Archives of Current Research International

Volume 24, Issue 11, Page 285-295, 2024; Article no.ACRI.126311
ISSN: 2454-7077

Integrating Morphological and Molecular Techniques to Identify Intraspecific Crosses in Citrus

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AKS and MKB conceptualized and designed the experiments. Author MKB preparation of the original manuscript. Authors AKS, MKB and MK analysis of data and interpretation. Author MK edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/acri/2024/v24i11970

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/126311

Original Research Article

Received: 07/09/2024
Accepted: 11/11/2024
Published: 15/11/2024

ABSTRACT

The morphological analysis and simple sequence repeat molecular studies were conducted with the objective for the development of novel promising citrus rootstocks with resistance to phytophthora and salinity stress. This study was established as a novel citrus breeding program at Dr. J.C. Bakhshi Regional Research Station, Abohar, Punjab Agricultural University. Here, rough lemon (*Citrus jambhiri*) was used as female parent while trifoliate orange (*Poncirus trifoliate*), Carrizo (*Carrizo citrange*) and rangpur lime (*Citrus limonia*) were taken as the male parent. Rough lemon is productive, vigorous with high tolerance towards active lime and diseases viz. *Citrus* virus exocortis

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Cite as: *Batth, Manveen Kaur, Anil Kumar Sangwan, and Maninder Kaur. 2024. "Integrating Morphological and Molecular Techniques to Identify Intraspecific Crosses in Citrus". Archives of Current Research International 24 (11):285-95. https://doi.org/10.9734/acri/2024/v24i11970.*

and *Citrus* tristeza virus, but it is susceptible to phytophthora pathogen. Trifoliate orange and carrizo, have been described as an “ultra-resistant rootstock” while rangpur lime is tolerant against salinity. Hybrids were derived from the cross between the *C. jambhiri* × *P. trifoliate*, *C. jambhiri* × *C. citrange* and *C. jambhiri* × *C. limonia*. The range of polymorphism information content (PIC) values ranged from 0.0 to 0.8 with 0.66 average PIC value. Seventy-three SSR markers were used for molecular analysis and the promising F50 marker showed maximum 0.87 PIC value that confirms the higher degree of polymorphism and diversity among the genotypes. The different combination of crosses undergo selection of 26 hybrid seedlings through both morphological and molecular screening utilizing Simple Sequence Repeats (SSR) markers. Thus, morphological and molecular approach proved to be effective for the differentiation of hybrids from nucellar seedlings among citrus rootstocks involving polyembryony cultivars.

Keywords: Citrus; hybrids; phytophthora; rough lemon; SSR markers.

1. INTRODUCTION

Citrus is an economically important fruit crop ranking third position in production after mango and banana throughout the world. The area under production is 0.95 million hectares with total production of 1166 million tonnes (Anonymous 2017). In citrus, rootstocks play vital role in growth, yield and fruit characteristics of a variety, growth and yield. Rootstocks enable the cultivation of a scion variety in different agro-climatic conditions by virtue of their resistance to various biotic (insect pests and diseases) and abiotic stresses (soil salinity and poor drainage). *P. trifoliate* is well known for its resistance to phytophthora and also imparts tolerance to low temperature and rangpur lime which is widely used in Brazil have tolerance to soil salinity and induce high yield into the scion. *C. limonia* is mainly used as rootstock in north western India because it is productive, vigorous, with high tolerance towards active lime and *Citrus tristeza* virus, but it is susceptible to phytophthora pathogen. *P. trifoliate*, *C. citrange*, has been considered as an “ultra-resistant rootstock” against phytophthora whereas rangpur lime is found to be resistance against salinity (Hutchison 1974; Wutsher 1974). Thus, there is an urgency for the development of phytophthora resistant rootstock of citrus. Therefore, rough lemon was taken as female parents and *P. trifoliate*, *C. citrange* and *C. limonia* as the male parent. This novel breeding program was initiated with the aim for the development of promising citrus rootstock resistance to phytophthora and salinity.

