American Journal of Plant Sciences, 2014, 5, 3089-3096
Published Online September 2014 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2014.520325

An Assessment of Genetic Relatedness between Soybean [*Glycine max* (L.) Merrill] Cultivars Using SSR Markers

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Received 4 July 2014; revised 23 August 2014; accepted 23 September 2014

In this present investigation, simple sequence repeat (SSR) analysis was used to determine the genetic relatedness among 32 soybean (*Glycine max* (L.) Merr.) cultivars from NRC for soybean, Madhya Pradesh. Among them 10 primer pairs showed 100% amplification. Mainly three primer pairs could amplify polymorphic SSRs from all of these cultivars. The polymorphic information content (PIC) among cultivars varied from 0.21 (S26) to 0.83 (S27) with an average of 0.51. Pairwise coefficients of genetic similarity between all genotypes ranged from 0.76 to 1.00. Unweighted pair-group method arithmetic average (UPGMA) analysis allocated the cultivars in 2 major clusters or groups and 6 sub-clusters. Of the two major clusters one contained 20 cultivars and the other contained 12 cultivars. The largest cluster was again divided into three sub-clusters I, II and III with 12, 2 and 6 cultivars respectively and the smallest cluster was divided into three sub-clusters IV, V, VI containing 7, 4 and 2 cultivars respectively. These results suggest that SSR markers are efficient for measuring genetic relatedness among soybeans irrespective of a wide agro-climatic zone. Genetic relationship assessments among soybean cultivars in India could provide useful information for efficient utilization of these materials, especially for widening the genetic base.

Keywords

SSR, UPGMA, Similarity Coefficient, PIC, Genetic Base

1. Introduction

Taxonomically, soybean belongs to the order Fabales, the family Fabaceae, the subfamily Faboidae and the genus glycine. The genus glycine is divided into two subgenera, glycine including 16 perennial species and *Soja*

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How to cite this paper: Ghosh, J., Ghosh, P.D. and Choudhury, P.R. (2014) An Assessment of Genetic Relatedness between Soybean [*Glycine max* (L.) Merrill] Cultivars Using SSR Markers. *American Journal of Plant Sciences*, **5**, 3089-3096.
http://dx.doi.org/10.4236/ajps.2014.520325

(Moench) F.J. Herm having two annual species, *Glycine soja* Siebold and Zucc (2n = 40) and *G. max* (L.) Merrill (2n = 40) [1]. This crop is aptly called as "Golden Bean" or "Miracle Crop" of the 20th century, because of its multiple uses. Apart from quality protein and oil, soybean also has many therapeutic components viz., lactose free fatty acids, antioxidants like vitamins C, K, and D and folic acid, vitamins of B complex group viz., nicotinic acid (23 µg/g), pantothenic acid (15 µg/g), thiamine (12 µg/g), pyridoxine (8 µg/g), riboflavin (3.5 µg/g) and biotin (0.7 µg/g) and isoflavones like genistein and daidzein [2]. Besides fixing the atmospheric nitrogen, this crop has the ability to grow in a range of environments, to reduce soil erosion, to suppress weeds and to suit inter as well as sequential cropping pattern.

With low input demand, this pearl of orient fetched high market price [3]. Yet, the crop is not cultivated over an area it deserves in India especially in the eastern states. This versatile natured crop and the wide yield gap, call for concerted pre-breeding efforts to widen the genetic base suitable to specific agro-climatic conditions.

Genetic diversity is normally assessed by common morphological traits. However, such traits are affected by environmental effects, developmental stages of the plant, and also the type of plant materials. Several replications require establishing the genotypic contributions. Hence, there is a need to go in for a highly reliable and precise method for assessment of genetic variability with no environmental effects. Most diversity studies on cultivated soybean published by now have focused on North American [4]-[6], Asian [7]-[12] as well as South American [13] soybean germplasms. Different PIC values were obtained using different genetic materials of soybean and have been reported by SSR marker studies [8] [14]. Likewise Indian cultivars are checked with high throughput markers like SSRs and SNPs to re-establish its varietal correlations.

