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Original article

Genetic identification and inference on genetic relationships of important *Citrus* rootstocks with microsatellite markers

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Summary

Introduction – **Rootstocks are the vital component of citrus production but a well-defined system for their characterization is lacking. Morphological markers to characterize and distinguish the citrus germplasm are scanty. Further, such markers prove inadequate to differentiate the rootstocks at seedling stage and become redundant soon after budding/grafting of a scion variety. This study aims to demonstrate how the use of molecular markers like microsatellites (SSR) can provide an accurate system for rootstock fingerprinting and diverse germplasm identification for citrus improvement program.** *Materials and methods* – **Forty-one citrus rootstock accessions were characterized with 49 SSR markers. The unweighted neighbor joining (NJ) tree and factorial analysis were used to decipher the genetic relatedness among the 41 citrus accessions.** *Results and discussion* – **The 49 SSR markers amplified a total of 260 alleles with range from 2 to 11 alleles per marker. A subset of 8 SSR markers was selected for their unique PCR amplification pattern, which were able to distinguish 35 of the 41 accessions. Among these, the SSR marker CS41 could differentiate trifoliate orange and its hybrids from other citrus accessions while SSR marker DY287851 produced a similar amplification profile for rough lemons, 'Volkamer' lemon, 'Nasnaran' and 'Ada jamir'. The combined use of NJ tree and factorial relationship helped in deciphering the genetic relatedness of the accessions. The NJ tree classified individuals in three different clusters. The acidic mandarins namely 'Cleopatra' mandarin, 'Pectinifera' and 'Shekwasha' accessions formed a separate group but showed closeness with phylogenetically related individuals in factorial analysis. Contrarily, sour orange and its relatives grouped with trifoliate hybrids in NJ tree but were well resolved in factorial analysis.** *Conclusion* – **The SSR based DNA fingerprinting has potential in proper identification of citrus rootstocks and the information generated about their genetic proximities will prove useful in breeding programs.**

Keywords
citrus, Simple Sequence Repeat (SSR), DNA fingerprinting, phylogenetic relationship, germplasm management

Significance of this study

What is already known on this subject?

- Rootstock is an essential component of citrus production. SSR markers are capable of detecting genetic variation and deciphering phylogenetic relationships.

What are the new findings?

- A subset of 8 unique SSR markers has been identified, which can distinguish the important available citrus rootstocks. The SSR marker CS41 differentiated trifoliate orange and its hybrids from other citrus accessions, while marker DY287851 differentiated rough lemon accessions from 'Rangpur' lime. The genetic relationships of different rootstocks, including indigenous accessions, have been estimated.

What is the expected impact on horticulture?

- The SSR identification scheme holds utility in rootstock identification and certification programs. The phylogenetic information will prove useful in citrus rootstock breeding.

Résumé

Identification génétique par marqueurs microsatellites et incidence sur les relations génétiques entre principaux porte-greffes d'agrumes.

Introduction – **Les porte-greffes sont une composante essentielle en agrumiculture, pourtant un système de caractérisation bien défini fait défaut. Les marqueurs morphologiques permettant de caractériser et de distinguer le matériel génétique en agrumes sont rares. En outre, de tels marqueurs se révèlent insuffisants pour différencier les porte-greffes au stade des semis et deviennent redondants peu de temps après le bourgeonnement d'une variété greffée. Cette étude vise à montrer comment l'utilisation de marqueurs moléculaires comme les microsatellites (SSR) peut fournir un système précis d'empreinte digitale des porte-greffes et d'identification de matériel génétique diversifié en vue d'un programme d'amélioration variétale des agrumes.** *Matériel et méthodes* – **Quarante et une accessions d'agrumes ont été caractérisées par 49 marqueurs SSR. L'analyse de l'arbre phylogénétique par neighbor joining (NJ) et l'analyse factorielle ont été utilisées pour déchiffrer la parenté génétique au sein des 41 accessions d'agrumes.**