The favourable feature of rootstock is nucellar embryony owing to the true-to-type plants production at a mimimal cost during propagation (Khan and Kender 2007). Besides, apomictic reproduction form is not a favourable character for breeders, mainly due to difficulty in distinguishing the zygotic seedlings from nucellar ones. The screening of maximum number of plants to sort out an ample number of hybrids is a tedious process. In citrus, this critical step has limitation because of the persistence of apomixes and polyembryony in most of citrus cultivars (Frost and Soost, 1968). The adventitious formation of embryos occurs from somatic nucellus tissue that surrounds the embryo sac, resulting in huge number of embryos vital for the mother plant (Kepiro and Roose, 2009). Early identification of hybrid seedlings is a crucial step to eliminate unwanted plantlets derived from nucellar embryo with the purpose of saving time, land with effective cost management.

Generally, citrus hybrids identification occurs through plant morphological characters. The derivation of hybrids from polymbryony cultivars result in easy identification, considering the genotypes as male parents with dominant character. Besides, the hybrids identification from the polyembryony citrus genotypes crosses owe a difficult approach when parents exclusive of dominant character. Under these circumstances, molecular markers serve as efficient tool for hybrid identification in citrus (Gaikwad et al. 2024). The identification of zygotic seedlings at an early stage is vital for a rapid propagation programme. Earlier, numerous biochemical methods were used i.e. gas chromatography (Weinbaum et al. 1982) and isoenzyme pattern analysis (Moore and Castle 1988) were used but these are not advantageous due to enzymatic darkening due to occurrence of polyphenols (Esen and Soost, 1974). Keeping in this account, the identification of reliable methods through molecular approach for distinguishing nucellar from zygotic seedlings at an early stage is the need of an hour.

In an early stage, the identification of hybrid seedlings has the potential to generate improvement in the efficiency of breeding program. In this perspective, the hybrids were identified both by morphological character and molecular markers. The DNA polymorphism for the hybrid seedlings identification is crucial in citrus breeding programs increase the efficacy of screening of progenies. Among DNA based methods, use of simple sequence repeat (SSR) markers is most promising approach for distinguishing hybrid from nucellar citrus seedlings. SSR markers are preferrable in citrus for assessment of genetic variability (Singh et al. 2023). Despite, these DNA-based methods are cost-intensive with more time consumption during development of crosses constituting polyembryony genotypes and its association with large numbers of nucellar seedling (Moore and Castle 1988). Thus, the method for the optimization of the hybrids screening from such crosses serves as preliminary step for initial selection of putative hybrids through morphological features, followed by the validation using molecular markers. This approach enables the identification of hybrid seedlings from crosses involving polyembryony cultivars through the combinatorial approach of morphological selection and SSR analysis.

2. MATERIALS AND METHODS

2.1 Controlled Pollination

The rough lemon was used as female and *P. trifoliate*, *C. citrange* and *C. limonia* were used as male parents, respectively. These parental plants were available at Punjab Agricultural University and controlled pollinations were conducted at Dr. J.C. Bakhshi Regional Research Station, Abohar.

2.2 Plant Material

The new citrus rootstocks hybrids developed from different cross combinations on five-year old trees planted in mother block of rough lemon during the year 2021 and 2022 at Regional Research Station, Abohar. The random selection of twenty rough lemon plants from each cross combination and two plants each of male parents were done. The selected rough lemon trees with three branches on each of four sides were covered with muslin cloth bags. The removal of opened flower and undeveloped buds undergo covering. This was done to reduce the chances of contamination by unknown pollen. The flowers at the verge of opening undergo emasculation in morning before dehiscence.

The collection of perfect pollen parents and freshly opened staminate flower in the petridish before dehiscence was done. The pollen shedding takes place in shade for 3-4 hours under 100watt lamp light. The emasculated flowers on tree undergo pollination. The coating of pollen grains on the stigma were done with hair brush, followed by bagging of the pollinated flowers. Then, after fruit setting these pollinated flowers were removed. Fruits were collected at full mature stage approximately from 210 to 300 days after pollination and extraction of seeds from each cross combination were done separately. Later on, these undergo washing under running tap water and placed in shade for the purpose of drying. These seeds were treated with bavistin and sown single seeded in sowing trays for raising F_1 hybrids. The seed germination started after 21 days of sowing. During the attainment of height of 20-25 cm in F_1 seedlings, fresh and young leaves were used for DNA extraction. The trifoliate seedlings were identified, and counting of all plants emerged from each seed was done.

