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Database Derived Microsatellite Markers (SSRs) of *Stevia rebaudiana* for Cross-transferability Testing Across Species in Family Asteraceae

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Authors' contributions

This work was carried out in collaboration between all authors. Author Poonam carried out the experiments and managed the literature searches. Author Shilpa performed the statistical analysis and wrote the first draft of the manuscript. Author NS assisted the experiments and author RK designed the study. Author Samriti helped to carry out the experiments. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Simple sequence repeat markers derived from expressed sequences tags (ESTs) were used to carry out transferability studies across members of Asteraceae family.
Study Design: NTSY Spc ver.2.0 was used to construct similarity matrix and dendrogram.
Place and Duration of Study: Present study was undertaken in Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India -173230 from 2013-2015.
Methodology: EST-SSRs were used for PCR amplification of genomic DNA of Asteraceae family members. The data was compiled in the form of per cent polymorphism depending on polymorphic and monomorphic bands. Similarity matrix was generated to find out per cent similarity between species and dendrogram represented visual phylogenetic tree of species used.

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Results: In this study we have studied the level of transferability of 45 *Stevia* EST-SSRs in 20 members of Asteraceae family, representing 20 genera of three subfamilies of Asteraceae. All selected 45 primers generated polymorphism. Transferability of the EST-SSRs ranged from 6.5% in *Dimorphothica sinuata* to 75.5% in *Tagetes erecta*, both of which belong to subfamily Asteroideae. Narrow base of studied material was depicted as similarity matrix values ranged from 0.00 to 0.30. Dendrogram was divided into two main clusters. Cluster 'I' contained only one genotype i.e. '*Dimorphothica sinuata*', while Cluster 'II' consisted rest 20 species.

Conclusion: Present study targeted those important members of Asteraceae family which were not undertaken all together for molecular studies. As number of sequences in database repository for Asteraceae family is low, thus utilizing the repository from one crop to other members of same family is the only way to carry forward molecular research. Overall our findings suggested that transferability of *Stevia* EST-SSRs across Asteraceae genera is varied, yet valuable, thereby providing a good set of markers for genetic diversity, molecular mapping, gene tagging and comparative mapping studies.

Keywords: Markers; amplification; polymorphism; transferability.

1. INTRODUCTION

The Asteraceae family is one of the largest and most diverse plant families comprising more than 23000 species [1]. Most recent molecular phylogenetic analysis of the family recognized 12 subfamilies, though three of these (Asteroideae, Cichorioideae and Carduoideae) account for nearly 95% of the species. Though medicinally very important, unfortunately Stevia could not catch the attention of molecular biologists. Molecular marker represent a valuable resource for genetic analysis of Stevia and related species and also have the potential to facilitate comparative map based analysis across of the species within the Asteraceae family. Simple sequence repeats (SSR) or microsatellites are regions of genome whereas a few bases are randomly repeated and are the markers of choice in genetics and breeding studies due to their multiallelic nature, co dominant inheritance, high abundance, reproducibility and transferability over genotypes and genome- wide coverage [2-3]. SSR markers are categorized into two classes, based on their origin: genomic which are developed from enriched DNA libraries and genic SSRs or expressed sequence tags (EST)-SSRs, derived from EST sequences originating from the expressed region of the genome which are submitted in public domain as cDNA clones. The development of genomic SSRs is tedious, expensive and time consuming, while EST-SSRs are easier to be searched *in silico* for a particular organism and these have been reported to be transferable across different relatives. Because of these issues related to genomic SSRs, many researchers have attempted to use EST-SSRs developed from one species for studies on related species and genera. In fact, ESTs are one of the powerful tools of genomics research. Transferability of markers from one genus to another genus helps in comparative genetic analysis such as comparative mapping and evolutionary studies. Comparative genetic analysis has shown that different plant genera often share orthologous genes for very similar functions and gene content and gene order among different plant genera are highly conserved. Therefore, data mined EST-SSRs were screened for polymorphism and tested for their transferability across the genera in Asteraceae family.