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Résultats et discussion **– Les 49 marqueurs SSR ont amplifié un total de 260 allèles avec une plage de 2 à 11 allèles par marqueur. Un sous-ensemble de 8 marqueurs SSR a été sélectionné pour leur modèle unique d'amplification par PCR, qui ont pu distinguer 35 des 41 accessions. Parmi ceux-ci, le marqueur SSR CS41 a permis de différencier l'orange trifoliée et ses hybrides d'autres accessions d'agrumes, tandis que le marqueur SSR DY287851 a produit un profil d'amplification similaire pour les citrons bruts, le citron 'Volkamer', 'Nasnaran' et 'Ada jamir'. L'utilisation combinée de l'arbre phylogénétique par NJ et de l'analyse factorielle a permis de déchiffrer les relations génétiques entre accessions. L'arbre NJ a classé les individus en trois clusters différents. Les mandarines acides, à savoir la mandarine 'Cleopatra' et les accessions 'Pectinifera' et 'Shekwasha' ont formé un groupe distinct, tout en montrant une proximité avec des individus liés phylogénétiquement dans l'analyse factorielle. En revanche, l'orange amère et ses proches ont été placés dans le même groupe que les hybrides trifoliés dans l'arbre NJ, alors qu'ils ont été bien séparés dans l'analyse factorielle.** *Conclusion* **– Les empreintes ADN à base de microsatellites offrent un potentiel pour l'identification correcte des porte-greffes d'agrumes et l'information générée au sujet de leurs proximités génétiques s'avérera utile dans des programmes de sélection.**

Mots-clés
agrumes, marqueurs moléculaires, empreinte génétique, relations phylogénétiques, ressources génétiques

Introduction

Citrus is a globally important fruit crop that ranks third in production after mango and banana in India. It is grown over an area of 0.95 Mha with a total production of 11.65 Mt (Anonymous, 2015). Rootstocks constitute an important component of citrus production. They influence the growth, yield and fruit characteristics of a variety (Castle, 1995). They also enable the cultivation of a scion variety in the different agro-climates by virtue of their resistance to various biotic (insect pests and diseases) and abiotic stresses (soil salinity and poor drainage). For instance, sour orange (*Citrus aurantium*) is known for *Phytophthora* resistance (Mourao Filho *et al.*, 2008), trifoliate orange (*Poncirus trifoliata*) exhibits resistance to *Phytophthora*, citrus nematode, citrus tristeza virus (CTV) and also imparts tolerance to low temperature (Benson *et al.*, 1997). Similarly, 'Cleopatra' mandarin (*C. reshni*) and 'Rangpur' lime (*C. limonia*) have tolerance to soil salinity (Storey and Walker, 1999). To suit the local agro-climates and depending on the stock scion compatibility, a range of rootstocks are being used over the world. In India, rough lemon (*C. jambhiri*) and 'Rangpur' lime are the most widely used rootstocks for inducing high yields in the scion varieties and for their tolerance to CTV (Kumar *et al.*, 2010; Sonkar *et al.*, 2002). 'Rangpur' lime is also a common rootstock in Brazil (Tazima *et al.*, 2013). The trifoliate orange is in use as rootstock in China and Japan whereas its hybrids like citranges and citrumelos find use in different parts of USA. Trifoliate hybrids are also gradually replacing sour orange in the Mediterranean countries (Castle, 2010).

The knowledge of genetic variability and inter-genetic relationships among different citrus rootstocks is essential for establishing correct genotype identity, reducing the redundancies, conservation of germplasm and for selection of parents in breeding programs (Kyndt *et al.*, 2010). The phenotypic variability present in various leaf and fruit related characters is useful to some extent in distinguishing individuals, but such characters are limited and their expression is also under the influence of environment (Fang *et al.*, 1998). Moreover, the morphological markers become redundant in rootstock identification, soon after budding/grafting of a scion variety over it. Thus, the growers do not have any proof about the authenticity of the rootstock used in the procured plants. As citrus cultivation is a long-term venture, the initial supply of scion material on an inappropriate rootstock, can adversely affect the returns of the growers in long run. Thus, there is a need to develop DNA based fingerprints for proper identification of important citrus rootstocks.

