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CHARACTERIZATION AND GENOTYPIC ANALYSIS OF CITRUS CULTIVARS IN PAKISTAN

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Abstract

Molecular analysis for varietal identification is the most genuine and widely accepted technique now a days. Assessment and characterization of genetic diversity in fruit crop species is fundamental for its improvement and development. Genetic diversity analyses were performed on 96 Pakistani local citrus genotypes. In the present study, different type of Simple sequence repeats (SSR) markers was identified but nine expressed sequence tags SSR markers and four simple SSR markers were used for final cultivar identification of 96 samples. Total twelve primer pairs were used to detect polymorphic markers. Several loci were assayed for allelic polymorphism. All SSR primer pairs were reproducible and showed several polymorphic alleles through genetic analyzer. Each marker detected approximately 3 to 5 alleles to assess the genetic variation among citrus genotypes from the Citrus repository of the Citrus Research Institute Sargodha**.** Results revealed that TAA45 showed more polymorphism than TAA15, TAA33, TAA41. Similarly in EST SSR markers, specially designed markers for Pakistani citrus varieties, PK2, PK3 showed two alleles, while PK4, PK5, PK6, PK8, PK9, PK10 showed several allelic polymorphisms ranging between 4-6 polymorphic bands per cultivar. Base pair amplification position of every marker, size range of amplicon, along with their repeating units were also identified. More than fifty alleles were detected, indicating a high level of genetic diversity within this cultivated citrus population. This information can be used in the development of citrus mapping populations, in the selection of parents to be used in cultivar development breeding programs through crossbreeding, and in determining the polymorphic level variation of SSR markers. Studies will provide useful information to assess genetic diversity and characterization of Pakistani Citrus varieties.

Key words: Citrus, Alleles, Molecular markers, SSR, Polymorphism, Germplasm, Genetic diversity.

Introduction

Pakistan produces more than thirty types of different fruits amongst which citrus leads the table with a share of about 30% of total fruit production of Pakistan (Siddique *et al*., 2018). Billions of dollars' worth of citrus is exported to various parts of world from Pakistan every year (Fig. 1). Citrus is cultivated mainly in Sargodha and Bhalwal regions of Punjab Province. Pakistan exports about 0.44 million tons of citrus annually, valuing around 166 million USD (Gmitter *et al*., 2012; Biswas *et al*., 2010; Chen *et al*., 2008). Pakistan produces sweet oranges, mandarins, grapefruit, lemon, lime, kumquat varieties of all six groups of *citrus*. Verification of proper citrus variety is a must for fresh fruit export. Buyers across the world often ask for identification of varieties or cultivars, but sometimes due to the unawareness of farmers or post-harvest mixing, we sell different citrus varieties by considering it some other citrus variety of different group. Sometimes fabricating brand names also sell kinnow as sweet orange to make more profit. It is indispensable for industry. To have a successful strategy for the verification of citrus; variety labeling, its composition, its traceability and validity, all parameters should be assessed (Distefano *et al*., 2013; Duhan *et al*., 2020; Elmouei *et al*., 2011). Due to unidentified citrus cultivars and genotypes, access of Pakistani citrus varieties to larger markets is very limited which results in great economical loss every year (Federici *et al*., 1998).

DNA markers based citrus authentication is the only way to overcome these problems. There are some locus specific markers currently being used in different countries for citrus authentication of genotypes from couple of years but none of such study is reported so far for Pakistani local citrus varieties. For the addition of citrus genotypes into the system and addition of new series of primers, it is vital to add new markers in the existing pool which are easily detectable and more polymorphic for all existing cultivated genotypes. For authentification, a number of methods are employed such as phenotypic markers, morphological characterization, DAr T marker and molecular marker analysis (Schulman, 2007; Nematolahi *et al*., 2013). Amar (2011) reported the use of fluorescent labeled simple sequence length polymorphism for SRAP, SSR and CAPS-SNP markers for genetic diversity of Citrus germplasm collection (Hu *et al*., 2014). Citrus varieties can also be distinguished by using TaqMan based real time PCR technique. Application of molecular markers have now been used mainly to address the problems in Citrus taxonomy. Development of agricultural biotechnology requires rapid and convenient methods for citrus crop plant genotyping. Therefore, the main objective of this study was to develop a panel of polymorphic markers with the ability to authenticate all the citrus genotypes. The aim of current study was to characterize genotypic markers of citrus germplasm present in Pakistan. Present research addressed SDG No. 2 Zero Hunger.