2. MATERIALS AND METHODS

2.1 Source Plant Material and DNA Isolation

The plant material i.e. 21 species of different genera of Asteraceae family, which include eight medicinal plants 12 ornamental plants and one vegetable crop (Table 1) was collected from experimental fields. Young and healthy leaves were excised from the plants in the field and brought to laboratory in ice box and stored in deep freezer at -80ºC till further use. Genomic DNA was isolated using CTAB method of Doyle and Doyle [4] with some modifications, followed by further purification.

2.2 PCR Amplifications and Gel Electrophoresis

Concentration of different components standardized for EST-SSR-PCR in 20 µl of reaction mixture is as follows: 1X PCR buffer, 2.2 mM $MgCl_2$, 1 mM dNTPs, 35 µM each primer (forward and reverse), 1U Taq DNA Polymerase,

50 ng template DNA following a thermal profile as: 5 min of initial denaturation at 95℃ followed by 40 cycles of 1 min denaturation at 94℃, annealing varied with Tm of each primer for 1 min and extension of 2 min at 72℃, further followed by final extension of 5 min at 72℃. The amplified DNA was electrophoresed in 2% agarose gel in 1X TAE buffer (40mMTris-acetate, 1.0 mM EDTA).

2.3 SSR Studies for Cross-transferability and Data Analysis

Out of 21 genotypes, *S. rebaudiana* was initially used for amplification of 45 EST-SSRs. Primers which gave polymorphism with *S. rebaudiana* genotypes were screened out to check their transferability across 20 species of Asteraceae family. Then the percentage of polymorphism (number of polymorphic bands/ total number of bands) and cross transferability (number of polymorphic primers/ total number of primers examined) was calculated. All Gel images were transformed into binary matrix and were analysed using NTSYSpc ver.2.0 [5]. Dendrogram was created for the results obtained and compared for the efficiency of generation of polymorphism by EST-SSRs in all the species under study.

The polymorphism information content (PIC) values provided 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 and was calculated using the formula: PIC= 1-Σpi^2, where *pi* is the frequency of the *i*th allele [6].

3. RESULTS AND DISCUSSION

3.1 Survey of Polymorphism and Cross Transferability Studies

45 EST-SSRs (Table 2) published by Kaur et al. [3] were tried for amplification on *S. rebaudiana.* All these primers generated amplification. After this, these primers were tried for cross-genera portability in 20 genera of Asteraceae family. *S. rebaudiana* was again used along with 20 genera to confirm amplification. Each of the 45 primers was able to amplify the genomic DNA of at least one genotype. Total 166 amplified bands were scored with 45 primer pairs in 21 genotypes of Asteraceae family including *S. rebaudiana*. Maximum number of amplified bands i.e. seven was produced by primer 'P4', 'P15' and 'P25' and minimum number of bands i.e. one was produced by primer 'P20', 'P37' and 'P40'. A total of 166 scorable bands were found (Table 2). Average number of polymorphic bands per primer was recorded to be 3.67. Average number of amplified fragments per accession was 24.09 and average number of amplified fragments per informative primer was 11.24. A total of 506 fragments were amplified in all the 21 genotypes. Primer 'P15' amplified maximum number of fragments i.e. 39 where as minimum number of fragments i.e. one was given by primers 'P37' and 'P40'. '*Tagetes erecta'* produced maximum number of amplified fragments i.e. 63, while '*Chrysanthemum coronarium*' produced minimum number of amplified fragments i.e. two with all primer pairs used. PIC value of *Stevia* EST-SSR primers ranged from zero in primer P20, P37 and in P40 to 0.83 in primer P15 with an average value of 0.415 (Table 2).

