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ORIGINAL ARTICLE

Fluorescein Amidite (FAM) based RAPD barcodes for genetic analyses of mango (*Mangifera indica*) genotypes

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ABSTRACT
Mangifera cultivars Alphonso, Totapuri, Amrapali, Kesar, and Langra are agronomically crucial as some of these cultivars are cultivated and exported. Each of these cultivars has characteristic economic values linked to molecular loci. In the present study, genetic variations of the mango cultivars are characterized by applying advanced techniques based on FAM (Fluorescein Amidite) assisted RAPD-PCR profiling. Three universal primers used in the study scored 132 loci, out of which 116 were found to be polymorphic, with an average of 54.53 polymorphic loci per primer. Average marker indices for Heterozygocity, Polymorphic Information Content, Effective multiplex ratio, Arithmetic mean, Marker Index, Discriminating power, Resolving power and Shannon's Information index was 0.49, 0.37, 58.80, 0.04, 0.80, 68.4 and 0.51, respectively. Primer 2 showed good efficiency with higher marker indices concerning all parameters. Genetic differences within and among the cultivars are described through AMOVA analysis. Barcodes for assessing the genetic diversity of the five mango cultivars are developed with FAM data from the present study.
Keywords: RAPD-PCR profiling, Molecular markers, Polymorphic Information Content, Barcodes, SCAR, Mango, FAM (Fluorescein Amidite).

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INTRODUCTION

India is well known for its Mango production and exports; hence the mango gene pool has attracted a lot of interest in molecular diversity analysis [51]. Conventionally, Mango cultivars (*M. indica*) have been genotyped, based on physical-chemical parameters, for crop improvement and future breeding and hybridization programs [24]. The differentiation executed in this study by conventional physicochemical parameters are significant but can be widely influenced by various environmental conditions such as climate, temperature, humidity, soil, etc. It is noted that even the morphology of the same plant could be extremely variable depending on the external growth conditions [19]. Molecular markers, with their unique ability, overcome the influence of environmental factors in resolving the limitations of conventional marker-based analysis [50]. Molecular markers are classified into PCR and non-PCR based markers. Restriction fragment length polymorphism (RFLP) represents the non-PCR based marker.

In contrast, PCR dependent include more reliable and advanced RAPD and ISSR markers to detect the genetic similarities or dissimilarities in various plants [5,19,32,54]. RAPD-PCR generates reproducible fingerprints from any organism without any need for DNA sequence information [56]. Molecular characterization has become an essential aspect in the field of genetics and breeding. In-plant genetic research, hybridization, and PCR based markers have been extensively utilized to analyze genetic variations, map genes, quantitative trait loci tracking in constructing phylogenetic trees [3]. RAPD has been effectively carried out in previous studies using RAPD primers to analyze genetic variations in Indian mango cultivars [26, 31, 1]. Since there was a dearth of information on fluorescence-labelled

molecular characterization of mangos, which is a very economical and novel approach [56]. The Consortium for Barcode of Life has stated: "DNA barcoding makes a huge difference to our knowledge and understanding of the natural world" [11]. The DNA barcodes are recognized as powerful additional tools for taxonomists to identify specimens to discover overlooked species [22, 30]. This study was carried out to barcode five mango elite cultivars.

Further, correct genotype tagging of plant material is essential in building genetic banks and species breeding [4, 9]. Considering this, the present study was carried out to develop DNA based SCAR marker specific to the Alphonso cultivar. All the works referred were more or less limited only to analyze genetic variations in mango cultivars.

MATERIAL AND METHODS

FAM assisted RAPD-PCR Analysis

Analysis of Molecular variation was performed by polymerase chain reaction (PCR) for barcoding marker generation. Genomic DNA was isolated and purified from 5 mango cultivars pulp; using a genomic DNA isolation kit (RKN11 purchased from Chromous Biotech, Bengaluru, India). It was amplified with three designed primers, tagged with fluorescent dye labelled 6-Carboxyfluorescein amidite (FAM) shown in Table: 1. With reference to Markus Schuelke (2000). Stability was tested using different amounts of DNA (5, 10 and 20 ng) as a template. The tagged DNA fragments were separated by capillary electrophoresis (C.E.), using Applied Biosystems® RAPD kit to generate enough informative alleles to distinguish between cultivars.