Material and Methods

The entire study was carried out in three steps; first step was the collection of different varieties of citrus, the second step was the extraction of their DNA and final step involved the evaluation of genetic diversity of citrus cultivars using genetic analyzer. Different tools and software were used to identify putative markers to identify different citrus varieties of Pakistan. Twelve SSR markers were used for analysis, markers labelled with PK are EST SSR markers. All of them were analyzed on all 96 varieties of citrus present in Pakistan through genetic analyzer.

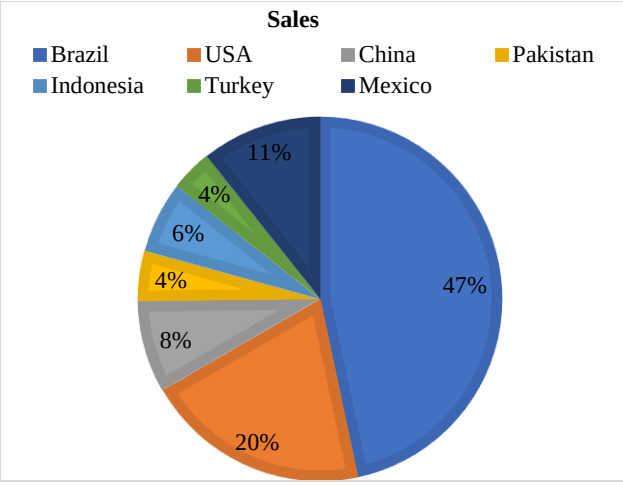

Fig. 1. Worldwide citrus production (Chavan *et al*., 2018).

Sample from true varieties were collected from citrus research institute CRI Sargodha for the identification and characterization of all citrus groups. A total of 96 samples representing various cultivars belonging to the six main groups of Citrus genus (maintained in Citrus Research Institute Sargodha at latitude 32.08 ºN and 72.67ºE) were used for present research.

The genomic DNA of citrus varieties was extracted from leaf using the Murray and Thompson modified protocol (Naz *et al*., 2013). The extracted DNA was stored at -80°C. Young leaves (3-4 weeks old) were collected and used for DNA isolation. 400 mg of fresh leaves were ground and extracted with CTAB protocol. After brief air drying, DNA pellets were re-suspended in 300 µl (300micro-liter) of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and kept at -20°C until use. Some of the designed primers were simple SSR and other were EST SSR specific primers. Products were amplified by PCR to visualize any allelic polymorphism. Genotyping of all varieties was done via genetic analyzer (Broccanello *et al*., 2018). Two TaqMan labelled probes were used. One probe labelled with HEX dye detects allele 1, other probe with VAM dye detects allele 2. Sequence specific forward and reverse primers were used to amplify polymorphic sequence of interest.

Results and Discussion

Molecular tools provide abundant information, they are highly efficient and are insensitive to environmental factors as compared to morphological data (Fernandez *et al*., 2002; Fujii *et al*., 2013). Molecular genetic markers of citrus have provided an ideal means for identifying genotypes, inheritance of economically important characters in fruit plants and following estimation of relatedness between different accessions (Garcia Lor *et al*., 2013; Gulsem *et al*., 2010). The present study investigated identification of 96 Citrus varieties and related cultivars using simple sequence repeat (SSR) markers (Table 1). These markers were applied to assess genetic variations. Results showed newly developed PK markers were valuable resource and provided powerful tools for genetic breeding of Pakistani citrus varieties. Genetic analyzer gave detailed description of allelic polymorphism within related group of all citrus groups, but PK-EST SSR markers specifically designed for Pakistani citrus varieties showed great polymorphism. Ninety-six well plate genetic analyzer can allow for high-throughput processing of citrus genotyping samples, which can be useful for large-scale studies (Fig. 2).