Table 1. List of genotypes used in present study

S. no.	Medicinal plants	Sub family	S. no.	Ornamental crops	Sub family
1.	*Stevia rebaudiana*	Asteroideae	10.	*Callistephus chinensis*	Asteroideae
2.	*Spilanthes acmella*	Asteroideae	11.	*Calendula officinalis*	Asteroideae
3.	*Eclipta alba*	Asteroideae	12.	*Chrysanthemum coronarium*	Asteroideae
4.	*Echinacea angustifolia*	Asteroideae	13.	*Tagetes erecta*	Asteroideae
5.	*Achillea millefolium*	Asteroideae	14.	*Helichrysum bractiatum*	Asteroideae
6.	*Artemisia annua*	Asteroideae	15	*Arctotis stoechodifolia*	Cichorioideae
7.	*Matricaria recutita*	Asteroideae	16.	*Dimorphothica sinuate*	Asteroideae
8.	*Silybum marianum*	Asteroideae	17.	*Acroclinum roseum*	Asteroideae
	Vegetable crop		18.	*Brachyscome dichromosomatica*	Asteroideae
9.	*Lactuca sativa*	Cichorioideae	19.	*Centaurea cyanus*	Carduoideae
			20.	*Bellis perennis*	Asteroideae
			21.	*Anthemis coluta*	Asteroideae

Table 2. List of SSR primer pairs for *S. rebaudiana*

Primer code	Primer sequence	Tm (℃)	GC%	PIC value	Number of alleles
P1	F: CTCATAATCTGCCGCTCACA	59.97	50.00	0.64	10
	R: CTTGCTAGGGTTCCACTTCG	59.87	55.00		
P2	F: ATGAAAGCGAGCCTGATGAT	59.80	45.00	0.22	8
	R: TCAAGCAACGATTCTTTCCA	59.40	40.00		
P3	F: CTCATAATCTGCCGCTCACA	59.97	50.00	0.54	17
	R: CTTGCTAGGGTTCCACTTCG	59.87	55.00		
P4	F: AATACAAACAGGGCGAGTGC	60.14	50.00	0.82	21
	R: TGGATATTGACTGCCACCAA	59.92	45.00		
P5	F: CTTCAGGACGATGGTGAGGT	60.11	55.00	0.2	9
	R: GGCGGATGATAGACTCGAAA	60.18	50.00		
P6	F: CTTTCCGTCAGGAGTTCAGC	59.99	55.00	0.19	13
	R: AATGGCAATTCCACGAAGAG	60.07	45.00		
P7	F: AGAGGCGAGCGGTGTATCTA	60.00	55.00	0.71	10
	R: GAAAACGCCTGGAATCAGAG	59.81	50.00		
P8	F: GCGGGTTTCAGTGTATTCGT	60.00	50.00	0.66	20
	R: TCCATACAGGATGTGCCTCA	60.07	50.00		
P9	F: CAAAGAAAGGCTCCCATCAA	60.18	45.00	0.76	33
	R: TTTCTGTGGAGTTGCAGGTG	59.87	50.00		
P10	F: GGGAAACATGGGAAGAACAA	59.77	45.00	0.69	7
	R: CCGGTGTGATTTGCCTTACT	59.99	50.00		
P11	F: TGGTGGCGTGTCATCATACT	59.99	50.00	0.80	20
	R: GCATGTCGCATAATGTGGTC	59.96	50.00		
P12	F: GCCCTGCTTAAGCTTTGATG	59.98	50.00	0.56	5
	R: TACAAGTTCCCGACGTTTCC	59.97	50.00		
P13	F: GAACAACTTCGCGTTTTCGT	60.29	45.00	0.58	14
	R:AGGGTGGTGTATGGAAGCAG	59.99	55.00		
P14	F: ACCACCACCGCTAATGAGAC	60.00	55.00	0.19	9
	R: GCACTCCTCGTCGCTAGAAC	60.16	60.00		
P15	F: GTAAACGGTACCCGCAAAGA	60.00	50.00	0.83	39
	R: CCACATGATCCCCCATAAAG	60.01	50.00		
P16	F: ACCACCACCGCTAATGAGAC	60.00	55.00	0.78	10
	R: GCACTCCTCGTCGCTAGAAC	60.16	60.00		
P17	F: CCATCATCATCCTCCTCCTC	59.41	55.00	0.80	23
	R: TCGTTTGGCAGCTAAAGGTT	59.88	45.00		
P18	F: ACCACCACCGCTAATGAGAC	60.00	55.00	0.77	7
	R: GCACTCCTCGTCGCTAGAAC	60.16	60.00		
P19	F: CGTCCTTTGTTTTGCAACCT	60.15	45.00	0.79	13
	R: GTCGAATTTGGAGGAAACGA	60.05	45.00		
P20	F: ACGGTTTTTCATGGCTCAAG	60.11	45.00	0	3
	R: TCGGTGATTAAAAGCGATGA	59.26	40.00		
P21	F: CTCATAATCTGCCGCTCACA	59.97	50.00	0.77	32
	R: CTTGCTAGGGTTCCACTTCG	59.87	55.00		
P22	F: CGGCCTCCTACAAACCCTAT	60.33	55.00	0.72	6
	R: GTTTGTTCTTCGCGGTTGAT	60.12	45.00		
P23	F: CTCATAATCTGCCGCTCACA	59.97	50.00	0.62	28
	R: CTTGCTAGGGTTCCACTTCG	59.87	55.00		
P24	F: TCTTGCAAGTGTGAGGAGCA	60.74	50.00	0.66	3
	R: TTTTGTTGACCAGCGTTGAC	59.74	45.00		
P25	F: TGGTCGAGTGGTGGTGACTA	60.15	55.00	0.82	26
	R: TTTGAGCCCCCATAGTTCTG	60.07	50.00		
P26	F: CCGTAAACCGAAAGGTCAAA	59.97	45.00	0.65	21
	R: CATGAACGAAATGGCTGAAA	59.66	40.00		