Natural hybridizations, introgressions and spontaneous mutations for the past many years are known to have contributed in the evolution of modern citrus and forms the basis of genetic variability (Wu *et al.*, 2014; Curk *et al.*, 2014, 2015). Molecular markers are able to detect the genetic variability, assess the phylogenetic relationships (Nicolosi *et al.*, 2000; Barkley *et al.*, 2006) and eventually can improve our understanding of *Citrus* taxonomy. In citrus, different types of molecular markers namely Randomly Amplified Polymorphic DNA (RAPD), Sequence Related Amplified Polymorphism (SRAP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) have been used (Hazarika *et al.*, 2014). However, owing to the desirable attributes like hyper-variability, co-dominant nature, random distribution throughout the genome and PCR amenability, SSRs have predominantly been used for characterization of the citrus germplasm (Barkley *et al.*, 2006; Froelicher *et al.*, 2008; Cristofani-Yaly *et al.*, 2011; Snoussi *et al.*, 2012; Polat *et al.*, 2012; Garcia-Lor *et al.*, 2015), for estimation of the diversity (Barkley *et al.*, 2006) and for establishing the phylogenetic relationships of different accessions (Barkley *et al.*, 2006; Gulsen and Roose, 2001). There are only a few studies, however, which have entirely focused on characterization of rootstock accessions with SSRs (Snoussi *et al.*, 2012; Polat *et al.*, 2012).

With the sequencing of *Citrus* genome (Wu *et al.*, 2014), the designing of the microsatellite markers has become relatively easy as compared to their earlier development from genomic libraries (Kijas *et al.*, 1997). The present study aimed to characterize and infer the genetic relationships of 41 rootstock accessions with previously published and new SSR markers designed from the *C. sinensis* (L.) Osbeck genome.

Materials and methods

Plant material and genomic DNA extraction

The 41 citrus accessions growing in the field gene bank of Punjab Agricultural University Regional Research Station, Abohar (30°9'0"N, 74°11'0"E) were selected for this study (Table 1). The accessions belonged to three taxa namely *Severinia buxifolia* (a primitive citrus relative), *Citrus*, *Poncirus* and their intergeneric hybrids. The genomic DNA of the accessions was extracted from the young leaves as per Doyle and Doyle (1987) method of DNA extraction with some modifications. Briefly, the CTAB buffer was supplemented with polyvinyl pyrrolidone (1.5%), the CTAB incubated samples

were treated with chloroform: isoamyl alcohol (24:1) twice and the nucleic acids containing supernatant was treated with RNase A (10 mg mL^{-1}) at 10 µL mL^{-1} to remove the RNA. The DNA obtained through this method was dissolved in 1X TE solution. The DNA was quantified in Bio-spectrometer (Eppendorf) and the final working concentration was set to 20 ng $µL^{-1}$ for use in PCR.

Table 1. List of 41 citrus accessions used for microsatellite characterization.

