein LOC101311158	F: TCTCCATTTTCCAGACCAAGAT	56.5	40.9	22	1	200	0
			R: CTACCTTCAGTTTCACCTTCGC	60.3	50	22			
29	Contig2744	Cysteine synthase	F: GGGTTCAAAGGTAATGTTTCCA	56.5	40.9	22	3	1000	0.48
			R: AAAGAAGGTTTGCTTGTGGGTA	56.5	40.9	22			
30	Contig2833	PREDICTED: uncharacterized protein At4g00950	F: TAGCCCTTCTATGCCTTGTGTT	58.4	45.5	22	4	300-600	0.47
			R: GGGTCCTTATGTGGGTAGATCA	60.3	50	22			
31	Contig2996	Trihelix transcription factor GTL1 isoform X1	F: GCTATCGGCTTCCTCAACTCTA	60.3	50	22	1	200-600	0
			R: AAATTGGATTCAGCCTCTCAAG	56.5	40.9	22			
32	Contig3187	Leucine-rich repeat receptor-like serine threonine- kinase At2g14440	F: GTTGTATGAGAGACCGTAGGGA	60.3	50	22	1	400	0
			R: ACTCAACCAGTCGTGTCCTAA	57.9	47.6	21			
33	Contig3605	kDa class V heat shock	F: GTCCTGCTAGGCTCTGTTGAG	61.8	57.1	21	2	200-400	0
			R: TGTCTTCCCCTCTCATCTTGTT	58.4	45.5	22			
34	Contig3606	Transcription factor VIP1-like	F: TTAAACCCCTGATGTTAATGGC	56.5	40.9	22	1	300	0
			R: CTGAGAGATGCCTTGAATGAAA	56.5	40.9	22			
35	Contig4208	PHD and RING finger domain-containing 1	F: CTCACCTCTTTCATTGCCTCTT	58.4	45.5	22	0	0	0
			R: AGACAGCTTTGGTGGTTTTGTT	56.5	40.9	22			
36	Contig4543	Cyclin-dependent kinase inhibitor 7	F: CAGTGTGTGCTTGTTGGTACAC	60.3	50	22	1	900	0
			R: TTAAAGCCATAGAAGAATCGCC	56.5	40.9	22			
37	Contig4580	CASP 2A1	F: CCACAATTACACAAGCACAACA	56.5	40.9	22	0	0	0
			R: CAACACCATTAAGAGCATCCAA	56.5	40.9	22			