The morphological features of the leaf apex were used for the selection of hybrids. Morphological characters of the identified hybrids were depicted using descriptor for citrus by IPGRI, Italy (Anonymous, 1999). Further, the germinated seedlings were selected and used for molecular analysis.

2.3 Genomic DNA Isolation

The different citrus rootstock genotypes were used and fresh, young, disease and insect free leaves were used for DNA extraction. The collection of leaf samples was done and proceeded for DNA isolation. DNA extraction procedure was done followed the procedure of Russel and Shamrock 2000 and Saghai-Maroof et al. (1984). DNA was quantified using 0.8 per cent agarose gel electrophoresis.

2.4 Molecular Analysis

A total of 73 SSR primers were used and 41 of them showed polymorphism with 56.61 percent of polymorphism. These polymorphic markers were subjected to polymerase chain reaction (PCR) reaction. PCR cycle constitutes pre-denaturation at 94°C for 5 min, denaturation step of 94°C for 30 sec 35 cycles, annealing

temperature at 55°C for 40 sec, and extension step at 72°C for 7 min (Kijas et al. 1995). The amplified PCR products were resolved on 2.5 per cent agarose gel electrophoresis system (Amresco 30175 Solon Ind. PKWY, solon, Ohio 44139) and PIC analysis was done.

3. RESULTS AND DISCUSSION

3.1 Morphological Screening of Hybrids

The hybrids of three different cross combinations were selected on the basis of their dominant trifoliate leaf shape morphological trait. Carrizo and trifoliate orange exhibited trifoliate leaf shape while rough lemon and rangpur lime showed single leaf structure (Fig. 1). The variation in leaf apex shape in rootstock and hybrids is shown in Fig. 2. This morphological difference at the leaf apex eases the hybrid identification at morphological level.

3.2 Cross Pollination

Three different cross combinations were attempted through controlled pollination to set hybrids during two years 2021 and 2022. The cross rough lemon × trifoliate orange resulted in 276 and 2131 seedlings, rough lemon × carrizo form 481 and 820 seedlings; and rough lemon × rangpur lime produced 238 and 2227 seedlings during the year 2021 and 2022 respectively. Owing to the perennial nature in citrus, we observed that during the year 2022, seedling number was higher than the year 2021.

3.3 Molecular Analysis

Seventy-three SSR markers were used for the selection of hybrids among three cross combinations. About 41 markers showed polymorphism among parents and hybrids (Fig. 3). PIC values of these four genotypes ranged from '0' (monomorphic) to '1' (highly discriminative with number of alleles in equal frequencies). The PIC value determines an estimate of the marker discriminating power considering many alleles at a locus and relative frequencies of these alleles in the genotypes. PIC value and the number of alleles detected using SSR markers presented in Supplementary Table 1. The PIC values fall in the range of 0 to 0.8 with an average 0.66 PIC value. We report that the marker F50 had highest PIC 0.87 value that determines greater level of polymorphism and diversity among the genotypes and could be used in plant breeding selection programmes. SSR markers viz. F20, CCSME42, F61, F40, CCSM147, CAG01, CCT01 with 0.6 PIC value also showed maximum level of polymorphism. Contrastingly, PIC value ranged from 0.32 (CCSME8, CCSMEC3, CCSME49, F16) to 0.828 (CCSME29), in rangpur lime with an 0.51 average PIC value among all the genotypes. Our results inferred that the PIC values vary with crop types and genotypes. Froelicher et al., 2010 also worked on 77 genotypes in citrus and found the PIC value from 0.05 to 0.70 over the four loci. PIC value in mandarin, lemon, grapefruit, sweet orange and natural hybrids were reported as 0.63, 0.68,0.61, 0.41 and 0.64 respectively (Novelli et al., 2000). Yoon et al. (2007) also found that the PIC values in peach and nectarine ranged from 0.326 to 0.779 with an average of 0.643. These SSR markers results were used for determination of dissimilarity coefficient from dendrogram in four genotypes. Among all these genotypes, the genetic distance ranged from 0.31 to 0.45 as shown in dendrogram (Fig. 5, Table 1).