Molecular markers have brought phenomenal changes in the area of plant biotechnology by their ability to produce unique DNA profiles in various crops. Simple sequence repeat markers (SSR) are being extensively used in genome studies, marker-assisted selection, and cultivar identification. These are also well-known for their versatility in providing a quick assay and for their highly informative data [15]-[17]. In India several groups are working on soybean varital studies. Ten soybean varieties were evaluated for seven seed traits. Seed coat color, hilum color, seed shape and seed size were stable traits and could be used for development of a varietal identification key [18]. The genetic diversity of the soybean genotypes of north-east region of India has been reported [19]. Soybean diversity pattern may serve as a valuable guide for finding and incorporating new lines into elite soybean genotypes [20]. The objective of this study was 1) to test the relationship among the 32 cultivars of India by SSR analysis, 2) to generate molecular fingerprints of cultivars currently used commercially in India and cluster them into groups according to genetic similarity by using marker analysis techniques. This information should be useful for soybean breeding programs and genetic studies.

2. Materials and Methods

The material used in this study comprised of 32 cultivars of soybean which includes most of the released varieties in India and belongs to different agro climatic zones provided in **Table 1**. The detailed information regarding experimental material along with their pedigree used in the study is given in the **Table 2**. The experiment was conducted during the period of 2010-2012 at Department of Plant Molecular Biology and Biotechnology, DSR, Mau and Department of Botany, University of Kalyani, West Bengal.

2.1. DNA Extraction

Leaf samples were collected from 30 days old plants of soybean varieties (as listed in **Table 2**) raised at Department of Molecular Biology, Directorate of Seed Research (DSR) Mau, Uttar Pradesh. 5 - 8 terminal leaves from each cultivars were collected in an ice box and the collected leaf samples were used to extract DNA following the CTAB [21] method. Isolated DNA was quantified using spectrophotometer (Cecil, Germany). 998 µl of TE buffer was taken in a quartz cuvette and 2 µl of extracted genomic DNA was added to it. The optical density (absorbance A) was taken at 260 nm (A260) and 280 nm (A280). The amount of the DNA present in the solution was calculated from absorption at 260 nm (A260) and the purity of DNA was calculated by A260/A280 ratio. For an ideal DNA preparation the A260/A280 ratio should be ≥1.8. Formula for calculating the DNA concentration:

DNA concentration = spectral reading (A260) × 50 µg/ml × dilution factor for double stranded genomic DNA, 1 O.D. corresponds to 50 µg/ml of DNA [22].

Table 1. Soybean agro-climatic zone in India.

Agro-Climatic Zone	States Covered under the Zone	Varieties
North Hill Zone	HP, Uttaranchal	Bragg, Palam Soya, Shivaleek, Lee, Punjab 1, Pusa 20, VL Soya
North Plain Zone	Punjab, Haryana, Delhi, NE Plains of UP, Western Bihar	Bragg, PK416, Shilajeet PS1024, Ankur, Punjab 1, Alankar
Central Zone	MP, Bundelkhand Region of UP, Rajasthan, Gujarat, Western Parts of Maharashtra	JS95-60, Ahilya 1, Durga, Gaurav, Gujarat 202, Kalitur, Type-49, Punjab 1, Bragg, NRC7, DS228
Southern Zone	Karnataka, TN, AP, Kerala, Southern Parts of Maharashtra	TAMS98-21, Bragg, Monetta, Hardee, Improved Pelican, ADT 1, Sneha, Co Soya 2
North Eastern Zone	Assam, West Bengal, Meghalaya, Eastern Bihar, Orissa, Chhattisgarh	Bragg, Birsa Soya

Table 2. List of experimental materials.