Sr. No.	Accession common names	Pedigree/scientific names[a]	Collection sources
Poncirus trifoliata and its hybrids			
1	Rubidoux trifoliate	*Poncirus trifoliata*	UOC[b], Riverside, USA
2	Rich 16-6	*P. trifoliata*	ICAR - CCRI[c], Nagpur, India
3	Carrizo citrange	*C. sinensis* × *P. trifoliata*	UOC, Riverside, USA
4	Troyer citrange	*C. sinensis* × *P. trifoliata*	UOC, Riverside, USA
5	Savage citrange	*C. sinensis* × *P. trifoliata*	UOC, Riverside, USA
6	Yuma citrange	*C. sinensis* × *P. trifoliata*	UOC, Riverside, USA
7	Benton citrange	*C. sinensis* × *P. trifoliata*	PAU[d], Ludhiana, India
8	C-35 citrange	*C. sinensis* × *P. trifoliata*	PAU, Ludhiana, India
9	C-32 citrange	*C. sinensis* × *P. trifoliata*	PAU, Ludhiana, India
10	Kuharske citrange	*C. sinensis* × *P. trifoliata*	PAU, Ludhiana, India
11	X-639	*C. reshni* × *P. trifoliata*	ICAR - CCRI, Nagpur
12	Sacaton citrumelo	*C. paradisi* (grapefruit) × *P. trifoliata*	UOC, Riverside, USA
13	Swingle citrumelo	*C. paradisi* (grapefruit) × *P. trifoliata*	UOC, Riverside, USA
14	Citremon	*C. limon* × *P. trifoliata*	UOC, Riverside, USA
Etrog Citron, Rough lemon and associated accessions			
15	Etrog citron	*Citrus medica*	UOC, Riverside, USA
16	Grambhir	*C. jambhiri*	UOC, Riverside, USA
17	Florida rough lemon	*C. jambhiri*	UOC, Riverside, USA
18	Italian rough lemon	*C. jambhiri*	UOC, Riverside, USA
19	Jatti khatti	*C. jambhiri*	Abohar, India
20	Jalandhari khatti	*C. jambhiri*	Jalandhar, India
21	Estes rough lemon	*C. jambhiri*	UOC, Riverside, USA
22	Sohmyndong	*C. jambhiri*	UOC, Riverside, USA
23	Rangpur lime	*C. limonia*	UOC, Riverside, USA
24	Volkamer lemon	*C. volkameriana*	UOC, Riverside, USA
25	Ada jamir	*C. assamensis*	North East India
26	Nasnaran	*C. amblycarpa*	UOC, Riverside, USA
Sour oranges and associated accessions			
27	Standard sour orange	*Citrus aurantium*	UOC, Riverside, USA
28	Karun jamir	*C. aurantium*	UOC, Riverside, USA
29	Chinotto	*C. myrtifolia*	UOC, Riverside, USA
30	Kinkoji	*C. obovoidea*	PAU, Ludhiana, India
31	Gou tou	Unknown *C. aurantium* hybrid	PAU, Ludhiana, India
32	Karna khatta	*C. karna*	UOC, Riverside, USA
33	Taiwanica	*C. taiwanica*	UOC, Riverside, USA
34	Aspal orange	*C. aurantium*	UOC, Riverside, USA
Acidic mandarins			
35	Cleopatra mandarin	*Citrus reshni*	UOC, Riverside, USA
36	Pectinifera	*C. depressa*	UOC, Riverside, USA
37	Shekwasha-1	*C. depressa*	UOC, Riverside, USA
38	Shekwasha-2	*C. depressa*	UOC, Riverside, USA
Others			
39	Box orange	*Severinia buxifolia*	UOC[b], Riverside, USA
40	Macroptera	*Citrus macroptera*	North East India
41	Gajanima	*C. pennivesiculata*	North East India

[a] Scientific names following Tanaka's (1977) system.
[b] UOC – University of California.
[c] ICAR-CCRI – Indian council of Agricultural Research - Central Citrus Research Institute.
[d] PAU – Punjab Agricultural University.

SSR markers and genotyping

Initially, 61 SSR markers were used for molecular characterization, but only 49 showed clear cut and unambiguous banding pattern and were chosen for further analysis. Of the 49 SSR markers, 39 were from previously published studies (Kijas *et al.*, 1997; Barkley *et al.*, 2006; Chen *et al.*, 2006; Novelli *et al.*, 2006; Palmieri *et al.*, 2007; Luro *et al.*, 2008; Froelicher *et al.*, 2008; Cristofani-Yaly *et al.*, 2011; Cuenca *et al.*, 2011; Shahzadi *et al.*, 2014) whereas 10 were new SSR markers, designed from genomic sequences of *Citrus sinensis* (available at https://www.citrusgenomedb.org). Microsatellites were identified with the MISA tool (www.pgrc.ipk-gatersleben.de/misa/) and primers were designed with Primer3 (www.bioinfo.ut.ee/primer3-0.4. 0/primer3). Of the 39 already published SSR markers, 31 were genomic-SSRs while 8 were derived from the expressed sequence tags (ESTs). The details of all these markers with respect to their sequences, annealing temperature and repeat motifs are provided in the supplementary data (Table T1 at https://doi.org/10.17660/th2017/72.6.3). All the markers were amplified through PCR in a 10 µL reaction volume containing 50 ng genomic DNA, 1X Go Taq Flexi PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of primers (forward and reverse each) and 0.75 units of Taq polymerase. The final reaction volume was made by adding sterile double distilled water. The PCR was performed in a Eppendorf thermal cycler programmed as: 5 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 46–60 °C (supplementary data, Table T1 at https://doi.org/10.17660/th2017/72.6.3) for 45 s, elongation at 72 °C for 90 s and a final extension step of 72 °C for 7 min. The amplified products were resolved in 2.5% agarose gel containing ethidium bromide and visualized under gel documentation system (*UV-P*, UK).