Scoring peaks binary data generation

Spectral data were then exported to GeneMapper® (Applied Biosystems), a software package; to generate a fingerprint for every sample analyzed[5]. Polymorphisms in the fragment profile were indicated by the presence or absence of a peak. These peak patterns were converted to a binary data table, "1" representing the presence of a given fragment while "0" representing the absence of the corresponding fragment, which were then used for phylogenetic analysis.

Genetic data analysis

The markers' applicability and effectiveness were evaluated to characterize genetic polymorphism based on Heterozygocity (He) indices and Polymorphic informative parameters following [42, 43]. The suitability and efficiency of RAPD markers are to be evaluated using the following four parameters. (a) Polymorphic information content (PIC), (b) effective multiplex ratio (EMR) (c) marker index (MI) and (d) resolving power (RP). Effective multiplex ratio (EMR) is calculated as the total number of polymorphic loci (per primer) multiplied by the proportion of polymorphic loci per their total number [45]. The marker index (MI) was calculated as described by [38]; the Marker index (MI) is a statistical parameter used to estimate the total utility of the maker system. The resolving power (Rp) of each primer was calculated according to [47]. Resolving power (Rp) is a parameter characterizing the ability of the primer/marker combination to detect differences between large numbers of genotypes [18, 47].

Phylogenetic relationship & Dendrogram construction

The genetic relationships were estimated using POPGENE software version 1.32. [57]. Genetic diversity was estimated by discerning the allelic frequency complying [35]. Consequently, Nei's genetic distance values Neighbour Joining (N.J.) tree was constructed based on distance methods for inferring phylogenies with the bootstrap approach by [16]using software PHYLIP Version 3.5. The optimal Dendrogram tree was generated, with branch lengths and bootstrap values, with 1000 replicates.

Principal Coordinate Analysis (PCoA) and AMOVA Analysis

Genetic distance and Clustering procedures were validated via multivariate statistical algorithms. Principal coordinate analysis (PCoA) was performed according to [20] to express the consistency of the genetic distances among cultivars with minimal distortion. PCoA complements cluster analysis [16, 34].

Analysis of molecular variance (AMOVA) was applied to infer metric distances among and within mango cultivars following [11]. On the whole, the significance of molecular analysis was recapitulated with Ewens- Watterson test following [59].

FAM labelled RAPD barcoding

Using an online barcode generator programme, three FAM-labelled markers binary data were utilized to generate RAPD barcodes for Alphonso, Totapuri, Langra, Kesar and Amrapali.

RESULTS AND DISCUSSION

Electropherogram analysis of five mango cultivars

DNA fragments generated from the three primers produced 52 allelic fragments shown in Figure:1. Primer: 2 efficiently separated 66 allelic fragments as shown in Figure: 2. Primer three isolated 14 alleles, as shown in Figure: 3. The results revealed that these markers could be considered for molecular barcoding of mango cultivars either singly or combined with other FAM markers.

A total of 132 alleles were scored, out of which 116 were polymorphic, as shown in Table 1. Accordingly, the polymorphic percentage was 89% which is in consent with the findings of [42] for Mangos**,** in *Moggridgei Rix* by [34] and *Cymbopogon* [1]. The high level of polymorphism (89%) reveals the story of outbreeding, which is on par with results obtained with RAPD in mango cultivar [22, 27, 43]. Similar results were reported in other fruits and nut species such as *Pistachio olive* by [15], walnut [44], and banana [24]and apple by [29]. The average Heterozygosity (Ho) estimated from primer 1, primer 2, primer 3 was 0.44 for 0.46 and 0.49 respectively, found to be in the moderate range and is similar to the findings in *Mangifera indica* [17] and *Mungbean* and other *Vigna* species [58]. The PIC value for 132 polymorphic loci was 0.21–0.38, averaging 0.353; PIC reflects the discriminating ability of the marker and usually ranges from 0-0.5 for dominant markers like RAPD and ISSR [6, 8]. Therefore, the PIC results of FAM markers used in this study can screen mango genotypes. Similarly, the average active polymorphic loci generated from primers 1, 2 & 3 were 21.5, 30.6 and 6.4, respectively. The highest effective multiplex ratio in this study was observed with primer two as 30.6. The average Marker Index MI was 0.040 for primer 1, 0.043 for primer 2 and 0.045 for primer 3. The higher MI corresponds to the better result [46, 39]. The resolving power (R.P.) of markers primer 1, 2, and 3 was 27.2, 32.8 and 8.4, respectively, the highest R.P. value observed with primer 2, and the lowest was with primer 3. Primer 2 with the highest resolving power is the most informative primer for distinguishing among the mango cultivars.