Sl No.	Name of Variety	Pedigree
1.	Gujarat 202	-
2.	Pusa 20	Bragg × Lee
3.	IP	Tanloxi × P.I60406
4.	PK 416	UPSM 534 × PK-317
5.	Shilajeet	Selection from EC 9309
6.	Lee	S100 × CNS
7.	Punjab 1	Selection from Nanking Variety
8.	Ankur	SPS from Composite of 22 Crosses
9.	TAMS 98-21	Mutant of JS 80-21
10.	Hara Soya	(Ankur × Himso 330) × Bragg
11.	Ahilya 1	Induced Mutant of Bragg
12.	Kalitur	Land Race
13.	JS-71-05	Selection for Lee Type Exotic Material
14.	Type 49	Selection from Indigenous Material
15.	PS 1024	PK-308 × PK-317
16.	Co Soya 2	UGM-21 × JS-335
17.	Hardee	D49-772 × IP
18.	Monetta	An Exotic Variety EC-2587
19.	Gaurav	EC-11437 × Bragg
20.	Vl Soya 1	Mutant of Bragg
21.	ADT-1	Selection from Hill Variety
22.	Durga	Selection from EC 172576
23.	Palam Soya	Land Race
24.	DS-228	JS 335 × DS181 (PI 4623133)
25.	JS-95-60	Selection from PS 73-22
26.	Alankar	D63-6049 × D61-4249
27.	Raus-5	-
28.	Birsa Soya 1	-
29.	Sneha	Hardee × Monetta
30.	Bragg	Jackson × D49
31.	NRC 7	Selection from S69-96
32.	Shivaleek	Selection from PK 7355

$$\text{Dilution Factor} = \frac{998\ \mu\text{l TE buffer} + 2\ \mu\text{l DNA}}{2\ \mu\text{l}}$$

DNA was run on 0.8 percent agarose gel stained with ethidium bromide following a standard method [23] and was visualized in a translluminator and photographed using Gel Doc (BioRad).

2.2. Polymerase Chain Reaction

The PCR (Polymerase Chain Reactions) were carried out in 25 µl volume with the reaction mixture. The quantified DNA diluted and prepared master mix with 10× taq buffer (2.5 µL), Taq DNA pol (.2 µL), Forward and Reverse primer(1 µL), dNTPs (25 µL) PCR was performed at an initial denaturation temperature of 94˚C for 4 min followed by 35 cycles of 1 min denaturation at 94˚C, 1 min annealing at a temperature 47˚C - 55˚C lower than melting point for each primer (**Table 3**) and 2 min extension at 72˚C with a final extension of 72˚C for 7 min using a thermal cycle (*Perkin Elmer gene Amp* 2400 *PCR system*). PCR products were run on 3% MetaPhor^{R} agarose gel (Lonza) [24] [25] with 1% TBE buffer. DNA was visualized via ethidium bromide added to the gels.

2.3. Data Analysis

Amplified fragments were scored as binary data, *i.e.* presence as 1 and absence as 0, for the homologous bands. Diffused bands or bands revealing ambiguity in scoring treated as missing data. Genotypes showing two allelic bands with equal intensity considered as heterozygous for the locus. The polymorphic information content (PIC) values for each primer were calculated using the following formula PIC = $1 - \sum Pij^2$ Where Pi is the relative frequency of j^{th} allele for i^{th} marker, and summed over n number alleles [26]. Genetic relationships among individuals were quantified by the Jaccard's coefficient [27]. It was used to calculate similarity between pairs of accessions, where, $[J = n_{x,y/}(n_t - n_z)]$, $n_{x,y}$ is the number of bands common to sample A and sample B; n_t the total number of bands present in all samples and n_z the total number of bands not present in A and B but found in other samples. Based on the similarity matrix obtained from Jaccard's coefficient, sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) and a principal coordinate (PCA) analysis according to the extracted Eigen vectors using NTSYS-pc software version 2.2 Statistical package [26].