C. citrange and trifoliate orange showing their close relation, which were further confirmed through the 0.31 low value of dissimilarity coefficient. Polymorphic markers determine the hybrid confirmation in all the three cross combinations were shown in Tables 2-4. All parents and their respective hybrids were analysed using 41 polymorphic SSR markers. The hybridity confirmation was done in the seedlings comprising of two amplicons derived from both the parents. The hybrid seedlings were selected from all the cross combinations with variability in leaf apex morphology has been shown in Fig. 5.

Dendrogram showed coincidence with pedigree information also as *C. citange* is hybrid of trifoliate orange and sweet orange. However, rough lemon and carrizo reported with 0.45 higher dissimilarity coefficient due to persistence of distinct species. Thus, rough lemon and rangpur lime categorised into two different groups. The genetic diversity of oat and tall fescue grass genotypes also observed through the combination of molecular markers and morphological methods (Arora et al. 2021; Sharma et al. 2019). Naliath et al. (2017) specifically reported that SSR marker CS41 and DY287851 differentiate rough lemon accessions from rangpur lime and trifoliate orange and its hybrids.

Table 1. Genetic distance showing dissimilarity coefficient values among citrus genotypes

Genotypes	Trifoliate orange	Rough lemon	Carrizo	Rangpur lime
Trifoliate orange	-	-	-	-
Rough lemon	0.44	-	-	-
Carrizo	0.31	0.45	-	-
Rangpur lime	0.40	0.40	0.43	

Table 2. Hybridity confirmation of cross between rough lemon and trifoliate orange using polymorphic SSR markers

Markers / Hybrids	F50	CCSMEc13	CCSMEc7	CCSMEc29	F43	CAC33	TAA33
H-1	AB*	A	A	A	A	A	A
H-2	A	A	AB	A	A	A	A
H-3	A	A	A	A	AB	A	A
H-4	A	A	A	A	A	AB	A
H-5	A	AB	A	A	A	A	A
H-6	A	A	A	AB	A	A	A
H-7	A	A	A	A	A	A	AB
H-8	A	A	AB	A	A	A	A
H-9	AB	A	A	A	A	A	A
H-10	A	A	A	A	A	A	AB
H-11	A	A	A	A	A	AB	A
H-12	A	A	A	AB	A	A	A
H-13	A	AB	A	A	A	A	A

**'A' shows rough lemon specific allele, 'B' shows trifoliate orange specific allele and 'AB' shows the heterozygous hybrid seedlings*

Table 3. Hybridity confirmation of cross between rough lemon and carrizo using SSR polymorphic markers

Markers / Hybrids	CCSME7	CCSMEc4
H-1	AB*	A
H-2	A	AB
H-3	A	AB

**'A' shows rough lemon specific allele, 'B' shows C. citrange specific allele and 'AB' shows the heterozygous hybrid seedlings*

Table 4. Hybridity confirmation of cross between rough lemon and rangpur lime using SSR polymorphic markers

Markers / Hybrids	F50	CCSME13	CAT01
H-1	AB*	A	A
H-2	A	AB	A
H-3	A	AB	A
H-4	A	A	AB
H-5	AB	A	A
H-6	A	AB	A
H-7	A	A	AB
H-8	A	A	AB
H-9	AB	A	A
H-10	AB	A	A

**'A' shows rough lemon specific allele, 'B' shows rangpur lime specific allele and 'AB' shows the heterozygous hybrid seedling*

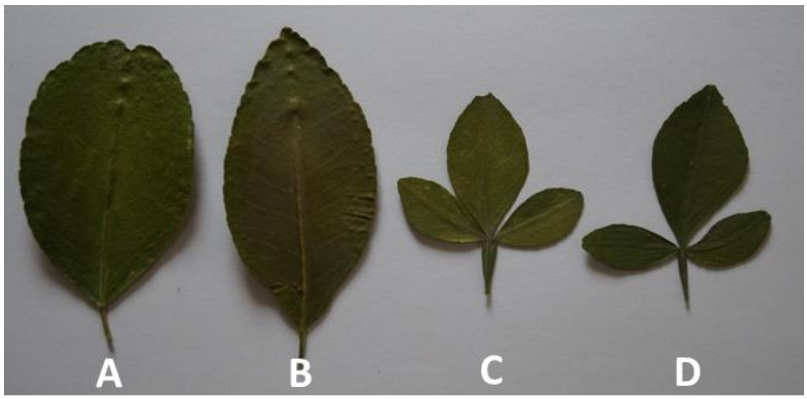

Fig. 1. Variability in leaf apex shape (A) Rough lemon (B) Rangpur lime (C) Carrizo (D) Trifoliate orange

