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Molecular characterization of different tea clones “*Camellia sinensis* L” grown at NTHRI, Shinkiari

A. Waheed^{*1}, G. Hina², F. S. Hamid¹, M. Khushi², A. Habib³, A. H. Shah⁴, M. Saqib⁵,
A. Naveed¹, A. Sohail¹, B. Madiha¹, A. Seemab¹, K. Nadia¹, A. Naseer⁶

¹National Tea and High Value Crops Research Institute, Horticultural Section (NTHRI) Shinkiari, Mansehra (PARC), Pakistan

²Department of Genetics, Faculty of Science, Hazara University, Mansehra, Pakistan

³Vice Chancellor Office, Islamia College University, Peshawar, Pakistan

⁴Department of Botany, Faculty of Science, Hazara University, Mansehra, Pakistan

⁵COMSATS Institute of Information Technology, Park road, Islamabad, Pakistan

⁶Plant Sciences Division, Pakistan Agricultural Research Council, Islamabad, Pakistan

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Abstract

Characterize selected tea varieties and clones based on molecular characteristics having maximum bands on using markers to find out the best tea variety/clone for high yield and drought tolerance. For DNA analysis 8 genotypes of tea were screened out with 10 SCoT (Start Codon Targeted Polymorphism) primer and 6 genotypes were screened with 4 RAPD primers. Out of these 10 SCoT primers used against genomic DNA of tea clones, however total 4 loci were generated by 3 SCoT primers with range of bands size at 200-2200bp. The S 11 proved the best primer as it showed maximum number of bands. Clone P3 and P8 resultant best for tea flush and shoot length but remained poor for genetic diversity. While clone Aa561 shows strong genetic diversity among other clones and placed out of that group. In view of different traits performance the clone Aa117/NTHRI was good comparatively on genetic diversification characters.

*Corresponding Author: A. Waheed ✉ abdulw900@gmail.com

Introduction

Tea (*Camellia sinensis* L.) belongs to family Camelliaceae, which consist of 23 genera and 80 species (Sealy, 1958). The basic components remain the same either it is control under a very strong genetic system. Young fresh tea shoots can be processed depending upon genotypes into different types of tea, biological characteristics and chemical/physical parameters of the shoots and concerned the genotype. Pakistan is a major tea consuming country ranking 2nd largest tea importer and per capita tea consumption is >01kg /annum in the world (Nathaniel, 1992) while it has a potential to produce best tea on its marginal and suitable land available in KP province (Waheed, 2009). Different tea clones and varieties have been grown at NTHRI, Shinkiari according to their capacity and habitats on the basis of their phenotypic characters. Morphological traits and characterization is the initial steps of classification and to identify characterization for any crop.

New approaches and innovations that facilitate the characterization of tea clones are direly need of time. Molecular markers on DNA based like RFLP, SSR, AFLP, RAPD and Scot can help in germplasm characterization (Sobral and Honeycutt, 1993; Volis *et al.*, 2001). Molecular markers on DNA based have acted as very prominent and useful tool in different kinds of fields *viz a viz* classification, embryology, physiology, ecology, genetic engineering and plant breeding. Applications of DNA based markers in determining the seed purity, finger printing genotype and in phylogenetic analysis through which the conservations of the plants can be made easy. DNA-based molecular markers are widely used for characterizing medicinally important plant species (Tharachand *et al.*, 2012).

Al-Qurainy *et al.* (2015) stated that a variety of DNA based molecular markers for analyzing the genetic diversity have been used for plant species. Recently a marker Sco Thas become the method to assess genetic variability. Al-Qurainy *et al.* (2015) used SCoT marker for assessment of genetic variability in Saudi Arabian cultivars of date palm. Within the population, the variation was 48% whereas 52% of the variation was present among the population shown by analysis of molecular variance (AMOVA).

Genetic variation among populations (52% of total variance, $P= 0.001$) was constant with the coefficient of gene differentiation ($G_{st}= 0.631$) as shown by a hierarchical analysis of molecular variance. Un-weighted pair group method of arithmetic averages (UPGMA) cluster analysis of the SCoT marker data distributed the eight clones into five main clusters at 0.95 genetic similarity coefficient level.

