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# An Assessment of Genetic Relationships in VP and Old Seedling Teas in Sri Lanka using RAPD Markers

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## ABSTRACT

Most of the vegetatively propagated (VP) teas that are used in current tea breeding programs and plantations in Sri Lanka are the selections made from open pollinated progenies of hybrids produced from an introduction, ASM 4/10. In addition, old seedling tea populations that are not currently used in tea breeding programs or tea plantations are distributed in different regions of Sri Lanka. The unrevealed genetic variability present in such populations could be used to enrich local tea genetic base. Systematic genetic variability assessment of those seedling populations is important to identify individuals with true genetic differences in order to utilize them in crop improvement programs. In this study, eighteen tea accessions including ten old seedlings obtained from Fairlawn Estate, four TRI hybrids generated from controlled hybridization, a Cambod hybrid - 'ASM 4/10', phenotypically distinct tea accession - 'China', and two Estate selections which are currently employed in tea breeding programs were assessed for their genetic diversities using RAPD PCR markers. A total of 208 polymorphic bands were scored. The resultant dendrogram based on RAPD separated these accessions into three major groups: (i) Old seedlings obtained from Fairlawn Estate and Estate selections (ii) Cultivars derived from Cambod hybrids (iii) Cultivars developed from controlled hybridization. The genetic diversity of the old seedlings of Fairlawn were found to be considerable (0.14-0.32) whereas genetic diversity values within different categories were comparatively higher than that of among categories, except in the case of 'Assam' and 'China'. Being the preliminary study to assess the genetic diversity of old seedling tea populations in Sri Lanka, this study generated useful information emphasizing the importance of exploiting the genetic variations present in the old seedling tea populations that are distributed in various regions of Sri Lanka.

**Key words:** *Camellia sinensis* (L.) O. Kuntze, Genetic diversity, RAPD, Tea.

## INTRODUCTION

Tea [*Camellia sinensis* (L.) O. Kuntze] is a beverage tree crop native to South-East Asia and has been introduced to Sri Lanka from India during 1840s. Breeding strategies in the tea depend on its predominantly out bred nature, long generation time from seed to flower and its potential for vegetative propagation (Hackett *et al.*, 2000). The tea-

breeding program in the Tea Research Institute of Sri Lanka (TRISL) started in 1938. Most of the tea cultivars developed by the TRISL (TRI cultivars) are originated from the selections made from the seedling progeny of a seed bearer identified as 4/10 at the Tocklai Experimental Station of the Indian Tea Association, Assam. Then, a progeny of seeds of Shan-Bansang No.777 from Indo-China was also used to originate certain tea cultivars and those Indo-China tea cultivars were used as parents in the tea breeding programs (Richards, 1965). In later stages, certain local seedling tea accessions, which perform better in the fields referred to as "Estate selections" were selected and used in the tea breeding programs (Gunasekare, 2008). Therefore, the genetic pool in Sri Lankan tea is mainly comprised of the Cambod hybrid ASM 4/10, Indo-China source Shan-Bansang No.777 and selections made from local seedling tea accessions. The old seedling teas are low yields with unknown pedigree. They are not mainly used in the current tea breeding programs whereas recommended teas commercially grown and known pedigree are effectively used in the current tea breeding programs.

Richards (1965) stated that over sixty percent of the replanted teas in Sri Lanka are vegetatively propagated and most of the growers have used a limited number of tea cultivars in their plantations. The most popular TRI cultivars of 2020 series (2021 up to 2027) were selections made from the open pollinated progenies of a single gene stock, ASM 4/10. Then, in subsequent tea breeding programs, cultivars of 2020 series and some Estate selections were used as parents to develop TRI 3000 and 4000 series. Hence, the available genetic diversity in Sri Lankan commercial tea plantations is low. This can cause detrimental effects in the sustainability of tea industry, as most of these popular cultivars are not adequately buffered against abiotic and biotic stresses. Hence, proper conservation of tea genetic resources and broadening of the tea genetic base are given high priority in the tea breeding programs. Identification of sources of genetic variability in the available germplasm and exploiting the utilizable genetic variability are important aspects in strategic planning of breeding and crop improvement programs. The old seedling tea populations located in various regions of Sri Lanka would be appropriate candidates with genetic differences that could use in tea breeding programs to broaden the existing tea genetic pool.