3. Result

Total 15 SSR primers screened and among them 10 SSR primers listed in **Table 3** have shown 100% amplification of all 32 cultivars and 3 primers given clear polymorphism. 8 alleles of length between 50 bp to 150 bp were identified across 10 primer pairs. Total 45 polymorphic bands have been detected across 32 cultivars. PIC a measure of the allelic diversity calculated for primer S26, S27 and S43 are 0.26, 0.83 and 0.46 and hence were the most informative for distinguishing among the soybean genotypes. In **Figure 1(A)** Primer S27 and in **Figure 1(B)** Primer S43 showing the 32 cultivars up lane 1 - 16 (soy1 to soy-16) and down lane 1 - 16 (soy17 to soy32) and M denotes the marker *i.e.* Low range Gene Ruler^{TM} DNA ladder ≠ SM1191/2/3/bp ng/0.5. The dendrogram based on genetic similarities between cultivars showed that (**Figure 2**) there are two major clusters and 6 sub-clusters. Three sub clusters of the larger cluster named as I, II and III with 12, 2 and 6 cultivars and the smaller cluster again divided into three sub clusters named as IV, V, VI with 7, 4 and 2 cultivars. In sub cluster I the cultivars groups together are soy1-Gujarat 202, soy2-Pusa 20, soy-3-Improved Pelican, soy7-Punjab 1, soy8-Tams 98-21, soy10-Hara soya, soy12-Kalitur, soy18-Monetta, soy29-Sneha, soy30-Bragg, soy32-Shivaleek. This is the largest among six sub clusters. In sub cluster II only varieties are soy27-Raus 5, soy28-Birsa soya. Sub cluster III consist of soy21-ADT-1, soy22-Durga, soy23-Palam soya, soy25-JS-95-60, soy26-Alankar, soy31-NRC 7. The smaller cluster consist of 7 members *i.e.* soy4-PK 416, soy5-Shilajeet, soy6-Lee, soy8-Ankur, soy13-JS-71-05, soy15-PS-1024 and soy17-Hardee. V^{th} sub cluster consist of soy14-Type-49, soy16-Co soya-2 soy20-VL Soya soy24-DS-228 and the last VI^{th} is the smallest having only one member soy19-Gaurav. Jaccard coefficient ranged between 0.76 to 1.00. From the Scatter plot expressing genetic distances among the cultivars it is observed that cultivars are genetically very similar. The contribution of first two coordinates (F1:F2) is 93.96%. Contribution of each coordinates (F3, F4) further was found less than 10%. Therefore, the best fittest coordinates has been given to explain the total variation among 32 cultivars (**Figure 3**).

Table 3. SSR primer details.

Primer Code	Sequence (5'-3')	Length (bp)	AT (˚C)
S26	F-5'GCCAAGTCACACACACACCAAG3' R-3'TTTGTTTGATCTATGCAATTGC5'	20 22	55
S21	F-5'AAAAAAGGTTTCACTGGCACT3' R-3'ATTTACGGATCTGCCATTCTC5'	21 21	50
S22	F-5'TTTGGAAATGAGTGAAATGGA3' R-3'GGATTAGATCAGGACACACATACA5'	24 24	50
S23	F-5'AAGGAGGTAAAATGAAACAAACTT3' R-3´TCCCTCCCAAGTTAAAATCAA5'	24 21	47
S27	F-5'GATTTTTTGCCTTCCTTTCTG3' R-3'TTGAACAGCAAGAGTTTGGAC5'	21 21	50
S12	F-5'TTTGGACTCTTTTTAGGGTTAGG3' R-3'GGGCATTTAAGAAGTTGCTCT5'	23 21	50
S43	F-5'CGTTTCATTCCCATGCCAATA3' R-3'CCCGCATCTTTTTCAACCAC5'	21 20	52
S13	F-5'GCTCAGTTATTTGGTTCATATGC3' R-3'TCACAATTAACTGCAAATTTCTTC5'	23 24	50
S1	F-5'TTGCAAAATAGATTCCAATG3' R-3'GACTCTCAGATTGATAATAATTTC5'	20 21	46
S30	F-5'CAGGCTTTTTTCTTTTTCTTCTT3' R-3'CCACACCACTGTCCCTTTGA5'	22 20	50

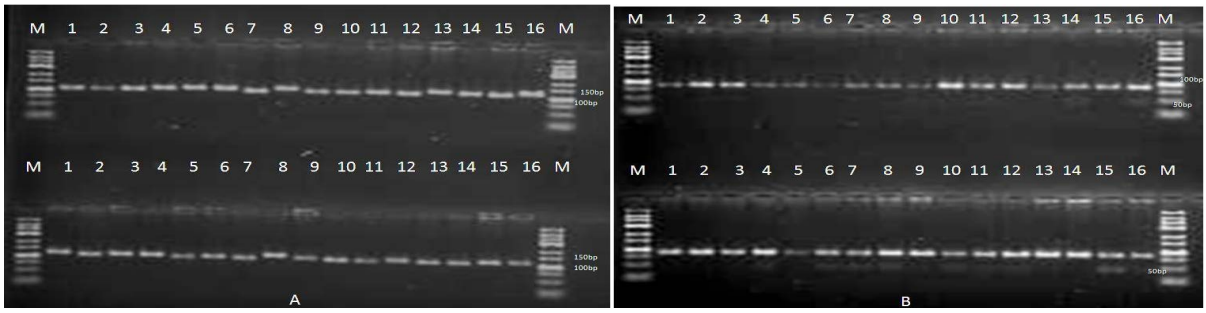

Figure 1. (A) Primer S27 and (B) Primer S43 showing 32 cultivars up lane 1 - 16 (soy1 to soy-16) and down lane 1 - 16 (soy17 to soy32) and M denotes the marker *i.e.* low range Gene Ruler^{TM} DNA ladder ≠ SM1191/2/3/bp ng/0.5.