Newly introduced marker known as Start Codon Targeted (SCoT) developed to assess purely for genetic diversity (Collard and Mac kill, 2009). This (SCoT marker) can reproducible while compared with RAPD because of longer primer sequence and molecular markers techniques has been targeted by this. The Start Codon is (ATG) and the flanking sequence that are high sealed/preserved in the genome of plants. ATG is the conserved regions of SCoT marker (Sawant *et al.*, 1999). Like SSR and RAPD markers, SCoT markers in a PCR amplification reaction are using as single primer. The SCoT markers have been used in most of the plant species such as date palm and crop such as rice (Collard and Mackill., 2009) and sugarcane (Que *et al.*, 2014).

Furthermore, to increase the metabolic activity clinical trials suggest that, green tea speed up insulin sensitivity, enhance oxidation of fats and glucose tolerance (Venables *et al.*, 2008). Epidemiological evidence also suggests that consuming green and black tea may be helpful in preventing diabetes (Iso *et al.*, 2006). According to another study, black tea can lower the level of cortisol (stress hormone) after a stressful activity (Steptoe *et al.*, 2007).

In addition to medicinal properties of tea, pigments and caffeine present in tea can serve as natural food additives (Zhongmao, 1996). Keeping in view of highly heterogeneity in phenotypic and genotypic characters of tea clones, the present study was designed to characterize some of the selected tea clones based on molecular characteristics for high yield, drought resistance, suitable for further multiplication. Evaluation through suitable molecular markers and estimation of genetic diversity among selected tea clones.

Materials and methods

Studied were carried out both at NTHRI, Shinkiar and Department of Genetics, Hazara University Mansehra during the year 2015-16. Analytical and molecular work was conducted with the application of molecular markers like RAPD and SCoT on to all the selected genotypes for PCR analysis.

Genotype selection

At present NTHRI has more than 12 different tea clones of *Camellia sinensis* L and *Camellia assamica* grown over there, out of that the following were selected with accession No. P-9, P-3, Aa-117, P-8, Aa-105, Aa-561, Aa-108, Cyto-1 for research study.

Molecular characterization

DNA extraction

The genomic DNA of tea clones was extracted from the leaves samples using standard CTAB protocol (Doyle and Doyle, 1987) with some modifications. Fresh tissue of young plants isolation of DNA was obtained. The DNA was isolated without gel but colour appeared as green which show the presence of leaf material and chlorophyll. About 35-50 mg of leaf samples was crushed into powder form using mortar and pestle after pouring liquid nitrogen on leaf sample. The genomic DNA was obtained by CTAB method, the again treated for removal of impurities and color. The extracted DNA was of good quality and high purity for 1% agarose gel. DNA extraction using CTAB protocol was not successful for all the leaf samples. Therefore, DNA extraction kit (TIANGEN Catalogue No: DP305-02) was used for the remaining samples according to the kit manufacturer's instructions.

Gel electrophoresis

Quality and quantity of extracted DNA was checked in 1% agarose gel in Tris-acetic acid-EDTA (TAE) buffer by electrophoresis, 0.5 gram agarose was added in 50 ml TAE. The mixture was completely boiled in flat bottom flask to completely dissolve the agarose. Then 25µl Ethidium Bromide was added to the mixture and gel was casted in gel tray with comb. Upon solidification, the gel was placed in a gel tank containing 1X TAE. From each sample 5µl DNA was taken and mixed with 2µl loading dye and then loaded in the wells. Gel was run at the voltage of 75 volts for about one and a half hour. Gel was visualized under "Uvitech" using UV light for gel documentation system.

Selection of molecular markers

PCR Markers, RAPD and SCoT (start codon targeted)

The SCoT (Start codon targeted) marker technique is based on a single primer, instead of specific for forward and reverse markers for PCR amplification single primer was used. The procedure is practically similar to SSR and RAPD markers. For amplification of PCR the thermocycler reaction performed by these markers to target gene region nearby ATG present on DNA strand (Wu *et al.*, 2013). Collard and Mackill's strategy is design for SCoT primer (Collard and Mackill, 2009). The primers of 18 nucleotides were designed consist on nucleotide region close to start codon "ATG".

Table 1. List of SCoT marker exercised during study (by BG1 Korea).