Different strategies, such as morphological descriptors (Gunasekare *et al.*, 2001), isozyme technique (Liyanage *et al.*, 1999) and molecular marker technique (Liyanage *et al.*, 2001) have been used to assess the genetic variability in the tea gemplasm in Sri Lanka. Tea germplasm characterization using morphological descriptors alone could lead to erroneous conclusions due to high plasticity and polygene effects in the morphological traits. Moreover, these traits are highly influenced by the environment, plant age and phenology. Detection of genetic variability using isozymes was also not successful because it failed to detect adequate polymorphisms present in tea populations

(Liyanage *et al.*, 1999). Molecular markers, such as Random Amplified Polymorphic DNA (RAPD) markers developed by Williams *et al.* (1990) have been successfully used to determine the genetic diversity in tea populations around the world (Wachira *et al.*, 1995; Wachira *et al.*, 1997). Many authors have used RAPD markers during last five years to assess genetic diversity in some tea cultivars in Sri Lanka (Liyanage *et al.*, 2001; Mewan *et al.*, 2005; Goonetilleke *et al.*, 2006). At present, powerful DNA techniques such as Simple Sequence Repeats (SSR), Single Nucleotide Polymorphisms (SNP) are frequently used for genetic diversity assessments in different crops. However, RAPDs are being used as attractive markers in assessing genetic diveristy of tea accessions in Sri Lanka due to low cost involvement, easy performance and no requirement of prior information on crop genome. Therefore, the objectives of this study were (i) to identify diverse old seedling tea accessions in Sri Lanka (ii) to obtain an understanding about the genetic relationships in old seedling teas and cultivated teas. Such information would be helpful to tea breeders in directing the breeding programs to utilize the available genetic diversity effectively, in determining progenitors for the future cultivar development programs and in genetic resource conservation.

## MATERIALS AND METHODS

### Plant Materials

Leaf samples of old seedlings obtained from Fairlawn Estate in Upcot, Sri Lanka and eight other tea cultivars maintained in the *ex-situ* field gene bank at the Tea Research Institute of Sri Lanka, Talawakelle, were used in this study (Table 1).

### DNA Extraction and Amplification

The flush (two tender leaves and the bud) were collected and stored immediately at -70$^0$C and used for DNA extraction. Genomic DNA was extracted according to the miniprep DNA extraction protocol as described by Chen and Ronald (1999) with few modifications to remove excessive amounts of polyphenols and proteins present in the tea leaves. One gram of tender tea leaves were cut into small pieces and placed in a 1.5ml eppendorf tube and then it was dipped into liquid nitrogen and crushed them into a powder. 700 $\mu$l pre-warmed (65$^0$C) isolation buffer [2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100mM TRIS (pH 8.0), 2% PVP, 5.0mM Ascorbic acid, 4.0mM DETC]. 7.5 $\mu$l Rnase A (10 mg/ml) was added and mixed properly by gentle inversions and incubated at 65$^0$C for 5 min. Then, 570 $\mu$l of chloroform: isoamyl alcohol (24:1) was added and mixed well for 5 min. and centrifuged at 13,000 rpm for 10 min. at room temperature (this step repeated if the supernatant was vicious). The supernatant was transferred into a new eppendorf tube and DNA was precipitated by adding 0.8 volume of isopropanol, mixing well for 1 min. and centrifuging at 13,000 rpm for 5 min. at room temperature. The DNA pellet was washed with 70% ethanol 500 $\mu$l, air dried for 1 hr. and suspended in 20 $\mu$l of TE. The extracted DNA was stored at -20$^0$C.

RAPD amplifications were performed according to the generally followed the method of Williams *et al.* (1990). Twenty decanucleotide primers from kits A, B, C, D and E of Operon Technologies, USA were used to detect polymorphism. The PCR amplifications were carried out on a Biorad-mycycler Thermal cycler (USA). A negative control with no DNA was included in each PCR run. The amplifications were repeated twice. The PCR products along with a 1kb DNA ladder (Promega, USA) were size fractionated in 1.5% agarose gel and electrophoresed in 1 X TAE buffer at 75 V for 3 hrs. and subsequently stained with ethidium bromide. The banding patterns were visualized and documented by Biorad Gel Documentation System (USA).