P27	F: GTGGGTGATGAGTCAGCAAA	59.68	50.00	0.71	9
	R: AGGCCTCAGCTTCTGACGTA	60.16	55.00		
P28	F: ACCACCACCGCTAATGAGAC	60.00	55.00	0.71	16
	R: GCACTCCTCGTCGCTAGAAC	60.16	60.00		
P29	F: GAGCAGGATGGGTGAAAAGA	60.20	50.00	0.77	11
	R: CAATCGCCTCTTTCTCTTGC	60.10	50.00		
P30	F: TCAAGTTAGGGTTCCGTTCG	60.10	50.00	0.44	3
	R: GCCGTTTTTCGTATCCTTCA	60.07	45.00		
P31	F: TGCTTTACACACGCACACAA	59.94	45.00	0.23	9
	R:GGCGGAGAGTATGAAGAGACC	60.23	57.14		
P32	F: GCGCACAATGAAACCCTAGT	60.14	50.00	0.50	2
	R: AAGCAGAAGGGGGATCATCT	60.04	50.00		
P33	F: CATGAACGAAATGGCTGAAA	59.66	40.00	0.62	4
	R: CCGTAAACCGAAAGGTCAAA	59.97	45.00		
P34	F: TCCAAAACCGTTTGTTTTCC	59.82	40.00	0.66	3
	R: CAGAACTATGGGGGCTCAAA	60.07	50.00		
P35	F: ACATGAAAAACCATGCGTCA	59.97	40.00	0.24	7
	R: ATGGTGTGGATTTTGGTGGT	59.95	45.00		
P36	F: TCAAGTTAGGGTTCCGTTCG	60.10	50.00	0.66	3
	R: GCCGTTTTTCGTATCCTTCA	60.07	45.00		
P37	F: TCAAGTTAGGGTTCCGTTCG	60.10	50.00	0	1
	R: AGCCGTTTTTCGGTATCCTT	59.97	45.00		
P38	F: CTCATAATCTGCCGCTCACA	59.97	50.00	0.66	3
	R: CTTGCTAGGGTTCCACTTCG	59.87	55.00		
P39	F: GTTCGGTTTGTTACGCGAAT	60.00	45.00	0.37	4
	R: GAATGAGGTAGTGGGGTCCA	59.78	55.00		
P40	F: AGGTAACACCTGCCCATCAG	59.99	55.00	0	1
	R: CCAGATGGAACCGAAGCTAA	60.21	50.00		
P41	F: CGGTCCACCAAGTCCTAAAA	59.96	50.00	0.44	3
	R: AAAGTATTCCAGCGGTGGTG	59.99	50.00		
P42	F: GTCGATCCCGTAGGAGGAAG	60.98	60.00	0.50	2
	R: CATCGCTGCTACCATCTGAA	59.97	50.00		
P43	F: CACTTCTTCCGGTTGTTGGT	60.01	50.00	0.37	6
	R: ACACTTGAACCTCCCATTCG	59.97	50.00		
P44	F: TATCACAACGCCCCAAAAAC	60.73	45.00	0.66	9
	R: TTCTGTTCAAACTCCGACGA	59.41	45.00		
P45	F: GGAAAGAATGCCGAATTTGA	48.00	40.00	0.44	3
	R: TGAGGATGAAGACGATGCTG	52.00	50.00		