S. No.	Primer Name	Sequence	GC Content (%)	Tm (°C)
1	S1	CAACA <u>ATGG</u> GCTACCACCA	50	54
2	S6	ACGAC <u>ATGG</u> CGACCAACG	60	58
3	S7	ACGAC <u>ATGG</u> CGACCGCGA	66	60
4	S10	CACC <u>ATGG</u> GCTACCACCAG	61	58
5	S11	ACC <u>ATGG</u> GCTACCACCGG	64	56
6	S12	ACC <u>ATGG</u> GCTACCACCGTC	61	58
7	S17	CC <u>ATGG</u> GCTACCACCGCAC	66	60
8	S18	<u>CATGG</u> GCTACCACCGGCC	72	62
9	S19	ACGAC <u>ATGG</u> CGACCGCG	66	60
10	S20	AACC <u>ATGG</u> GCTACCACCGC	61	58

S9CoT-PCR amplification and detection.

These primers are different from one another by at least one location, the difference at 3' end, through which precise annealing and amplification. Collard and Mackill (2009) suggested two basic conditions of SCoT primers for bp sequence, i.e. ranges from 50-80% GC content mostly used to avoid palindromic sequence. Markers selection to amplify the sensitive genotype. These primers were purchased for study from BG1 Korea.

By using the conditions which are Standard, to amplify the DNA template PCR (Polymerase Chain Reaction) was carried out.

The mixture of PCR was prepared in 200 μ l tube. The amplification was carried out in thermal cycler (applied biosystem) DNA with the modified version of condition used (William *et al.*, 1990).

Amplified product of PCR was resolved on 1.5% TAE Agrose gel, and visualise under a UV Tech Trans Illuminatoreach reaction mixture was contained 20-40ng of genomic DNA of each tea clone. The total volume for each reaction mixture was adjusts to 25 μ l with distilled water. All the images were captured and reserved for further analysis of the data.

It was programmed as initial single denaturation step at 94°C for 4 minutes followed by 30 cycles of 30 second adjusts for denaturation at 94°C, 30s annealing of primer at 52.°C (depending upon the best amplification of decamer primers), and extension for 30 second at 72°C. Final extension step at 72°C for 5 minutes followed by holding adjust at 4°C.

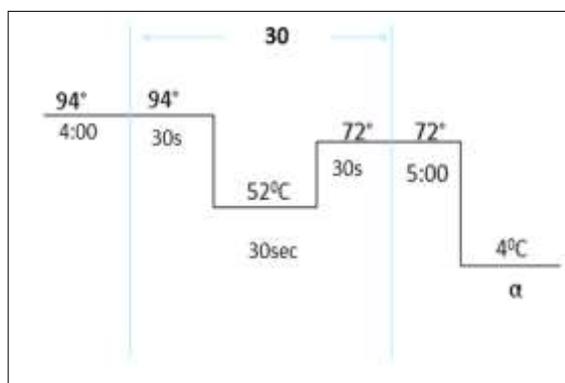


Fig. 1. PCR reaction condition for best amplification of product.

Resolving of PCR product

The PCR products were electrophoretically separated on 1.5% TAE/Agarose gels described in this section.

Scoring and data processing

The PCR product was resolved by an electrophoresis along with DNA marker on 1.5% of agarose gel, was prepared in 1X TAE buffer (Tris/Acetic acid/EDTA). An electrophoretic image was saved by using gel documentation. The amplified product of PCR was performed two or three times for reproducibility of scoring of the bands. The size of amplified DNA fragments (bp) RAPD and SCoT was estimated to a recognize DNA markers. These amplified DNA fragments was compared and was used in statistical processing. The absence or presence of fragments were recorded as if1 (present) or o (absent). The set of binary data was recorded on a separate spreadsheet for further more processing. To draw dendrogram based on Pale ontological Statistics (PAST) version 3.11 software was used.

Results

Extraction and analysis of genomic DNA

By using standard protocol the isolation of DNA for clones of both the varieties *Camellia assamica* and *Camellia sinensis* were practiced. The DNA extracted was pure and free from other impurities in all the eight clones of both genotypes. After extraction the quantity and quality of genomic DNA was checked by gel electrophoresis, the better quality of DNA was observed in the ratio of 1.80-2.00.