**Statistical Analysis**

Only intensely stained, unambiguous and reproducible polymorphic bands were scored for presence (1) and absence (0). The binary data were used to compute similarity matrix among tea accessions and this matrix was used in an unweighted pair group method using arithmetic averages (UPGMA) cluster analysis (Nei and Li, 1979). RAPDistance software developed by Armstrong *et al.* (1994), ANU, Canberra, AUS was used to construct the dendrogram. Nei's genetic diversity (Nei, 1973) for each tea

Table 1. Tea accessions and their pedigree used in this study

| Accession | Identity | Pedigree |
|---|---|---|
| FL 9* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 22* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 23* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 30* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 34* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 46* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 62* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 76* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 101* | Old seedling from Fairlawn Estate | Pedigree unknown |
| FL 103* | Old seedling from Fairlawn Estate | Pedigree unknown |
| TC 10 | Seedling selection from Tilicoultry | Estate selection |
| TRI 2016 | Seedling selection from St. Coombs | Estate selection |
| ASM 4/10 | Introduction from India | Cambod hybrid |
| China | Introduction from China | China accession |
| TRI 2043 | Selection from Indo- china seed source | Shan Bansang No.777 O.P. |
| TRI 3014 | TRI developed cultivar | TRI 2025 O.P. |
| TRI 4001 | TRI developed cultivar | ASM 4/10 X TRI 777 |
| TRI 4076 | TRI developed cultivar | N2 X DN |

* Fairlawn old seedling selections are not 'recommended tea culitvars'. Their suitability for planting is under evaluation; O.P.: Open Pollinated

accession category was calculated using the computer program POPGENE 1.31 (Yeh *et al.*, 1999) assuming population is in Hardy-Weinberg equilibrium.

## RESULTS

### RAPD Analysis

Out of the 20 primers used, 13 produced intensely stained, reproducible bands (Table 2). A total of 208 polymorphic bands were scored from the amplification products of those 13 primers. The average number of polymorphic bands per primer was 16. It was possible to distinguish between all tea accessions used in the study by using these polymorphic-banding patterns.

Table 2. Primer sequences and the polymorphic fragments recorded in RAPD analysis

| Primer | Sequence 5' to 3' | No. of Bands | Band size (bp) |
|---|---|---|---|
| OPA 07 | GAAACGGGTG | 26 | 300-2000 |
| OPA 09 | GGGTAACGCC | 21 | 500-1500 |
| OPA 10 | GTGATCGCCG | 21 | 300-1750 |
| OPB 04 | GGACTGGAGT | 08 | 500-2000 |
| OPB 10 | CTGCTGGGAC | 21 | 250-2500 |
| OPB 13 | TTCCCCCGCT | 10 | 175-1500 |
| OPB 17 | AGGGAACGAG | 13 | 300-2000 |
| OPC 09 | CTCACCGTCC | 18 | 200-2500 |
| OPC 10 | TGTCTGGGTG | 16 | 300-2500 |
| OPC 17 | TTCCCCCCAG | 18 | 200-2500 |
| OPD 03 | GTCGCCGTGA | 12 | 300-2250 |
| OPD 15 | CATCCGTGCT | 12 | 200-2150 |
| OPE 06 | AAGACCCCTC | 12 | 200-3000 |
| Total | | 208 | |

According to the results of the similarity index, the accessions used in this study were clustered into three major groups at the similarity index of 0.97 (Figure 1). The first group consisted of old seedling populations (old seedlings obtained from Fairlawn Estate and Estate selections originated from other estates). Group two comprised of the tea accession 'ASM4/10' and old seedling from Fairlawn Estate (FL 101). The third group consisted of hybrids produced from controlled hybridization by TRISL (TRI 4076, TRI 4001), cultivar derived from Indo-Chinese sources (TRI 2043) and phenotypically distinct accession 'China'.