Table 3 — Details of EST-SSR primers used in present study — Continued

Sr. No	Sequence Id	Sequence Id	Primer sequence	Tm* (°C)	GC%*	Length in bp	Total number of amplified alleles	Size range of amplified fragments (bp)	PIC value
38	Contig4630	Kynurenine formamidase	F: TCGGAGTTATTAGCGAGAGACC	60.3	50	22	1	1000	0
			R: CCAAATTCCAACTGACCACTC	57.9	47.6	21			
39	Contig4686	kDa class I heat shock -like	F: AGTCGCACACCTCATCCTTTAT	58.4	45.5	22	0	0	0
			R: CCTGATGTTAAGGCCATTCAG	57.9	47.6	21			
40	Contig4779	Subtilisin-like protease	F: ACTCTGCATCACAAACCAACC	57.9	47.6	21	1	100	0
			R: AGCTTTCGCCTGGAAGTAGTAG	60.3	50	22			
41	Contig4898	ethylene-responsive transcription factor ERF034-like	F: TAATACTCCCACAAGAACGGCT	58.4	45.5	22	1	500	0
			R: CTTCTGCTTCTCCCAAAGACAT	58.4	45.5	22			
42	Contig4999	GDSL esterase lipase At5g18430-like	F: TCGGTGGAGAACTGGAGATAGT	60.3	50	22	0	0	0
			R: AACTTGTGCCCTAACAGAGGTC	60.3	50	22			
43	Contig5053	Ethylene-responsive transcription factor 4	F:AACTTTACTTAGGTGCTCTCAACG	59.3	41.7	24	1	900	0
			R:TCATCAGCAGTACCATCAACAA	56.5	40.9	22			
44	Contig5116	Gamma-interferon-inducible lysosomal thiol reductase	F: CTAGAACTCGAACCATTGTGGA	58.4	45.5	22	0	0	0
			R: GGGTTTTCAGGTAATTCACAGC	58.4	45.5	22			
45	Contig5166	Myb-related 306-like	F: CTAGAACTCGAACCATTGTGGA	58.4	45.5	22	0	0	0
			R: GGGTTTTCAGGTAATTCACAGC	58.4	45.5	22			
46	Contig5178	Calcium-binding EF-hand	F: AGAACCCGTAGGTCTCTTCACA	60.3	50	22	0	0	0
			R: CTCCCATCTCCTTCTCTTCGTA	60.3	50	22			
47	Contig5266	Dof zinc finger -like	F: TATGCACCATTTGTTGTAGGGA	56.5	40.9	22	1	700	0
			R: TGAGTCTTCTGGAGCTGGAAAT	58.4	45.5	22			
48	Contig5509	Probable WRKY transcription factor 35	F: TTGGATCAAGAAGGGACAAAAG	56.5	40.9	22	0	0	0
			R: CACATCCCCTGTGTTACTCTCA	60.3	50	22			
49	Contig5732	Transmembrane emp24 domain-containing p24delta3-like	F:TTGGTGAGTAAAGGTGCTAAACTG	59.3	41.7	24	1	200	0
			R: ATTGGGAATCAGTAGCGAGAAA	56.5	40.9	22			
50	Contig6002	Xylosyltransferase 1	F: TCCCAGTTGTTTGTTCTTACACC	58.9	43.5	23	1	400	0
			R: GATTTGATGATGGATGTGATGG	56.5	40.9	22			
51	Contig6005	Target of Myb 1	F: CTGCTGAAGGTATTGTGCTTGT	58.4	45.5	22	0	0	0
			R: GTCCAAGGGTCATCTATGGGTA	60.3	50	22			
52	Contig6066	EXORDIUM-like 3	F: GACGGTTATATCGGCAGTTAGG	60.3	50	22	1	400	0
			R: CACTCTCTCTCCTCTCGACTCC	64.0	59.1	22			