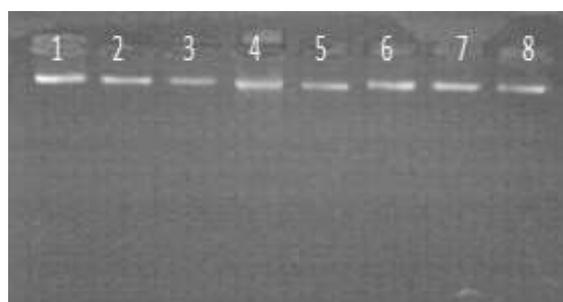


Fig 2. Represented the genomic DNA of 8 different tea clones and the presence of DNA bands showing the similarity among all the genotypes; however genome 3-Aa 117 and 5-Aa 105 show the strong bands.

1=P9, 2=P3, 3=Aa117, 4=P8, 5=Aa105, 6=Aa561, 7=Aa108, 8=Cyto 1.

Amplified product of RAPD marker

The RAPD marker (Random Amplified Polymorphic DNA) was used to find out DNA bands for presence or absent of tea clones through amplification of marker to check out the genetic diversity. The amplification was carried out in a thermal cycler (applied biosystem), set at 94°C for (4 min), 52°C for (30 sec), 72°C for (30sec) final extantion for 72°C for 5 minute and 40 cycles. Amplified productwas resolved on 1.5% agrose gel, and visualise under the UV Tech Trans Illuminator. Where genomic marker J05 gave band of 500bp in sample 1. Marker 109 shows band of 500bp in all samples. K15, G7 markers having no results inall samples. These RAPD primers was purchased from BIONEER. Primer 109 shows 100% polymorphism.M represents the Marker ladder mix (Enzymomics; cat # BMool).



Fig. 3. Gel photograph 1.5% presents amplified product of 8 different clones.

Against the use of primer gel 1= P9 and 7= Aa108 showing the DNA bands presence While the rest of the genotypes have nil bands. Whereas, the second part of the graph showing no band at all against.

The primer used. 1= P9, 2= P3, 3= Aa117, 4= P8, 5= Aa105, 6= Aa561, 7= Aa108, 8= Cyto 1.

Table 2. Polymorphic and Monomorphic bands detail produced by 4 RAPD Markers in 8 Tea Clones Genotypes.

Primer Name	Sequence	TB	PB	MB	PP	Range of bp	
J05	CCCGATTTCGG	1	1	0	100	500	
109	TGGAGAGCAG	1	0	1	0	500	
K15	CTCCTGCCAA	0	0	0	0	0	
G7	GAACCTGCGG	0	0	0	0	0	
Total		2					

The table shows the primer used and their sequence at base pair 500 for polymorphic and monomorphic bands , however it was resultant 0 in primer used k15 and G7 at all. The sequence J05 and 109 have been showed the 1 band in TB, BP and MB.

Amplified product of SCoT marker

The purified genomic DNA samples from eight selected clones of tea (Fig. 2.) was subjected to characterization and analysis of genomic diversity with the help of SCoT-PCR in between them. Ten SCoT primers were selected from Enzymomic cat# DMooLand applied to the genomic DNA of eight samples of tea for preliminary screening.

The ten selected SCoT markers produced total 34 bands, in the eight tea clones. The gained data was provided for results to Pale ontological Statistics (PAST) version 3.11 software, this software shows genetic difference and similarities in clones of tea on the bases of given data.

Polymorphism and properties of SCoT

The polymorphism is the most important property of DNA markers, which can be use to understand the genetic based characters in crops/yield.