The genetic diversity values of different accession categories are presented in the Table 3. It shows that, 'China'- Estate selections category has the highest genetic diversity

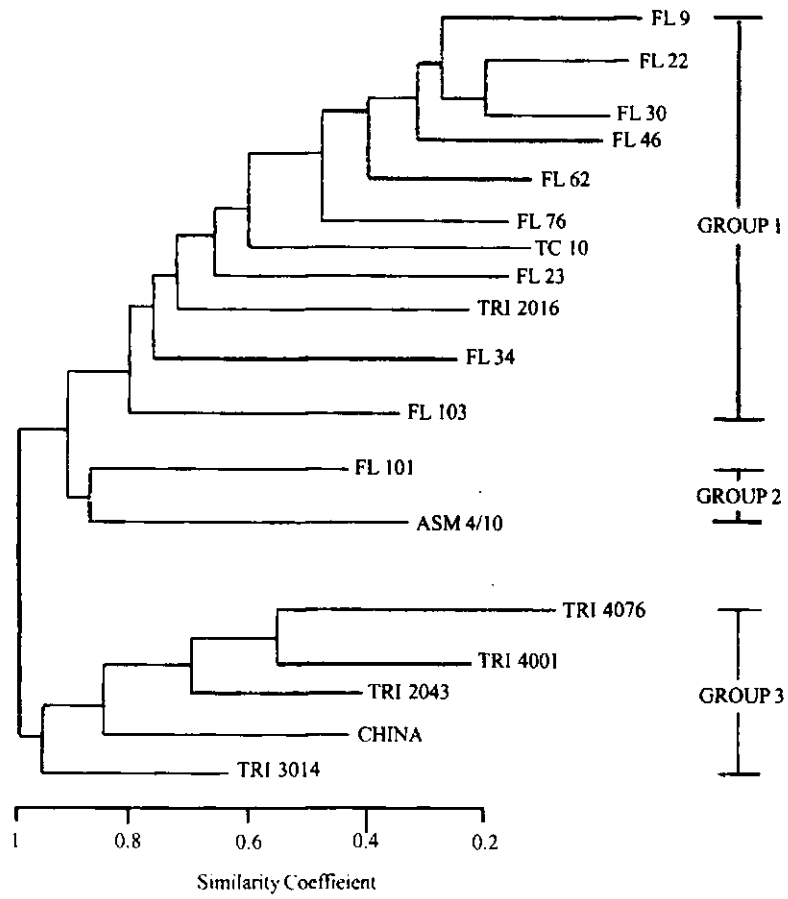


Figure 1. Dendrogram based on RAPD data

Table 3. Genetic distances among accessions with different origin obtained from the POPGENE analysis

| Accession category | Fairlawn | TRI hybrids | Assam | China | Estate selections |
|---|---|---|---|---|---|
| Fairlawn | _ | | | | |
| TRI hybrids | 0.04 | | | | |
| Assam | 0.22 | 0.25 | | | |
| China | 0.34 | 0.35 | 0.40 | | |
| Estate selections | 0.18 | 0.20 | 0.38 | 0.43 | _ |

Table 4. Most diverse and least diverse accessions identified in this study

| Accession | FL 9 | FL 22 | TRI 4001 | TC 10 |
|---|---|---|---|---|
| FL 30 | 0.14 | 0.14 | 0.40 | _ |
| FL 101 | _ | _ | 0.46 | 0.37 |

(0.43) whereas old seedlings obtained from Fairlawn Estate - TRI hybrids category has the lowest value of 0.04. These results are comparable with the results generated from RAPD distance analysis.

The possible combinations of the accessions with high genetic diversities that could be used as potential parents in cultivar development programs are summarized in the Table 4. Old seedlings obtained from Fairlawn Estate (FL 30, FL 101) have the potential of generating high genetic diversity values of 0.40, 0.46 respectively with the TRI 4001.

**DISCUSSION**

The RAPD based cluster analysis clearly divided old seedlings obtained from Fairlawn Estate and other local seedling tea accessions (Estate selections) into one group while TRI hybrids developed from controlled hybridization into another group. These results strengthen the previous research findings that TRI cultivars and Estate selections have distinct origins (Mewan *et al.*, 2005; Goonetilleke *et al.*, 2006). Besides, this indicates the genetic relatedness of the local selections made from the elite bushes that grown in tea estates in different regions of the country.