Phylogenetic relationships & tree construction

Nie's pair-wise genetic identity and distance values for five mango cultivars are shown in Table 4. This shows the genetic similarity ranges from a minimum of 0.507 to a maximum value of 0.674 in the same way as the distance values ranged from a minimum of 0.394 to a maximum of 0.678. The data are par with the findings of [1, 27, 26, 31 and 51] in mango cultivars. Accordingly, the optimal tree generated with branch lengths and bootstrap values with 1000 replicates displayed in Figure: 4 shows that the Dendrogram Cluster Analysis divided the five cultivars into 2 clusters. Cluster 1 comprised Alphonso, Kesar, and Amrapali. This cluster1 was further divided into two clusters displaying Alphonso and Kesar and Amrapali in another cluster. Similarly, cluster 2 displayed Totapuri and Langra cultivars.

Principal Coordinate Analysis (PCoA) and AMOVA Analysis

The PCoA analysis complements cluster analysis [25]. The consistency of the differentiation among the cultivars defined by the cluster analysis indicated that the three RAPD primers used in this study depicted in Figure 5 revealed 81.96 % of the genetic variations among the five cultivars. The first two major coordinates accounted for 60.65 % of the total variation. The contribution of the remaining coordinate was less than 21.31% and is in the same lines as the results obtained through ISSR markers in cultivars *Cymbopogon* by [2]. Significantly, the results of PCoA analysis were consistent with the results of cluster analysis. The Kesar and Amrapali cultivars were grouped in one cluster; Totapuri, Langra and Alphonso cultivars were segregated into three clusters.

The AMOVA results indicated in Figure 6 revealed that 0% of the total molecular variance was within the cultivars. It was expected since the samples selected were from the same geographical area, where most cultivars have arisen through clonal selection [26], showed similarity to the highest degree and indicated their clonal purity. In homogeneous populations, higher amounts of geitonogamy would increase selfing frequencies to approach 100% similarity [21]. Conversely, 28.7% of the variance was attributable among the cultivars; therefore, 100% of the total molecular variance was attributed to the genetic variation among the varieties. This resulting difference may be due to the heterogeneity prevailing between these cultivars.

The significance of molecular analysis was validated and concluded by the Ewens-Watterson Test for Neutrality [10, 55] by **c**omparing the expected homozygosity with the observed homozygosity, based on the within-species variation. The statistic data was calculated using 1000 simulated samples in which the observed F values were 0.52 and the mean value was 0.61. They all are significantly smaller than the simulated mean for all the scored alleles of the genotypes analyzed. Results were evenly distributed to fit into neutral expectation (Wei-gang, 1997), indicating balancing selection for different alleles.