Fig. 2. Hybrids seedlings emergence with (A) Rootstock with single leaf (B) Hybrid with trifoliate leaf shape

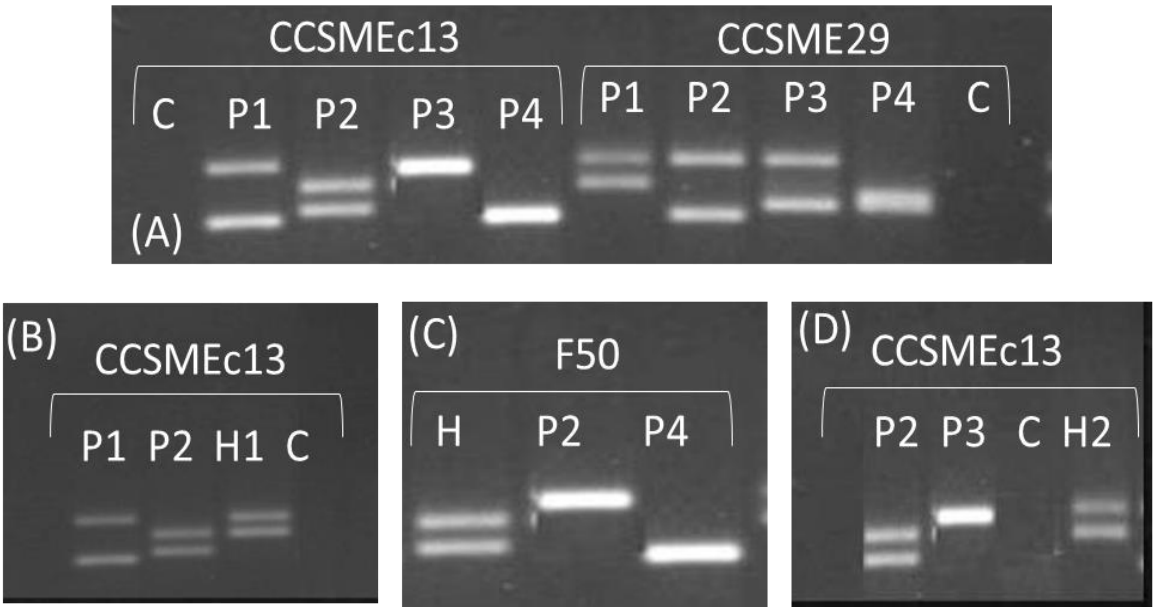

Fig. 3 Parental Polymorphism using SSR markers i.e. CCSMEc13, CCSMEc29, F50 (A) citrus rootstock genotypes and (B-D) parents and hybrids viz. P1; trifoliate orange, P2; rough lemon, P3; Carrizo, P4; rangpur lime, H; Hybrid, C; Control