4. Discussion

In this present study the genetic closeness and low levels of genetic diversity were clearly established and similarity coefficient range (0.76 to 1.00) and scatter plot expressing genetic distances supported this fact. In **Figure 3** the genetic distance proved to be very less *i.e.* maximum cultivars almost showing similar plot area on the scatter plot diagram. An early report had been established between the 15 certified soybean varieties under cultivation in Thailand. Pair wise coefficients of genetic similarity between all genotypes ranged from 0.73 to 1.00, which is consistent with present study. Among them 64% were ≥0.87 [28]. The analysis of allelic profiles at 20 SSR loci produced an average of 11.9 alleles and a mean genetic diversity of 0.782 in 131 soybean accessions introduced from 14 Asian countries [8]. This low level of genetic diversity and such a highest level of similarity may be ascribed to the emphasis on direct introductions, selection from introduced germplasm and single cross hybrids (some of which shared common parents) in the soybean breeding programs. Therefore, inclusion of more diverse germplasm in the soybean breeding programs may provide the genetic variability necessary to permit continued progress and broad adaptation. A previous report showed that higher genetic diversity could be found among exotic soybean introductions from different countries [29]. The genetic relationships among soybean genotypes may facilitate the selection of parents in breeding programs with the hypothesis that the more genetically diverse the parents, the more likely they are to possess unique alleles for traits of interest [5]. This study will help to restructure the Indian soybean cultivars to widen the genetic base to introduce new varieties.

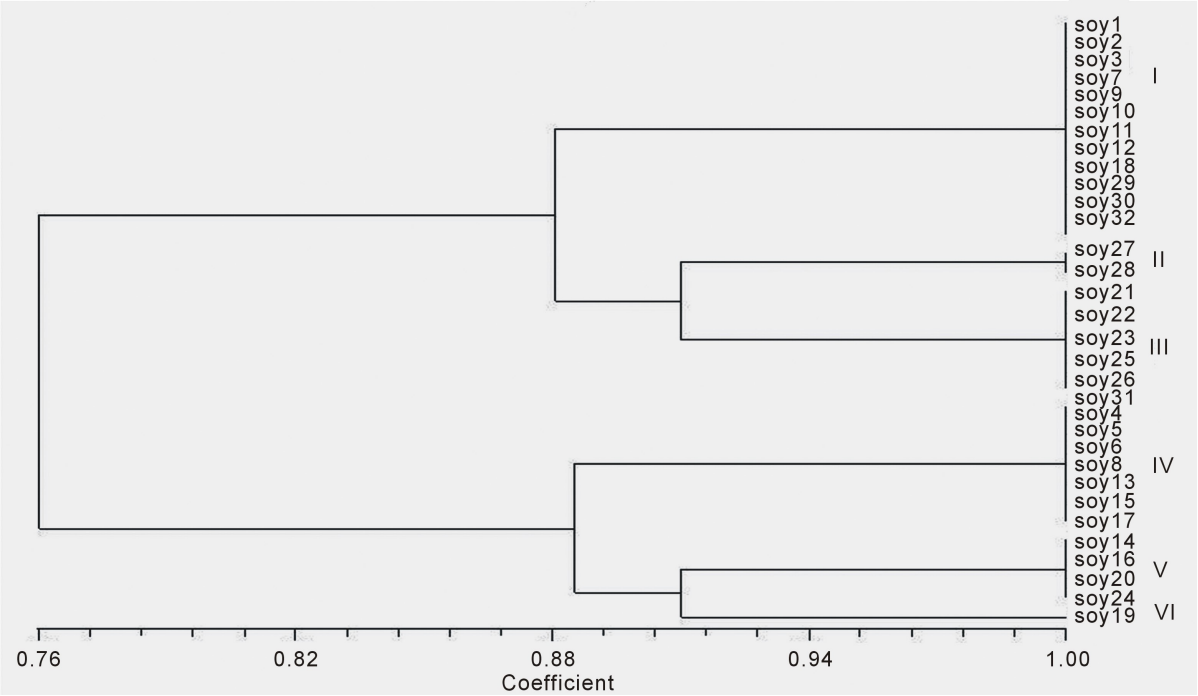

Figure 2. Dendrogram showing similarity coefficients and genetic relationships among 32 soybean cultivars.