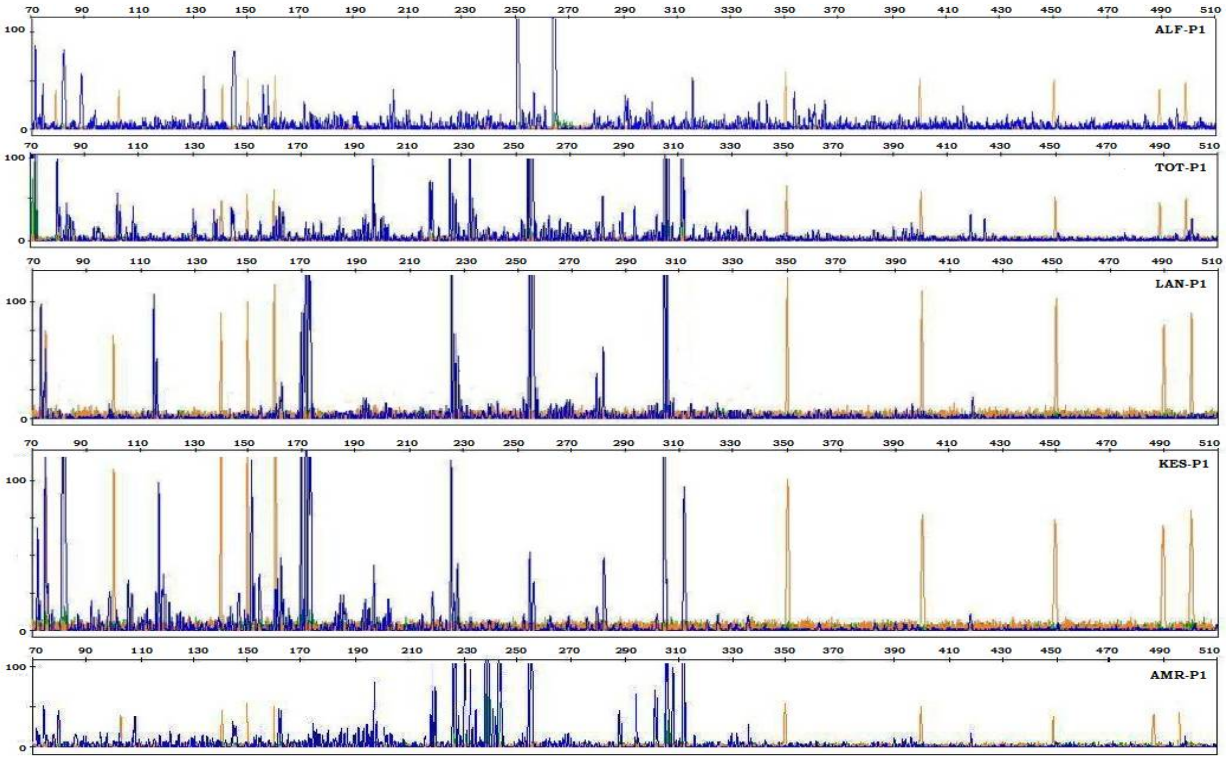

Figure 1: Depict FAM assisted RAPD electropherograms peaks profile of Alphonso, Totapuri, Langara, Kesar and Amrapali generated by primer 1.

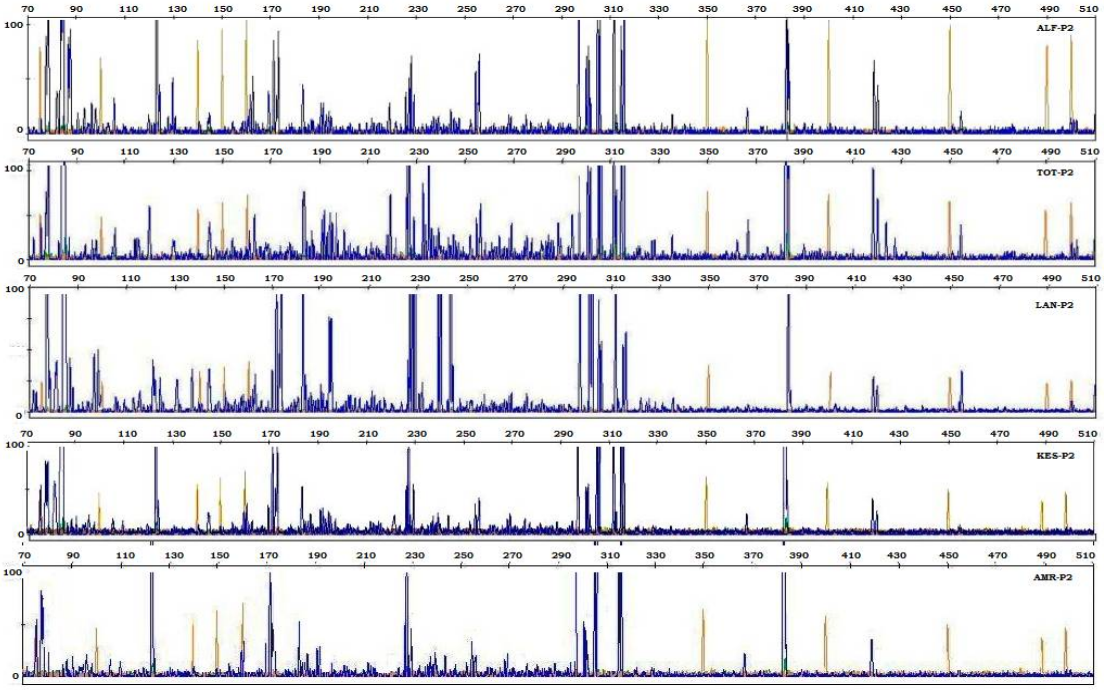

Figure 2: Depict FAM assisted RAPD electropherograms peaks profile of Alphonso, Totapuri, Langara, Kesar and Amrapali generated by primer 2.