53	Contig6098	3-ketodihydrosphingosine reductase-like	F: TCCATTGATCCTTCTCCTCCTA	58.4	45.5	22	1	700	0
			R: AGGTTCTTCCTGGTATGGTTGA	58.4	45.5	22			
54	Contig6191	Probable WRKY transcription factor 31	F: CCAAAACCCTGATTGACACTTT	56.5	40.9	22	1	600	0
			R: GCCATCTTCATTTGCTGATACA	56.5	40.9	22			
55	Contig6296	Clathrin heavy chain 1	F: AAGGTTGATTCGGCATACTCAT	56.5	40.9	22	1	500	0
			R: CTGTTCGCTTTTGAGAGGGTT	57.9	47.6	21			
56	Contig6536	Calcineurin B 3 isoform X1	F: ATTTCGGGGTCTTCTAATCCTC	58.4	45.5	22	1	900	0
			R: GTAACACCACCATCGCCA	56.0	55.6	18			
57	Contig6584	Inorganic phosphate transporter 2-chloroplastic	F: GCCATATAAAACCCAAACAGGA	56.5	40.9	22	0	0	0
			R: GTGAATCCCATTATCTCCCAAA	56.5	40.9	22			
58	Contig6662	Scopoletin glucosyltransferase-like	F: AATGCAACGTACAACACAGACC	58.4	45.5	22	1	1000	0
			R: CTATTCGGATCTCAAACGAAGG	58.4	45.5	22			
59	Contig6728	Homeobox-leucine zipper ANTHOCYANINLESS 2 isoform X1	F: CACCACATTTACTCTGCACACC	60.3	50	22	1	100	0
			R: CCTGACGATGACCTTTCAGATT	58.4	45.5	22			
60	Contig6849	Secoisolariciresinol dehydrogenase-like	F: CCGGCCAGTTCTTATCAATCT	57.9	47.6	21	1	700	0
			R: GACAGTACGGGATACGTGTGAA	60.3	50	22			
61	Contig6961	BEL1-like homeodomain 4	F: CATGACTATCTGCTCTCACCCA	60.3	50	22	1	200	0
			R: CCTCCAGCTCACTCAACTTCTT	60.3	50	22			
62	Contig7145	C2 domain-containing At1g53590 isoform X2	F: TCAAGTTCACCCATCTTCACC	57.9	47.6	21	1	800	0
			R: AAGGCATGGCAAAGAGTTTCTA	56.5	40.9	22			
63	Contig7190	Peptidyl-prolyl cis-trans isomerase	F: TGCTTCCATTCTAACTGTGACG	58.4	45.5	22	1	500	0
			R: CCCAACCAACGAATCAAGTATT	56.5	40.9	22			
64	Contig7226	PTI1-like tyrosine- kinase 1 isoform X1	F: AGCTCCAGCAACAAAGAACTTG	58.4	45.5	22	3	200-400	0.52
			R: GTTCCGTCCAAATATGAGCATT	56.5	40.9	22			
65	Contig7348	CD2 antigen cytoplasmic tail-binding 2-like	F: AGTAAATCTCTTTGGCCCTTCC	58.4	45.5	22	1	300	0
			R: TTTGATGAGGCTTCTGGGTACT	58.4	45.5	22			
66	Contig7410	kxDL motif-containing 1	F: ATATCTTCTAGGGCGGAGCAA	57.9	47.6	21	1	100	0
			R: ACACACGCCTTTTACGGTCTAT	58.4	45.5	22			
67	Contig7555	Eukaryotic translation initiation factor 3 subunit H	F: ATAACCAGTCCCTCGATCTGAA	58.4	45.5	22	0	0	0
			R: CCTCCTTCAATTTCTCTTTGGA	56.5	40.9	22			
68	Contig7736	Nudix hydrolase chloroplastic	F: TTTGGGTTCTCATAGGCAATCT	56.5	40.9	22	0	0	0
			R: TCTCCTTCATTTCTTCCGTCTC	58.4	45.5	22			
							341		