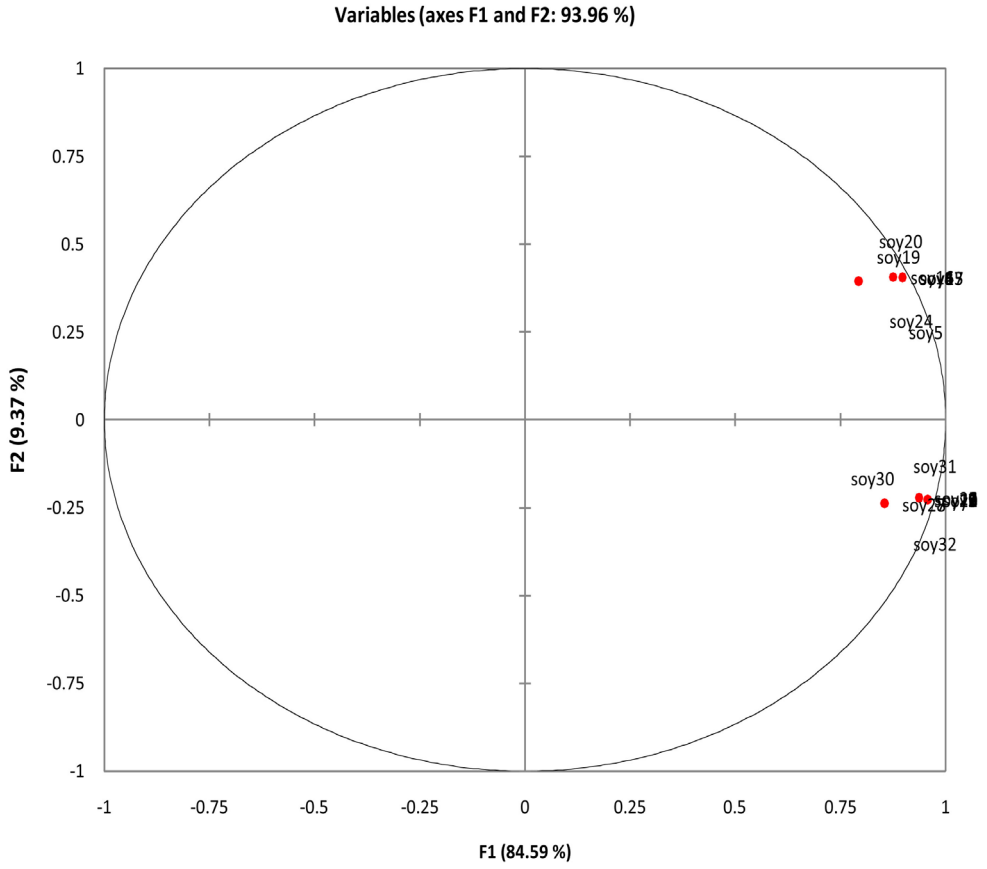

Figure 3. Scatter plot expressing genetic distances among the cultivars.

Acknowledgements

The authors express their gratitude to Ex. Project Director Dr. Asit B. Mandal for providing the field and some early screening at Molecular Biology Lab in Directorate of Seed Research, Mau. They are also very appreciative to NRC Soybean, MP, for providing the seeds and helpful information. This research was supported by project grant from ICAR.

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Abbreviations

SSR: Simple Sequence Repeats
UPGMA: Unweighted Pair Group Method with Arithmetic Mean
PIC: Polymorphic Information Content
CTAB: Cetyl Trimethylammonium Bromide
TBE: Tris/Borate/EDTA
SAHN: Sequential Agglomerative Hierarchical Non-Overlapping

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