Genetic analysis of SSR data

The bands were recorded as present (1) or absent (0) and data was compiled into a two-way matrix. The alleles in each primer were enumerated according to their size. The easily scorable amplified DNA fragment (band) at the top of the gel was numbered as first and the lowest band as the last one. The allele size was estimated through Alpha Viewer SA software by comparing the bands with the 50 or 100 bp DNA size marker, loaded at both the sided of the samples in the gel. The basic population statistics such as effective number of alleles (*Ne*), observed heterozygosity (*Ho*), expected heterozygosity (*He*) were estimated using Popgene software (Yeh and Boyle, 1997). The polymorphism information content (PIC) of the markers was estimated using the formula given by Nei (1978):

$$PIC = 1 - \Sigma Pij^2$$

where *Pij* is the frequency of j^{th} allele in i^{th} primer and summation extends from 1 to '*n*' patterns. The genetic relatedness among the different accessions was calculated with dice coefficient of association and the tree was constructed through unweighted neighbor joining (NJ) tree method of software package DARwin 6.0 (Perrier and Jacquemoud-Collet, 2006). The stability of the tree was ascertained through 1,000 bootstraps. In addition, factorial analysis was also performed using this software to ascertain the genetic relationships.

Results and discussion

SSR polymorphism and genetic diversity

The 49 SSR markers amplified a total of 260 alleles across the 41 accessions. The number of alleles ranged from 2 to 11 with 5.30 as mean number of alleles per marker (Table 2). Most of the SSR markers exhibited high polymorphism information content (PIC). The PIC value of the SSR markers varied from 0.166 to 0.878 with an average of 0.653. The 39 SSR markers had PIC values more than 0.500 whereas 27 exhibited PIC values even more than 0.700. Among the 10 newly designed SSR markers, 3 markers showed PIC values more than 0.500 (Table 2). The high PIC value reflects high allelic variation and their distribution across different accessions. The number of effective alleles (*Ne*) tells about the number of alleles that would be expected in a locus in each population. The *Ne* in present study ranged from 1.1 to 7.9 with the majority of accessions showing more than 2 effective alleles. The observed heterozygosity (*Ho*) ranged from 0.0 to 0.927, whereas the average expected heterozygosity (*He*) ranged from 0.095 to 0.885. Except 10 SSR markers (CAC33, CAC39, CAC15, ATC09, CCSM70, DY281040, CCSME41, CS02, CS09 and CS80), the *Ho* was lower than the expected *He* (Table 2). Citrus in general is considered to be highly heterozygous as many of the species have been developed through hybridization and further diversified through spontaneous mutations. The different natural hybrids like rough lemons, 'Rangpur' lime and sour orange and man-made hybrids like citrumelo have high heterozygosity (Luro *et al.*, 2008; Cristofani-Yaly *et al.*, 2011) while, the pure *generas* apparently exhibit low heterozygosity (Cristofani-Yaly *et al.*, 2011). The presence of pure accessions like 'Box orange' (*Severinia buxifolia*), trifoliate orange (*Poncirus trifoliata*), *Citrus medica* and acidic mandarins, especially *C. reshni*, coupled with a small number of accessions could have reduced the level of *Ho*.