was also subdivided into two sub-clusters, i.e. B1 and B2 at 0.69% similarity. The sub-cluster B1 was found to contained four cultivars viz 'Fern', 'Pride', 'Belrubi' and 'Gorella' whereas sub-cluster B2 contained only three cultivars viz Chandler', 'Shasta', 'Confectura'. Among these maximum similarity of 94% was found between 'Shasta' and 'Confectura' (Fig. 1, 2 & 3).

Combined dendrogram of morphological and molecular data based on Euclidian distance matrix also form two groups; Group A contained 17 cultivars *viz* 'Selva', 'Red coat', 'Addie', 'Etna', 'Majestic', 'Douglas', 'Dilpasand', 'Katrain Sweet', 'Sweet Charlie', 'Dana', 'Brighton', 'Tioga','Fern', 'Pride', 'Shasta', 'Confectura' whereas 'Belrubi', 'Gorella' and 'Chandler', placed into a separate group (Fig. 4).

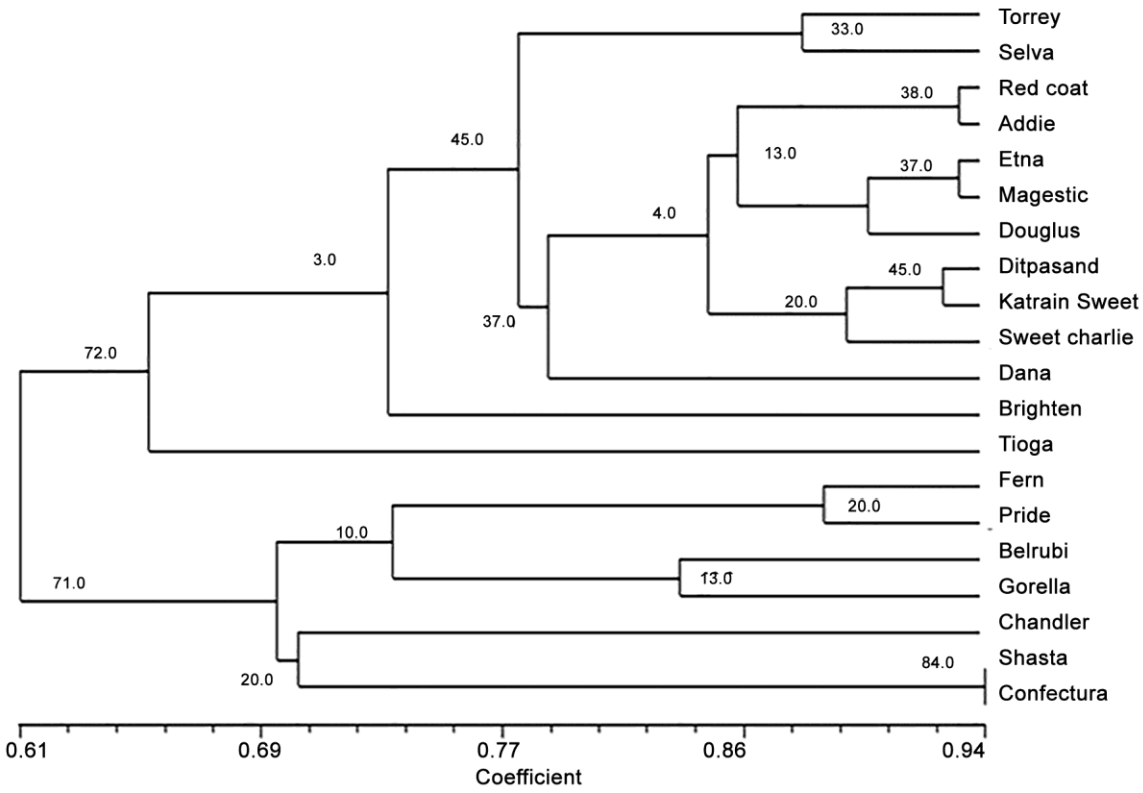

Fig. 1 — Dendrogram of 20 varieties of strawberries based on 68 EST-SSR primers of *Rubus ellipticus*

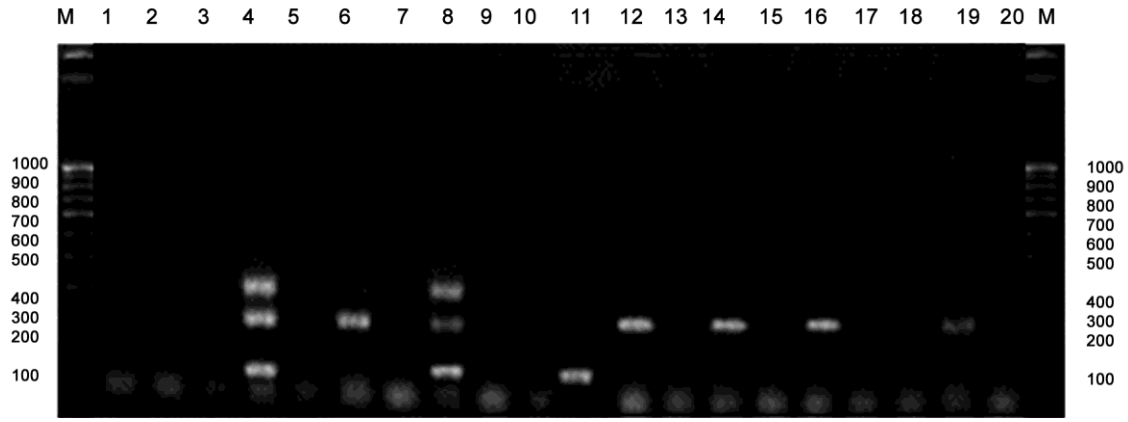

Fig. 2 — Gel image of EST-SSR contig 397 primer

Bootstrap analysis was also carried out to check the robustness of the generated individuals. Robust clusters (60%) were discovered between many cultivars. The number at the fork in the dendrogram represented the number of times the group consisting of the cultivars below that fork occurred (Fig. 4). Maximum similarity was found between 'Shasta' and 'Confectura' but our main concern is to find out genetically distinct cultivars. Categorization of cultivars using molecular analysis determines genetic similarities and dissimilarities which help in the selection of genetically diverse parents (selected from different clusters instead from same cluster) for breeding lines development from multiple crosses. Such recombination breeding program will increases the yield and also broaden the genetic base for wider adaptability due to the involvement of already adapted cultivars.

From this study, it was revealed that EST-SSRs based molecular characterization serve as a potential