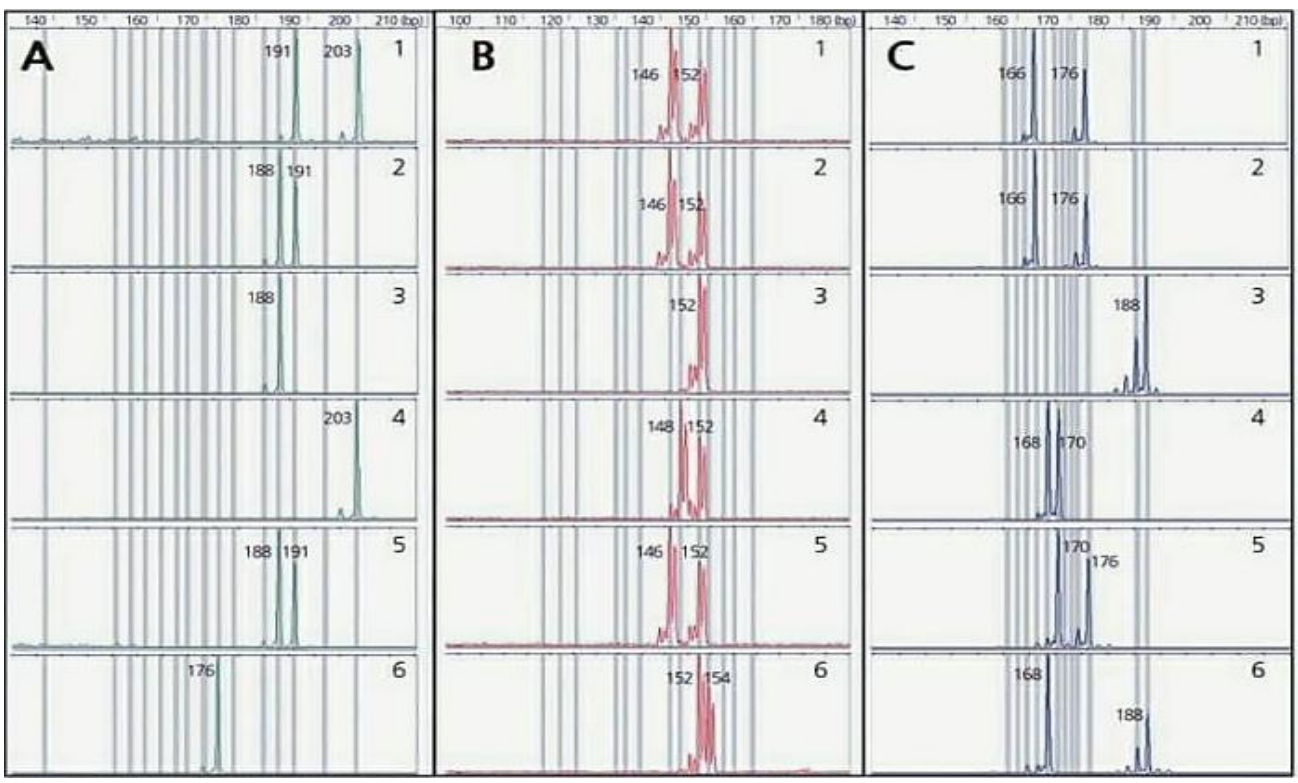

Fig. 2. Amplified fragment pattern of some SSR markers (TAA15 (A), TAA33(B), TAA41(C) in six citrus varieties. PCR products were separated and detected using 3130 xl genetic analyzer (Applied biosystems) with 36cm array and polymer.

Table 1. Names of citrus cultivars, 96 samples of Pakistani citrus varieties from citrus research institute Sargodha.

1. Pomelo White	33. Nova	65. Frost Navel
2. Shamber	34. Dancy	66. Cutter Orange
3. Chakotra	35. Taracco	67. Campbell Valencia
4. Star Ruby	36. Washington Navel	68. Campbell Valencia
5. Ruby Red	37. Valencia Late	69. Atwood Early
6. Flame Seedling	38. Salustiana	70. Atwood Early
7. Tar Ruby grafted on ray rubi	39. Jaffa	71. Midsweet
8. Ray Rubi	40. Musambi	72. Navellina
9. Red Blush	41. Succari	73. Lanelate
10. Link Ruby	42. Pineapple	74. Rhodred Valencia
11. Shaddock	43. Marrs Early	75. Sunstar
12. Meiwa (sweet)	44. Olinda Valencia	76. Blood Red
13. Naghmi (Oval)	45. Parson Brown	77. Moro Blood
14. Marumi (Round)	46. Hamlin	78. Taracco Rose
15. Lemon	47. Murrccot	79. Kala Blood
16. Volka Meriana	48. Casa Grande	80. Kala Blood
17. Desi lemon	49. Taracco-N	81. Local Blood
18. Bara Masi	50. Hinkley	82. Cara Cara
19. Lake Land Lemon	51. Kozan	83. Bouqet-De-Fleurs
20. Mesero Lemon	52. Westin	84. Degrasse
21. Lisbon Lime	53. Natal	85. Minneola
22. Peshawari Mittha	54. Robel	86. Sunbright
23. Local Mittha	55. Peera Rio	87. Pearl
24. Tahiti Lime	56. Frost Rose	88. Orlando
25. Persian Lime	57. Spring Navel	89. Fremont
26. Eustis Lime	58. New Hall	90. Fairchild
27. Dancy	59. Ruby Blood	91. Fallglo
28. SeedlessKinnow	60. Ruby Sweet	92. Ambersweet
29. Nagpuri Sangtra	61. Eamy Gold	93. C-35
30. Feuterell's Early	62. Lane Navel	94. Rangpur Lime
31. Honey	63. Glen Navel	95. Gada Dehi
32. Kinnow	64. Navelate	96. Flying Dragon

Table 2. Primer sequences of different type of TAA-SSR markers.