Unique alleles and rootstock identification

Twenty-one of the 49 SSR markers amplified 30 unique alleles specific to different rootstocks (Table 3). Among these 21 SSR markers, the marker CCSME41 produced a maximum of 3 unique alleles while cAGG9, mCrCIR01F04a, DY292105, CT21, DY287851, mCrCIR01D06a and CCSM75 amplified 2 unique alleles each (Table 3). Across the accessions, the frequency distribution of unique alleles was according to the degree of primitivity. The maximum number of eight unique alleles were recorded for 'Box orange', which according to Swingle and Reece (1967) system of classification, is a primitive citrus fruit and is distinct from the genera *Citrus* and *Poncirus*. The next highest number of unique alleles were observed in Macroptera (5), which is a member of group *Papeda* and is also a wild accession. Among the other accessions, 'Gajanima' (3), 'Taiwanica', 'Karna khatta', 'Nasnaran', 'X-639' (2 each) and 'Savage' citrange, 'Benton' citrange, 'Swingle' citrumelo, 'Gou tou', 'Rangpur' lime and 'Estes' rough lemon (1 each) also exhibited unique alleles (Table 3). The presence of unique alleles even in secondary *Citrus* species like *C. karna* cv. Karna khatta, *C. taiwanica* cv. Taiwanica and hybrids like 'X-639' indicated either the absence of their direct ancestors in the present study or it is also equally likely that the unique allele could arise *de novo*. The variation in the SSRs is due to the differences in the number of short tandem repeat (STR) units, which could be due to the unequal crossing over during meiosis, retro-transposition mechanism or strand-slippage during DNA replication (Fan and Chu, 2007). The genotype-specific alleles have been reported as rare alleles in previous studies (Barkley *et al.*, 2006). It is probable that during the process of domestication, these unique alleles could have remained unaltered in the wild species, landraces and primitive cultivars and may show association

Table 2. Summary statistics of the used SSR markers. Sr. No. 1–31 = Genomic-SSRs, 32–39 = EST-SSRs, both are previously published while 40–49 are new Genomic-SSR markers designed in this study (*na* = observed number of alleles; *Ne* = effective number of alleles; *Ho* = Observed heterozygosity; *He* = Expected heterozygosity; *PIC* = Polymorphic information content).