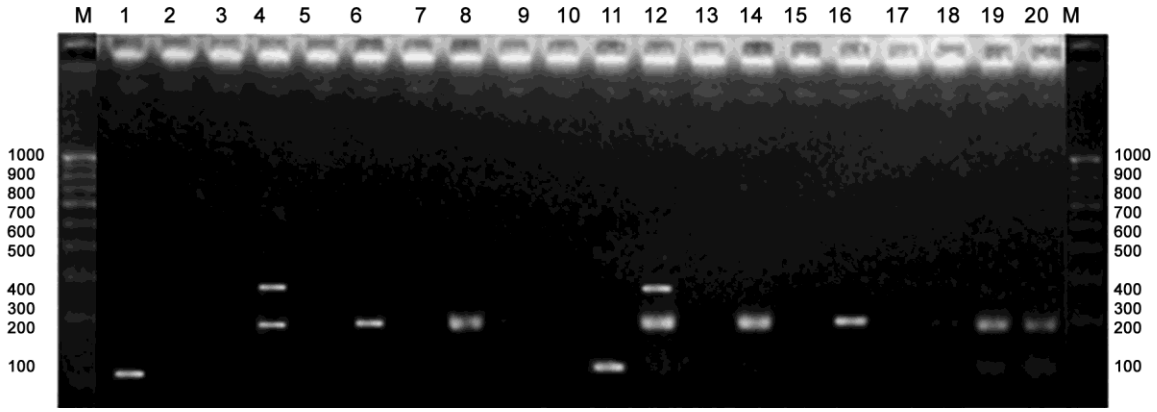

Fig. 3 — Gel image of EST-SSR contig 11 primer

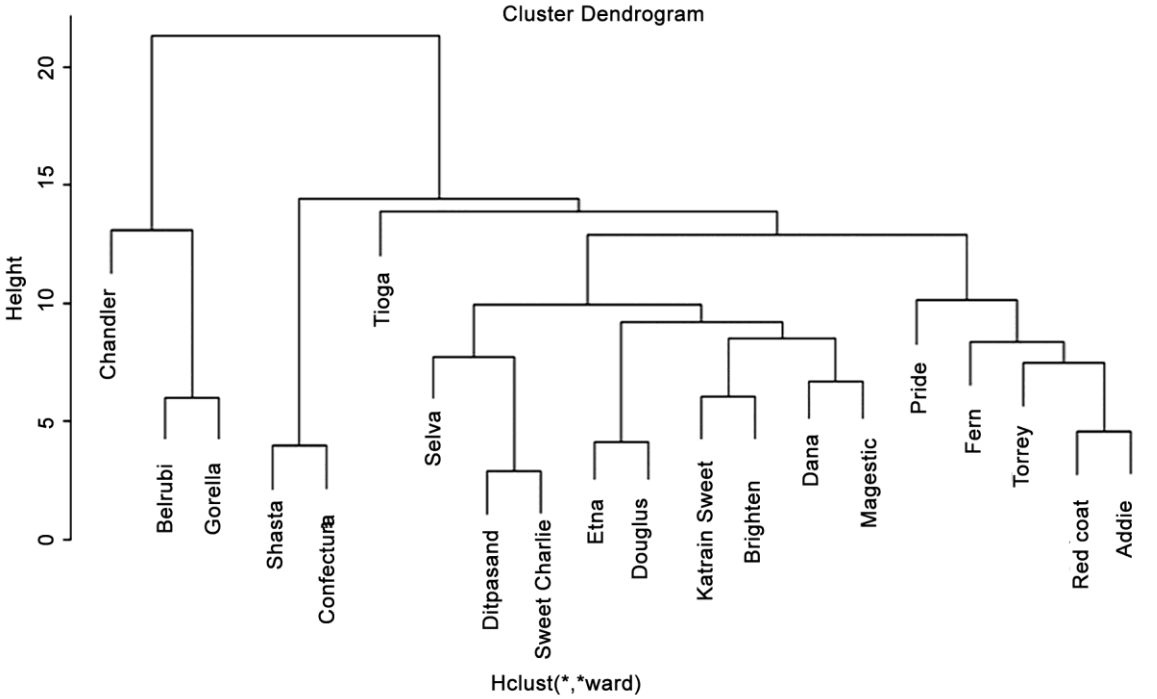

Fig. 4 — Morphological and molecular based Euclidian dendrogram

source in the detection of genetically distinct cultivars as well as well as sorting of duplication for morphological close cultivars. The utilization of 68 EST-SSR markers revealed a high level of genetic polymorphism and these 51 EST-SSR markers gave sufficient information for unambiguous identification of 20 strawberry cultivars.

Conclusion

It is important to assess the genetic background of germplasm for better utilization of genetic resources. For well planned conservation and management strategies in strawberry, information related to genetic differences between cultivars is essential. Sometimes, uncertain diversity and artificial groups of species create confusion during classification of strawberry cultivars, both at scientific and commercial levels. So, the use of microsatellite markers and morphological descriptors showed major impact on genetic analysis of strawberry cultivars. Our results show that the strawberry cultivars of present study were relatively untapped. There is no doubt that these unique EST-SSR markers of *R. ellipticus* will be helpful in cultivar identification, QTL analysis and population structure

detection. In addition, the genetic diversity analysis is a pre-requisite for exploration and utilization of cultivars for breeding programs.

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