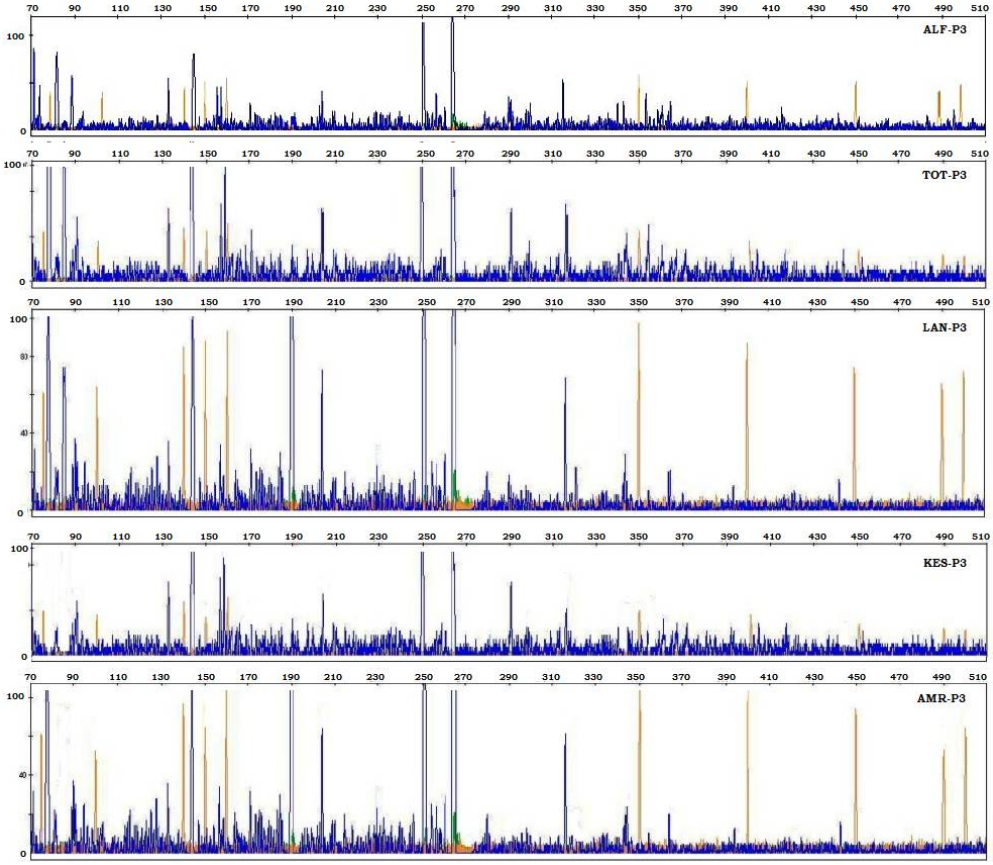

Figure 3: Depict FAM assisted RAPD electropherograms peaks profile of Alphonso, Totapuri, Langara, Kesar and Amrapali generated by primer 3.