Marker name	Fluorescently labelled dye	Forward primer sequence	Reverse primer sequence
TAA15	6-FAM	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC
TAA33	6-FAM	GGTACTGATAGTACTGCGGCG	GCTAATCGCTACGTCTTCGC
TAA45	HEX	GCACCTTTTATACCTGACTCGG	TTCAGCATTTGAGTTGGTTACG
TAA-41	HEX	AGGTCTACATTGGCATTGTC	ACATGCAGTGCTATAATGAATG

Data analysis: Twelve primers were selected for PCR amplification (Tables 2 and 3). EST-SSR markers were further used to assess the genetic diversity and the population structure of 110 cultivated citrus cultivars. PK primers were EST SSR marker, sequences are given in Table 2. The amplification products were analyzed for presence and absence of alleles for all primer sets. Citrus varieties of all different groups show different results and band sizes. Allelic variation was also observed almost in all cultivars. Data was further used to calculate the similarity and dissimilarity between cultivars reflecting the genetic distance between them. PCR Products of selective amplifications were separated by Capillary Electrophoresis on an ABI Prism 310 Genetic Analyzer.

Due to the absence of basic knowledge about genetic diversity it becomes difficult for the farmers and breeders to utilize, improve and conserve the economically significant crops (Jannati *et al*, 2009; Jarrell *et al*., 1992). Detailed and clear understating of diversity enables the farmers in order to choose clones correctly for the conservation, improvement and elimination of repetition. This also helps them to maintain cost profit ratio of the crop's germplasm. That's why determination and identification of genetic diversity is referred as the basic and important step for utilizing plants resources (Jin *et al*., 2020; Graham *et al*., 1999).

Our study screened 12 pairs of primers with highest level of polymorphism within species and lack of polymorphism among species, good repeatability and easy and clear recognizable bands. Heterozygosity was also observed in some of the primer pairs as heterozygosity is important for both cultural and natural populations because it gives greater spectrum of genotypes for adaptive responses to external conditions and also because heterozygous individuals are normally preferable as compared to less heterozygous individuals due to various economically important traits like fertility, disease resistance and growth. Below is the result of banding pattern observed by different markers in different citrus varieties.

Table 3. Fluorescently labelled Primer sequences, size range of amplicon of different markers, and repeating units of different type of EST-SSR PK markers.

Marker name	Fluorescently labelled dye	SSR	No. of repeats	bp position	Forward primer sequence	Reverse primer sequence	product E. size
PK-3	6-FAM	agc	8	87-110	TTCAAGCCAAAGCAAGAGGT	ACCCAAATGCTCAAAACACC	269
PK-5	6-FAM	taa	9	227-253	GCAGCAATTCTGAAGGAAGG	ACGGCCTCAATGGAACCTAT	140
PK-4	HEX	tct	8	312-335	ACGATGACCAAGAATCCAGC	AAGATCCCACAAGCCATCAC	248
PK-2	6-FAM	gaa	9	66-92	GGTGTTGTTCTCGCAACAGA	CGGCAGCCTATTGCTACTTC	286
PK-6	6-FAM	ctt	10	145-174	ACTGCTGTTCACCCTGTTCC	GAGAGCTTTCGAGCCTTTGA	140
PK-8	6-FAM	taa	16	262-293	TCAGCACTGAATCCAATCCA	GTGAGAGCTTGAGGCTGACC	292
PK-9	HEX	at	13	1094-1119	GGCTGCTTTAGCAATTGAGG	AACATTATCCCCGGTTGACA	252
PK-10	6-FAM	at	33	155-220	AATTTCCAATTGAAACCCCC	GGGCAAGTCTCTTTTTCCCT	182

TAA15: Genetic analyzer data reveals that by TAA15, thirteen citrus varieties showed 3 allelic bands ranging in size from 147bp-199bp, while the rest of the varieties shows 2 bands. Like, *Shaddock* (grapefruit) showed 3 bands at 159,162,199 bp, all other grapefruit cultivars like *Red Blush, Ruby Red* showed 2 bands one at 159 and the other at 183 bp position. Among lemon and lime cultivars, only desi lemon and Eustis lime showed 3 bands all other varieties show similar bands at 159,183 bp position.