Maximum 21 and minimum three polymorphic primers were produced in '*Chrysanthemum coronarium'* and '*Arctotis stoechodifolia'*, respectively. Same pattern was followed in others parameters like total number of scorable bands and total number of amplified fragments. While '*Spilanthes acmella'* scored maximum two average number of amplified fragments per informative primer (Table 3).

Per cent transferability varied from 6.6 % to 75.5 % representing a broad range obtained with same primers in different members. Some earlier findings also supported the transferability of SSRs is high between species of the same genus and between closely related genera, as compared that between distant genera of the same family [7-10]. For low level of transferability i.e. 6.6% in *Dimorphothica sinuate*, 8.8% in *Calendula officinalis* and 15.5% in *Bellis perennis* missing homologous genic regions in these members may be the reason. Reports with 16% - 19% transferability by Folta et al. [11], Sargent et al. [12], Mnejja et al. [13] and Lewers et al. [14] were in agreement with present findings. While presence of some homologous regions may be the cause for transferability not too high nor too low i.e. 20% in *Echinacea angustifolia, Silybum marianum* and *Acroclinum roseum*, 26.6% in *Spilanthes acmella, Callistephus chinensis*, 31.1% in *Centaurea cyanus,* 33.3% in *Achillea millefolium*, *Arctotis stoechodifolia,* 35.5% in *Anthemis coluta,* 37.7% in *Chrysanthemum coronarium*, 40.0% in *Brachyscome dichromosomatica*, 44.4% in *Eclipta alba*, *Helichrysum bractiatum* and 46.6% in *Lactuca*

sativa. Findings of Gasic et al. [15] and Yasmin et al. [16] with 28 and 28.7% transferability supported these results. High level of transferability was observed in three members 51.1% in *Artemisia annua*, 64.4% in *Matricaria recutita* and 75.5% in *Tagetes erecta* which may be due to high degree similarity in sequences. Almost same percentages of transferability were obtained by various workers in their respective crops i.e. 44.4% to 68% between peach and apricot by Vendramin et al. Hormaza et al. Romero et al. and Zhebentyayeva et al. [17-20], 63.9% of peach 59.1% of apricot to other members of same family by Mnejja et al. [13], 20% by Messina et al. [21] from apricot SSRs to *Prunus* species.

In general EST-SSRs have been found to be significantly more transferable across taxonomic boundaries and perhaps this is one of the most important features of the EST-SSR markers [22-23]. Transferability of EST-SSR markers to related species and genera has been demonstrated in several studies [2,24-25]. The transferability of microsatellites within related species is extremely beneficial for the research community as it speeds up the process of generating linkage maps. Vanwynsberghe et al. [26] and Mnejja et al. [13] also reported significant transferability of EST-SSRs to members of same family.

3.2 Data Analysis

The coefficient values ranged from 0.00 to 0.300. This indicated a low range of genetic variability, suggesting a narrow base of the genotypes taken. The highest value of similarity 0.300 was found between genotypes '*Silybum marianum*' and '*Echinacea angustifolia*'. Minimum similarity was 0.00 obtained between many genotypes. In the dendrogram (Fig. 1), the 21 genotypes separated into two main clusters, 'I' and 'II', at two per cent similarity. Cluster 'I' was found to contain only one genotype i.e. '*Dimorphothica sinuata*' and Cluster 'II' accommodated rest 20 genotypes. Cluster 'II' was further subdivided into two clusters at similarity value of 9% (Fig. 1). It was concluded that '*Echinacea angustifolia*' and '*Silybum marianum*' were closely related as they showed 30 per cent similarity.