Sr. No.	SSR markers	*na*	Allele size range (bp)	*Ne*	*Ho*	*He*	*PIC* value
1	TAA15	10	174–248	7.9	0.732	0.885	0.878
2	TAA27	5	208–241	3.4	0.561	0.718	0.730
3	TAA33	4	98–125	2.0	0.244	0.501	0.566
4	CAC33	8	134–206	2.9	0.707	0.668	0.729
5	CAC23	3	240–255	2.2	0.171	0.551	0.598
6	CAC39	3	156–176	1.4	0.317	0.299	0.550
7	cAGG9	5	103–120	2.8	0.317	0.650	0.658
8	CAC15	3	157–176	1.6	0.415	0.389	0.456
9	ATC09	7	174–235	5.0	0.927	0.811	0.793
10	CAG01	4	123–150	3.0	0.342	0.675	0.714
11	AG14	6	131–182	3.5	0.098	0.723	0.735
12	CT02	3	130–153	2.0	0.049	0.515	0.530
13	CT19	5	141–181	4.1	0.342	0.768	0.780
14	CT21	6	136–171	2.2	0.098	0.550	0.574
15	GT03	6	178–196	4.0	0.439	0.759	0.765
16	CCSM147	7	104–133	4.5	0.512	0.789	0.792
17	CCSM06	4	226–266	2.9	0.585	0.669	0.691
18	CCSM40	9	124–221	5.5	0.634	0.829	0.833
19	CCSM146	7	71–109	6.3	0.756	0.851	0.841
20	CCSM156	7	82–128	5.6	0.634	0.832	0.824
21	CCSM46	6	106–140	3.6	0.000	0.731	0.722
22	CCSM68	6	86–116	3.5	0.561	0.726	0.738
23	CCSM70	6	103–139	4.1	0.854	0.768	0.759
24	CCSM75	9	100–160	5.6	0.366	0.832	0.834
25	CCSM77	5	94–129	3.6	0.180	0.730	0.719
26	CCSM95	6	114–142	6.0	0.275	0.843	0.824
27	mCrCIR01D06a	5	222–262	2.5	0.122	0.601	0.614
28	Ci01H05	3	131–147	2.8	0.220	0.651	0.647
29	mCrCIR01F08a	6	117–145	3.8	0.561	0.749	0.743
30	mCrCIR01F04a	11	166–222	7.4	0.537	0.876	0.869
31	mCrCIR07D06	4	166–296	3.9	0.561	0.755	0.749
32	DY292105	5	114–133	1.6	0.390	0.393	0.488
33	DY279967	3	101–111	1.9	0.073	0.469	0.453
34	DY281040	6	179–204	5.1	0.829	0.815	0.807
35	DY287851	7	160–434	4.5	0.000	0.787	0.777
36	CCSME15	7	102–133	4.2	0.293	0.774	0.788
37	CCSME41	7	244–277	2.3	0.610	0.570	0.626
38	F17	8	100–141	5.7	0.707	0.834	0.834
39	BQ624796	6	230–267	3.9	0.585	0.752	0.743
40	CS02	2	206–241	1.5	0.342	0.318	0.397
41	CS03	8	230–952	3.1	0.275	0.688	0.743
42	CS09	3	228–250	1.1	0.098	0.095	0.166
43	CS12	3	229–241	2.5	0.220	0.613	0.639
44	CS17	2	259–279	1.4	0.024	0.303	0.308
45	CS18	2	195–215	1.2	0.000	0.159	0.198
46	CS41	4	200–326	1.8	0.293	0.461	0.491
47	CS42	3	197–202	1.7	0.220	0.426	0.462
48	CS49	3	243–263	2.2	0.000	0.549	0.543
49	CS80	2	220–250	1.2	0.195	0.178	0.273
	Mean	*5.3*	–	–	*0.373*	*0.630*	*0.653*

with a number of economically important traits. The unique/rare alleles also hold relevance to use as fingerprints (distinct markers) due to their genotype specificity and rare occurrence (Sarao *et al.*, 2010).

Other differentiating alleles

Apart from these unique alleles, we shortlisted a subset of 8 SSR markers (CS41, DY287851, TAA15, CCSM70, CCSM95, CCSM156, mCrCIR01F04a and CS02), which can genetically distinguish 35 of the 41 genotypes from one another (Table 4). Of these 8 SSR markers, the newly designed genomic SSR marker, CS41 was sufficient to differentiate the trifoliates and its hybrids from rest of the accessions. The marker amplified a ~200 base pair (bp) allele specific to tri-

Table 3. List of primers with unique alleles and their genotype specificity.

Primers	Number of unique alleles	Unique alleles specific for genotype(s)
CCSME41	3	'Box' orange, 'Gajanima' and 'Karna khatta'
cAGG9	2	'Rangpur' lime and 'Nasnaran'
mCrCIR01F04a	2	'Box' orange and 'Nasnaran'
DY292105	2	'Gajanima' and 'Box' orange
CT21	2	'Macroptera' and 'Benton' citrange
DY287851	2	'Savage' citrange, 'Taiwanica'
mCrCIR01D06a	2	'Macroptera', 'Taiwanica'
CCSM75	2	'Gou tou', 'Box' orange
CCSM147	1	'Box' orange
CS41	1	'Box' orange
DY279967	1	'Box' orange
BQ624796	1	'Box' orange
CS03	1	'X-639'
TAA15	1	'X-639'
CS42	1	'Estes' rough lemon
CAC33	1	'Karna khatta'
ATC09	1	'Macroptera'
CCSM77	1	'Macroptera'
CCSM46	1	'Macroptera'
TAA27	1	'Swingle' citrumelo
F17	1	'Gajanima'