TAA33: Four citrus varieties showed 3 allelic bands ranging in size from 112bp-121bp, while the rest of the varieties shows 2 bands. Like, Chakotra and cutter orange varieties showed high polymorphism (3 alleles). Atwood early and sunstar from sweet orange they show 3 bands (112,112,118 and 109,115,121), rest all other citrus cultivars showed 2 bands.

TAA41: Mesero lemon, Tahiti lime and glen navel orange they show 4 bands at 130-150bp. all other citrus varieties show 3 polymorphic allelic bands ranging in size from 130-150bp. Desi lemon, shaddock, Persian lime they show bands at 128,141,165,bp positions.

TAA45: With TAA45 marker, many citrus varieties showed 5,4,3 bands ranging in size from 129bp-136bp.TAA45 showed high polymorphism. *Bara masi* (lemon) showed 6 bands ranging in size from 129-151.*Meiwa and Marumi* (kumquats) showed 5 bands ranging in size from 127-142bp.*Shaddock* (grapefruit) showed 4 bands one at 121 bp others at 127,129,133 bp, whereas all other grapefruit cultivars show 3 bands at 129,133 and 136 bp.

PK2: Ruby red grapefruit shows 4 bands between 275-290 bp position. Shamber, Minneola, Cara Cara, New Hall, Taracco – N,Kozan, Casa Grande, Shamber, Jaffa they all show three alleles 269,275,281 bp position. Rest all varieties showed 2 bands.

PK3: Red blush,sweet meiwa, local mittha, flying dragon, fallglow, they show no bands with EST SSR marker PK3. Rest all other varieties showed double band at 139 bp position. Only grapefruit cultivars show bands at 145 bp position. PK3 marker does not show more polymorphism.

PK4: Desi lemon and rubidox grapefruit show 3 allelic bands ranging in size from 240-250bp. All other varieties show 2 bands at 247,250 bp position.

PK5: Desi lemon, cutter orange and Atwood early sweet orange they show 4 polymorphic alleles ranging in size from 256-280bp. Flame seedling, Tahiti lime and navellate they show 3 polymorphic bands. Rest all varieties show 2 Bands at 266 bp position.

PK6: PK6 EST SSR marker shows that Tahiti lime and flame seedling show 4 bands (131-149bp). 3 different type of allelic polymorphism was exhibited by rubidox, Jattikhatti, Ambersweet, Navelate, Desi lemon, Shaddock. All other citrus varieties shows 2 bands at 131 and 137 bp position almost in every group.

PK8: Tahiti lime shows 4 bands. Lake land lemon, Natal, robel, moro blood (sweet orange) they show 3 bands at 290-299 bp position. All other Pakistani citrus cultivars show two bands.

PK9: Glen navel orange and westin sweet orange they show 3 bands between 240-256 bp. All other citrus varieties show 2 bands one at 250bp, the other at 256 bp with slight change in bp position as all groups of citrus are different. Many varieties did not show any band with EST SSR marker PK9.

PK10: Feuterell's Early mandarin and flame seedling, chakotra, pomelo white grapefruits they show 3 polymorphic bands ranging in size from 123 -158 bp. In grapefruit citrus group, Pomelo white and chakotra showed similar result compared to flame seedling. Rest all shows 2 polymorphic bands between 120-150 bp. Therefore, the impact of having more polymorphic alleles in plants depends on the specific traits being considered and the balance between the benefits and costs of maintaining genetic diversity.

Molecular markers have provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters. Having more polymorphic alleles can increase genetic diversity within a plant population, which can enhance the plant's ability to adapt to changing environmental conditions, resist pests and diseases, and improve overall fitness. This is because a more diverse gene pool provides more opportunities for beneficial traits to arise through recombination and mutation.

Conclusion

Markers are very useful for evaluating the genetic diversity and relationships between Citrus species. Analysis of citrus germplasm (maintained in Citrus Research institute, Sargodha, Pakistan) by genetic analyzers provide more accurate and efficient results, especially in cases where the differences between plants are subtle or difficult to detect. Studies will also help to understand the distinctive biological features to gain insight into the cultivar ID of citrus for improving export in Pakistan. Overall, the use of polymorphic markers in citrus has been critical for understanding the genetic diversity and relationships among different citrus varieties, which can be used to guide breeding programs and conservation efforts.

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