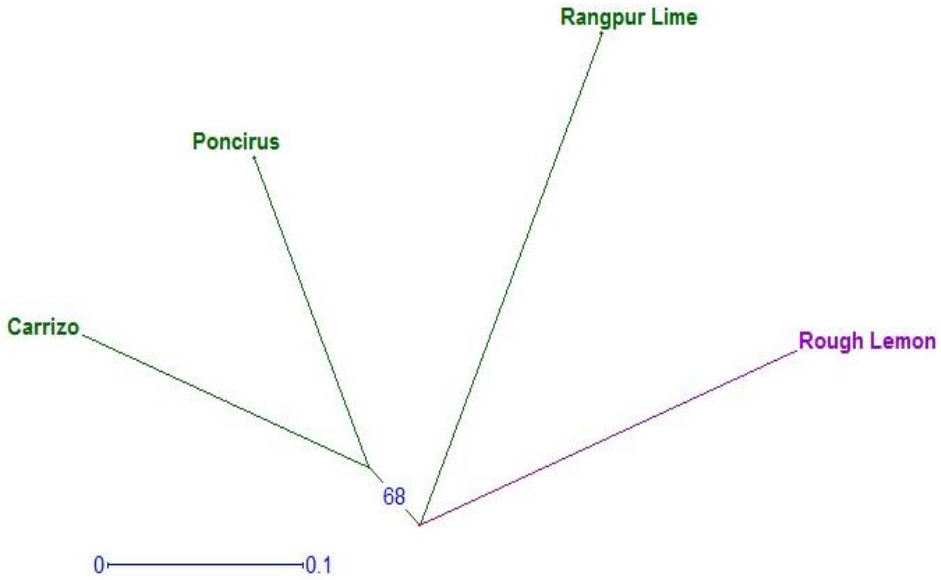

Fig. 4. Dendrogram showing genetic distance among citrus rootstocks

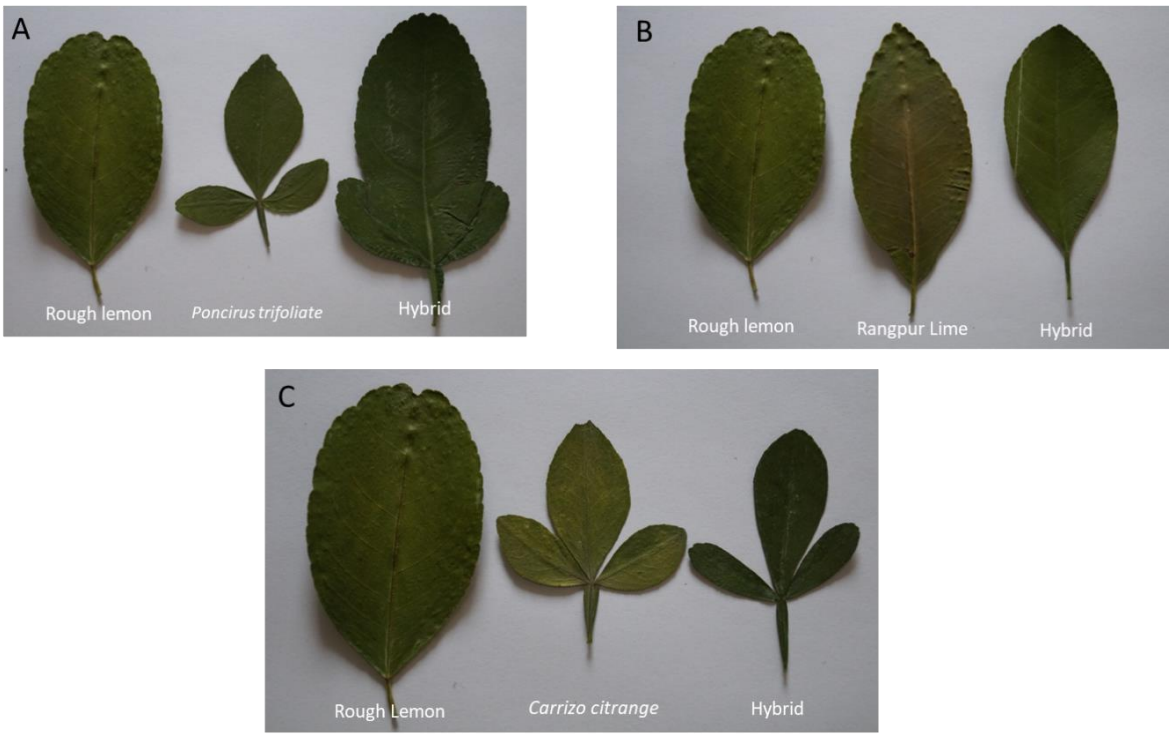

Fig. 5. Variability observed among parents and the hybrid of (A) rough lemon x trifoliate orange, (B) rough lemon x rangpur lime, (C) rough lemon × carrizo

In cross combination of rough lemon × *P. trifoliate*, the F50, CCSMEC13, CCSMEc7, CCSME29, F43, CAC 33 and TAA33 were identified as promising SSR markers that identify a total of thirteen hybrids. Similarly in the second cross combination rough lemon × *C. citrange*, three hybrid seedlings were confirmed with CCSME7 and CCSMEc4. Whereas in third cross rough lemon × *C. limonia*, ten hybrid seedlings were confirmed by SSR marker F50, CCSME13 and CAT01.

Similarly, Ahmad et al. (2012) also used SSR markers for the identification of citrus hybrids.