According to the results, some combinations between old seedlings from Fairlawn Estate and TRI accessions (FL 30 and TRI 4001, FL 101 and TRI 4001) have the potential of generating hybrid vigor in the future breeding programs. The clustering behavior of these accessions indicates that they are from different sources of origin. Hence, these accessions could be used as potential candidate parents in the future hybridization programs to generate hybrid vigour.

When considering the lowest genetic diversity values elucitaed for potential crosses: old seedling combinations of Fairlawn Estate (FL 9 x FL 30 and FL 22 x FL 30) have the lowest value of 0.14. This depicts that their origin would be from the same stock of planting material and would be having similar genetic base. But the overall genetic diversity in old seedling of Fairlawn Estate varied from 0.14 to 0.32. This indicates that a considerable genetic variation is present within old seedlings of Fairlawn Estate and these accessions could be effectively used in the breeding programs and in tea germplasm conservation.

Genetic diversity values of TRI hybrids lie between 0.21- 0.32. The TRI hybrids used in the present study are generated from controlled hybridization. TRI 3014, TRI 4001 are closely related to ASM 4/10 by pedigree, TRI 3014 originated from open pollinated progeny of TRI 2025 and female parent of TRI 4001 is ASM 4/10. Clustering behavior and low genetic diversity value (0.22) of these accessions indicates that they have

common genetic base. The genetic relatedness reflects the lower chances of achieving a further genetic improvement by making crosses, by selecting either male and female parent within open pollinated half-sib or full sib progenies of TRI cultivars unless breeding for known inherited economic traits governed by qualitative traits.

Tea accessions categorization based on their origin depicted that, the genetic diversity within category is higher than that of among category, except in the case of 'Assam' and 'China'. This is because the origin of ASM 4/10 and China are from different sources (ASM 4/10 is a tea accession belong to cambod hybrid with green leaves with pink pigmentation on the base of the petioles particularly when young and China is phenotypically different type with very small thick leaves with high content of polyphenols). According to the genetic diversity assessment results reported by Mewan *et al.* (2005) tea accession 'China' is identified as an accession with high genetic distinctiveness.

The highest genetic diversity values were observed among Assam and Estate selections, Estate selections and China, Assam and China, *viz.* 0.38, 0.43, and 0.40, respectively. Hence, prioritization of crosses that belong to different origins is the effective method to generate hybrid vigor in the tea breeding programs.

The lowest genetic diversity value of 0.04 was observed among TRI cultivars developed by controlled hybridization (TRI hybrids) and Fairlawn seedlings. One of the reasons for this could be due to the limited representation of the genetic variation existing in the TRI hybrids as this study deals with a very few TRI hybrids.

TRI 2043, a cultivar developed from the seed material of Indo-chinese sources (Shan Bansang No.777), contain red anthocyanin pigment, buds are covered by glossy hairs, with relatively long internodes (Richard, 1965) clustered in group 3 of the dendrogram closely connected to the TRI 4001. By pedigree male parent of TRI 4001 is TRI 777 which is originated from Indo-china seed sources. This grouping behavior indicates that they have similarities in their gene composition (genetic diversity value: 0.21) although they are phenotypically different. Morphological analysis dose not elucidate the true genetic diversity of the tea germplasm, which is the information required for the breeder to shorten the breeding cycles.Therefore, molecular markers would be the appropriate method to unveil the true genetic diveristy present in the germplasm.

The results of this study suggest that Fairlawn seedling accessions, which categorized as old seedling tea populations, bear considerable genetic diversity and can be used to broaden the available genetic diversity in Sri Lankan tea germplasm. The present study can be considered as an initiative to exploit the genetic diversity present in different old

seedling populations in Sri Lanka. Furthermore, old seedling tea populations should not be neglected as they have the potential of generating considerable genetic variability when crossed with cultivated teas. Therefore, such tea germplasm need to be conserved and could efficiently and effectively utilize in the future tea breeding programs and crop improvement programs.

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