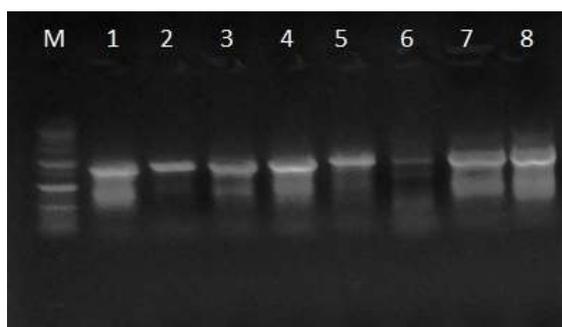
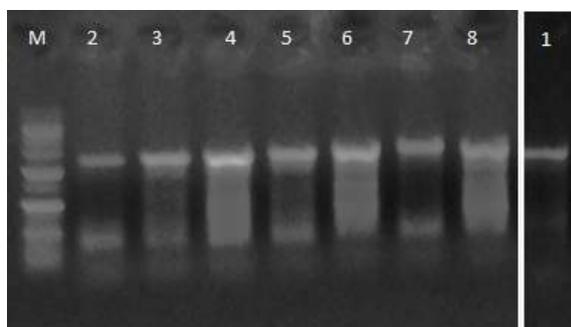
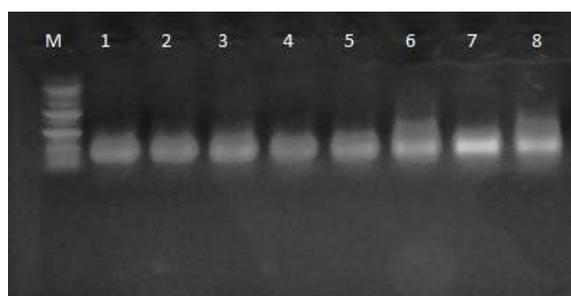
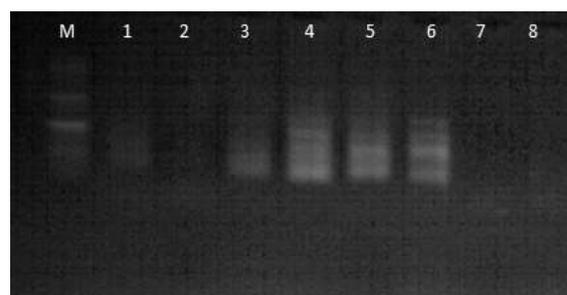
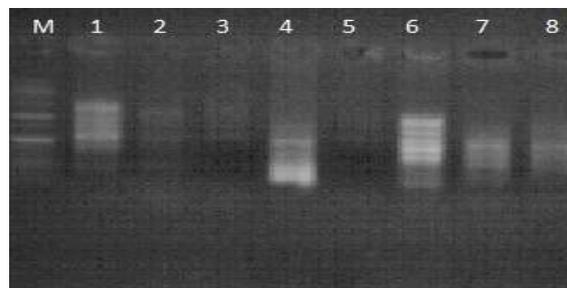
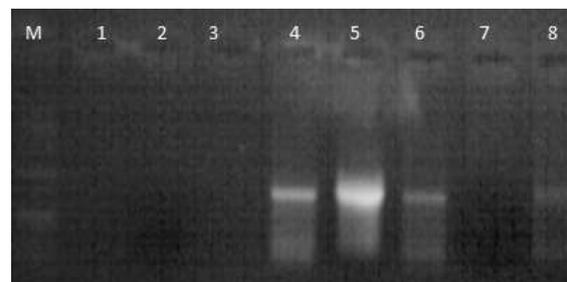
Ten designated SCoT primers produced 34 bands in total, with eight clones of tea. The produced band size of the SCoT polymorphic DNA marker was ranged from 200 to 2200 (Table 2).

The maximum number of bands/loci were produced by S11 (6 bands) while minimum were produced by S19 (1 band). The polymorphism of DNA marker was recorded and four markers namely, S10, S11, S17, and S18 were produced 100% while No polymorphism was observed in S12 and S19 (Table 3).

Polymorphic loci observed could be used as a presence and absence of bands on the base of absence (0) and presence (1) of specific locus.

Table 3. Monomorphic and Polymorphic bands detail produced by 10 SCoT markers in 8 tea clones genotypes.