The five SSR markers (TTA15, TTA27, TTA33, CCSM 18 and CCSME147), were used and 99 hybrids from the crosses NARC 05-17x Sanguinello, NARC 05-18x Tarocco, and kinnow × Tarocco were screened. Among these markers, two SSR markers viz. TTA15 and CCSM147 determine the hybrid identification. Rao et al. (2008) employed the use of RAPD and expressed sequence tag- SSR markers for characterizing the nucellar and zygotic seedlings in the introgression crosses of pummelo (*C. maxima*) and mandarin (*C. reticulate*). Moreover, Ruiz et al. (2000) also gave emphasis on the use of SSRs markers for distinguishing sexual from nucellar citrus seedlings.

4. CONCLUSION

The present study concluded that the variability exists in the plant materials and selection of hybrids from both morphological and molecular methods definitely would be beneficial in plant breeding and hybridization programmes. The genotypes used in the generation of crosses showed genetic divergence and they could be used for the generation of transgressive segregants. The preliminary morphological screening serves as a primary step to reduce the screening load of larger population of seedlings. Further, molecular screening paves towards the crop improvement programmes through selection of hybrids conferring resistance to phytophthora and salinity conditions. The screening for the development of phytophthora resistant rootstock of citrus is underway. These identified hybrids explain sufficient genetic variability and may be recommended for future citrus breeding programmes.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ACKNOWLEDGEMENT

The corresponding author acknowledges the members at Dr. J.C. Bakhshi Regional Research Station, Abohar, Punjab Agricultural University, Ludhiana, Punjab, India for providing support and infrastructural facilities to conduct this experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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SUPPLEMENTARY DATA

Supplementary Table 1. Polymorphic information content (PIC) and number of alleles amplified using SSR markers

Sr. No.	SSR marker	Number of alleles amplified	PIC value
1	CCSMEc 10	1	0
2	CCSMEc 15	1	0
3	CCSME 26	2	0.5
4	CCSME41	2	0.37
5	CCSME50	3	0.64
6	Ci03C08	2	0.5
7	F17	1	0
8	F38	2	0.44
9	F53	1	0
10	F98	1	0
11	CCSMEc8	4	0.69
12	CCSMEc14	1	0
13	CCSME23	3	0.66
14	CCSME33	3	0.66
15	CCSME49	1	0
16	CAC15	1	0
17	F13	2	0.5
18	F33	2	0.37
19	F50	2	0.87
20	F90	3	0.61
21	CCSMEc7	6	0.81
22	CCSMEc13	4	0.72
23	CCSME8	1	0
24	CCSME31	1	0
25	CCSME46	1	0
26	CCSME06	1	0
27	F07	1	0
28	F29	1	0
29	F46	1	0
30	F87	1	0
31	TAA52	2	0.5
32	CAGG9	2	0.5
33	TAA1	2	0.37
34	TAA15	1	0.37
35	CAC23	1	0
36	282(DY294129)cds	3	0.65
37	TAA33	1	0
38	CAC33	6	0.78
39	CCSMEc4	5	0.78
40	CCSMEc12	1	0
41	CCSME5	3	0.56
42	CCSME29	4	0.69
43	CCSME43	1	0
44	TTA41	4	0.72
45	F02	4	0.66
46	F23	1	0
47	F43	3	0.55
48	F77	1	0
49	CCSMEc3	3	0.66
50	CCSMEc11	1	0
51	CCSME1	3	0.65

Sr. No.	SSR marker	Number of alleles amplified	PIC value
52	CCSME27	2	0.37
53	CCSME42	3	0.64
54	67(DY268562)	2	0.5
55	C102D09	4	0.40
56	F20	3	0.61
57	F40	3	0.61
58	F61	3	0.57
59	mCrc1RE01H05	1	0
60	MCrc1R07D06	1	0
61	CCSM75	1	0
62	AG14	1	0
63	CAG01	3	0.62
64	CAT01	1	0
65	CCSM40	2	0.5
66	CCSM147	3	0.61
67	MCrc101F04a	1	0
68	CCSM111	4	0.72
69	CT21	1	0
70	CCT01	3	0.62
71	ATC09	1	0
72	CCSM95	2	0.5
73	CT02	2	0.5
Average		4.19	0.66

Peer-review history:
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