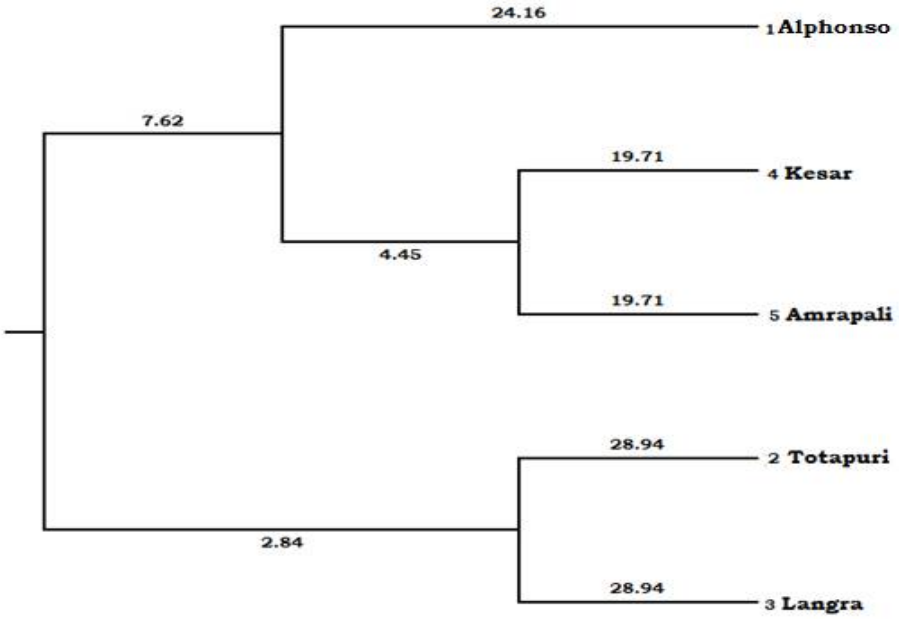

Figure 4: Dendrogram of five mango cultivars based on Nei's Genetic distance

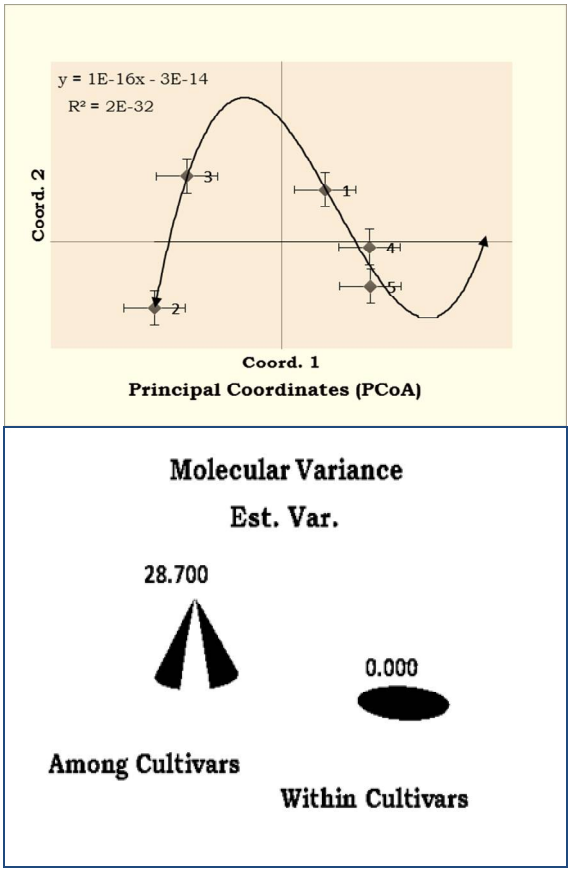

Figure 5: Shows the distribution of variance between and among cultivars

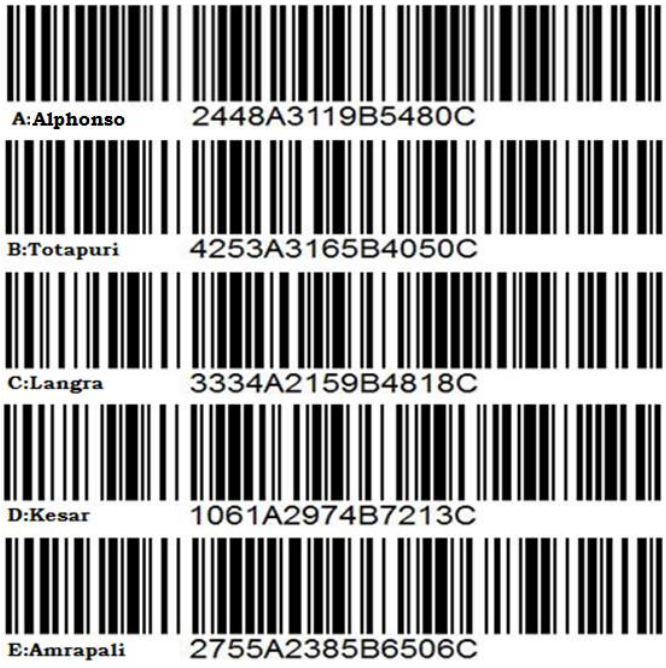

Figure 6: Barcodes for the Mango Cultivars

CONCLUSION

To increase the accuracy, reproducibility of markers, to characterize mango cultivars. Through this study, we have developed three FAM-labelled barcoding primers for five elite mango cultivars. Alphonso, Totapuri, Langra, Kesar and Amrapali, would serve as a genetic key to identify and characterize mango cultivars species and populations. These markers can also help detect various adulterants' presence, as DNA barcodes with standardized DNA sequences can act as tags for several fingerprinting techniques [53].

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