Primers Name	Sequence	TB	PB	MB	PP	Range of bp
S1	CAACAATGGCTACCACCA	4	3	1	75	500-1200
S6	ACGACATGGCGACCAACG	3	1	2	33.3	450-1400
S7	ACGACATGGCGACCGCGA	3	2	1	66.6	500-1000
S10	CACCATGGCTACCACCAG	4	4	0	100	500-1000
S11	ACCATGGCTACCACCGG	6	6	0	100	300-2200
S12	ACCATGGCTACCACCGTC	3	0	3	0	4000-1000
S17	CCATGGCTACCACCGCAC	3	3	0	100	650-1000
S18	CATGGCTACCACCGGCC	4	4	0	100	400-2000
S19	ACGACATGGCGACCAGCG	1	0	1	0	200
S20	AACCATGGCTACCACCGC	3	2	1	50	400-1500
Total		34				

**Fig. 3.** Agarose gel 1.5 percent electrophoretic picture showing the PCR product of eight tea clones 1 to 8, Results are present in all samples in marker S1.**Fig. 4.** Agarose gel (1.5 %) electrophoretic picture for all the eight tea clones where 1- 8 samples have results in marker S6.**Fig. 5.** The (1.5%) agarose gel electrophoretic picture shows the result of PCR for S7 marker found in all samples.**Fig. 6.** Agarose gel (1.5%) electrophoretic picture showing the PCR results of eight tea clones 1,2,7 and 8 represents no results while rest of the clones have shown results with marker S10.4.**Fig. 7.** The PCR amplified product for eight tea clones in (1.5%) agarose gel electrophoresis photograph. Marker S11 results observed in all the samples except No.5.**Fig. 8.** Agarose gel (1.5%) electrophoretic picture showing result of amplified product of o8 tea clones. The results with Scot 12 marker shown by 4, 5, 6 and 8.

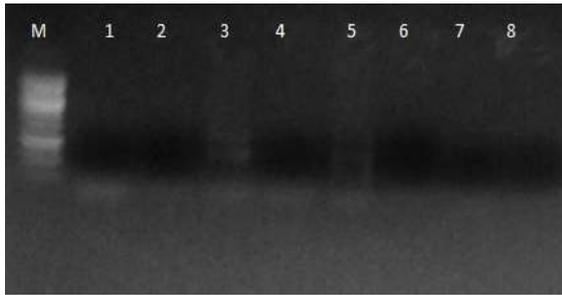


Fig. 9. The (1.5%) agarose gel electrophoretic pictures showing result of amplified product. The results with S17 in sample 3 and 5 were shown.

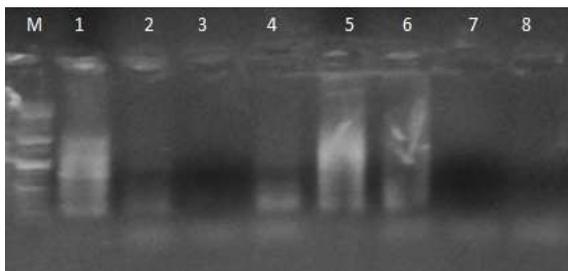


Fig. 10. S 18 marker PCR 3, 7 and 8 sample have no results among all 8 different tea clones. 1st Sample showed polymorphic & other showed monomorphic band.

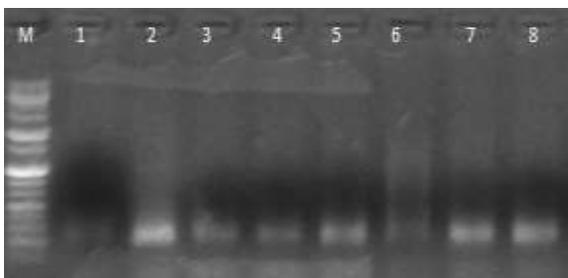


Fig. 11. Monomorphic results photograph with marker S 19 PCR amplified product of 8 different tea clones on 1.5% agarose gel.

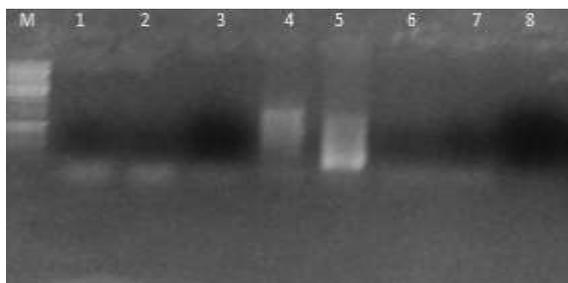


Fig. 12. S 20 marker PCR result (8 different tea clones) Sample 4 and 5 showed polymorphic bands. Where M represents Marker ladder mix (Enzymomics; cat # DMool).