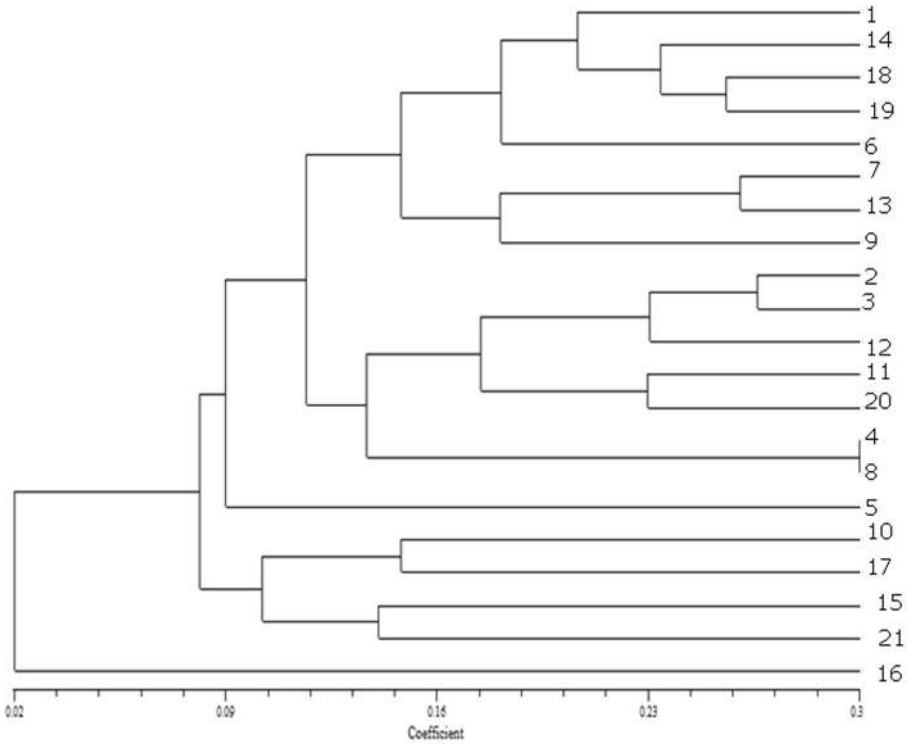

Fig. 1. Dendrogram of 21 species of different genera of *Asteraceae* family based on EST-SSR analysis; 1-21: Labels as described in Table 1

Table 3. Summary of EST-SSR amplified products obtained from 20 species of different genera of Asteraceae family examined in study

	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Total no. of primers examined	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45	45
Number of informative primers	12	20	9	15	23	29	9	12	4	17	34	20	15	3	9	18	14	7	16	21
Number of polymorphic primers	12	20	9	15	23	29	9	12	4	17	34	20	15	3	9	18	14	7	16	21
Total number of scorable bands	24	34	13	19	27	55	13	15	6	30	63	32	16	2	8	22	26	8	26	35
Total number of amplified fragments	24	34	13	19	27	55	13	15	6	30	63	32	16	2	8	22	26	8	26	35
Average number of amplified fragments per informative primer	2	1.7	1.4	1.2	1.1	1.8	1.4	1.2	1.5	1.7	1.8	1.6	1.0	0.6	0.8	1.2	1.8	1.1	1.6	1.6
Per cent transferability of stevia primers to other genotypes	26.6%	44.4%	20%	33.3%	51.1%	64.4%	20%	26.6%	8.8%	37.7%	75.5%	44.4%	33.3%	6.6%	20%	40%	31.1%	15.5%	35.5%	46.6%

2-21: Labels as described in Table 1

4. CONCLUSION

This is the very first study of its own kind which targeted different members of same family which were otherwise underestimated for molecular studies. Present studies demonstrated that cross transferability of EST-SSR producing amplification and polymorphism across members of same family can often be transferred across relatively large taxonomic distances, crossing not just species within a genus, but in some cases across the genera within a family.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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