Specificity of SCoT (Start Codon Targeted) marker

For specificity of SCoT (Start Codon Targeted) marker, eight tea clones were selected to characterize on the basis of molecular markers and identification of linked DNA locus. For identification of markers, ten SCoT markers were amplified PCR products in tea clones and only 3 out of 10 SCoT primers produced 4 specific loci were generated in clones against genomic DNA within the range of band size from 400-2200bp (Table 4).

Primer S-1 produced 650bp band, in Aa 561. Primer S-11 produced two bands of 2200bp in P-9 and 400bp in P 8. Similarly primer S 20 produced 400bp. Results indicated that these markers could be utilized to identify best tea clones.

Table 4. Selective SCoT marker against different tea clones produced specific loci.

S.No	Primer	Locus	Linked to clone No.	Clone
1.	S1	650	6	Aa561
2.	S11	2200	1	P9
		400	4	P8
3.	S20	400	5	Aa105

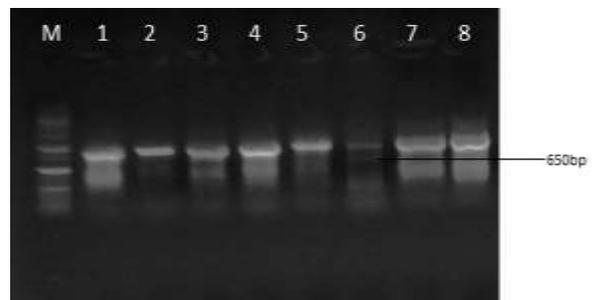


Fig. 13. Primer S1 produce 1 band of 650bp at (1.5%) gel electrophoretic photograph represents Scot pattern among 8 tea clones.

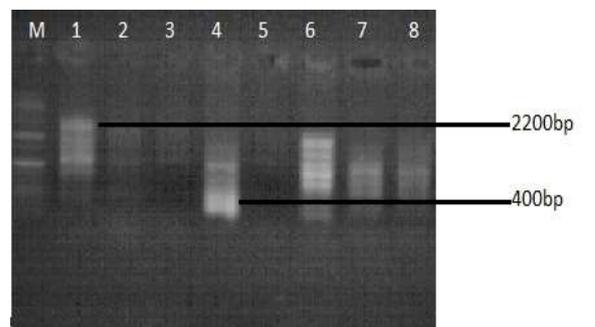


Fig. 14. Primer S11 produced 2 bands of 2200bp & 400bp in sample 1 and 5 on (1.5%) gel electrophoretic.

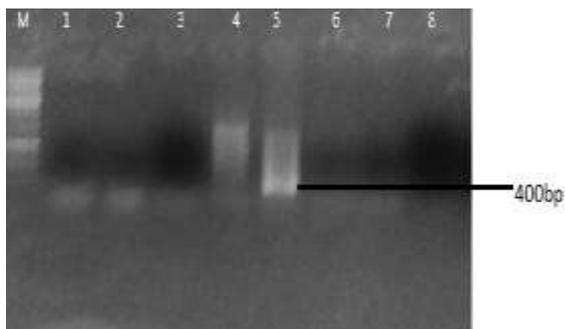


Fig. 15. Primer S 20 produce 1 band in sample 5 of 400bp on (1.5%) gel electrophoretic .

Cluster analysis

Bands produced by SCoT primers were counted on the basis of absence (0) or presence (1). The data provided to the Pale ontological Statistics (PAST) version 3.11 software for CA. The software make a homology dendrogram based on a separation matrix using the neighbor-joining method (NJ), this shows

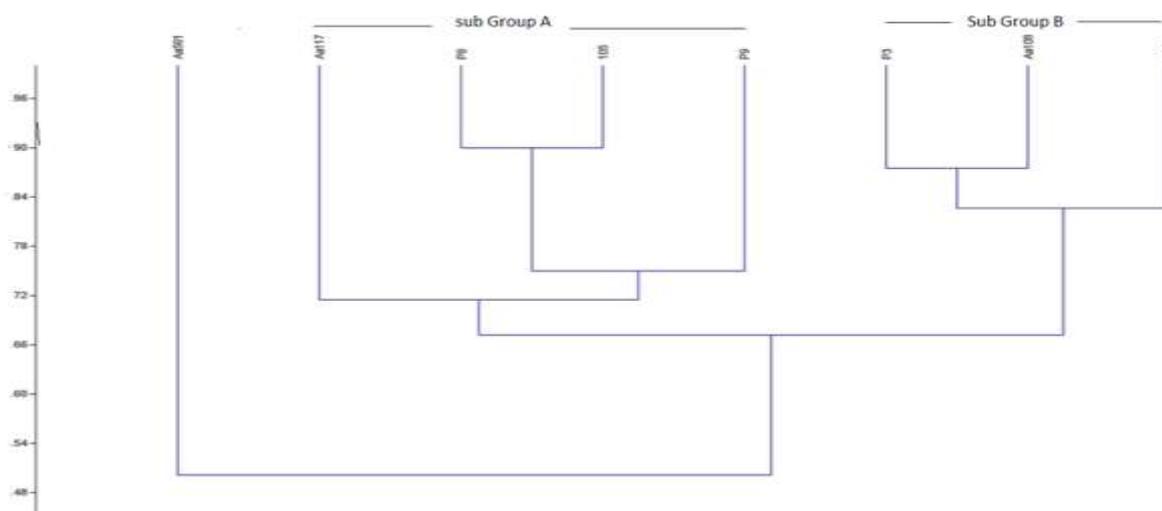


Fig. 16. Homology dendrogram constructed showing the genetic similarity among eight tea clones from NTHRI with 10 SCoT primers by the Pale ontological Statistics (PAST) version 3.11 software.

Discussion

Molecular characterization

To analyze genetic diversity among different tea genotypes grown at NTHRI, 4 RAPD markers and 10 SCoT markers were used for research findings. On the average, 4 fragments were amplified as per primer genotype which was greater.

Results are similar to the findings of (Chen *et al.*, 2005a), where about 3.5 alleles per primer per genotype were reported.

the genetic resemblances differences between eight tea clones (Saitou and Nei, 1987).

Eight tea clones genotypes show two major groups, sub group A and sub group B based on 72 % homology. Group one includes clones i.e., P9, Aa 105, P8, and Aa 117, P-8 and Aa 105 shows 88% homology between them. Aa 117 shows 72% and P9 show 74% homology among group A. Group B contains clone P3, Aa 108 and Cyto 1. Tea clone Cyto-1 shows 82% homology with group B. Tea clones P3 and Aa 108 have 86% homology with rest of the group. Similarly about 68% homology was present in sub group A and B. Tea clone Aa-561 indicates no similar diversity among all the clones groups. The genetic assortment among the eight tea clones, genotypes show diverse arrangement, according to the polymorphism relationship of SCoT primer sand genetic distances.

This particular difference between the two studies may be due to the reason as aforementioned studies (Chen *et al.*, 2005a) only elite genotypes were selected, the most favored alleles are retained instead of rare ones. Furthermore, relatively higher genetic distances were observed during the present study could be due to that plant materials obtained from different geographical regions as opposed by Chen *et al.*, (2005b).

An efficient mean to distinguish intra- or inter-specific variation of tea germplasm, focus is on the use of different molecular markers which are sufficient and beneficial to characterize and differentiate the varieties of tea and clones (Chen and Yamaguchi, 2002; Mondal, 2002; Chen *et al.*, 2005b). Here in this case the use molecular markers for its DNA were ensured genetically.

Variation between the tea clones observed at a molecular level using SCoT and RAPD markers resolved eight genotypes in two main clusters. Therefore, SCoT marker is highly reliable and extremely important to indicate the unexplored genetic diversity in tea clones and their usefulness. It has been successfully detected polymorphism between the genotypes from present research findings. The results also reflect that this method can be used for taxonomic classification and genetic fingerprinting of tea genotypes.

Conclusion

It is concluded that molecularly, high diversity exists among all the tea genotypes and extensively different from each other on the basis of genetic polymorphism. Clone Aa561 proved highly genetic variations diversity and placed out of group. Out of ten SCoT primers used against genomic DNA, 4 loci were generated with the range of 200-2200bp band size. The S11 resulted best primer for its maximum number of bands and polymorphisms.

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