foliate orange rootstocks ('Rubidoux' trifoliate and 'Rich 16-6'), whereas it amplified a 276 bp allele in addition to the 200 bp allele in *Citrus* × *Poncirus* hybrids like various citranges, citrumelos and 'X-639' (Figure 1, panel A). The marker, therefore, can be used for identification of hybrids in the crosses of *Citrus* × *Poncirus* and *Poncirus* × *Citrus*. The use of additional markers, TAA15, CCSM70, CCSM95 and mCrCIR-01F04a can help in differentiation of trifoliate orange and its hybrids (except 'Carrizo-Troyer') from one another (Table 4), which is otherwise difficult morphologically.

Similarly, the EST derived SSR marker DY287851 amplified a ~434 bp allele for different accessions of rough lemon like Grambhir, Florida rough lemon, Italian rough lemon, Sohmyndong, Estes rough lemon, Jatti khatti and Jalandhari khatti, 'Volkamer' lemon, 'Nasnaran' and 'Ada jamir' and therefore, could resolve them from 'Rangpur' lime and other citrus accessions (Figure 1, panel B). The rough lemon, 'Rangpur' lime and 'Volkamer' lemon are known to be very close to each other. The rough lemon and 'Rangpur' lime have been proposed to be derived from hybridization between mandarin and citron (Scora, 1975; Nicolosi *et al.*, 2000; Barkley *et al.*, 2006; Curk *et al.*, 2016) whereas, different opinions have been expressed about the origin of 'Volkamer' lemon (Deng *et al.*, 1996; Nicolosi *et al.*, 2000; Curk *et al.*, 2016). Froelicher *et al.* (2011) through their mitochondrial marker based study demonstrated that acidic mandarins were the maternal ancestors of these rootstocks. The three acidic mandarins and 'Etrog' citron were the part of the characterized samples. However, the 434 bp allele was present neither in the acidic mandarins nor in the 'Etrog' citron. However, the amplification profile of 'Rangpur' lime for this marker matched to 'Etrog' citron, which indicated that the 'Etrog' citron in the development of rough lemon and 'Etrog' citron could be different. Earlier, Carvalho *et al.* (2005) also reported that rough lemon and 'Volkamer' lemon had slightly different karyotypes than that of 'Rangpur' lime. They observed that rough lemon and 'Volkamer' lemon had two 45S rDNA sites in the euchromatin region, one each in D and F type chromosomes, which was absent in 'Rangpur' lime. However, they ruled out the possibility of their origin from different crosses, whereas Curk *et al.* (2016) reported that rough lemon and 'Rangpur' lime come from a cross between an acid mandarin (female parent) and citron (male parent). Nonetheless, due to more conserved nature of EST-SSR markers, the DY287851 has direct utility in differentiating the rough lemons from the 'Rangpur' lime and other rootstocks.

The genomic-SSR markers, CCSM70, CCSM95, CCSM156 and CS02 were able to resolve the 'Etrog' citron and different rough lemon accessions including 'Jatti khatti' and 'Jalandhari khatti'. Similarly, SSR marker, mCrCIR01F04a differentiated 'Rangpur' lime, 'Cleopatra' and 'Pectinifera' from each other, whereas, the SSR markers, mCrCIR01F04a and CS02 differentiated 'Cleopatra', 'Pectinifera' and 'Shekwasha' (except between the two 'Shekwasha' accessions). The SSR markers, CCSM70 and CCSM95 were able to differentiate 'Standard' sour orange and related accessions (except 'Standard' sour orange from 'Karun jamir'). 'Box orange', 'Macroptera' and 'Gajanima' could be distinguished with the help of SSR markers CCSM70 and CCSM156 (Table 4).

The global citrus industry is based on a limited number of rootstocks. The rough lemons, 'Rangpur' lime, trifoliate orange and its hybrids like citranges and citrumelo occupy the majority of world citrus area. The SSR based fingerprinting has potential in distinguishing these rootstocks at seedlings stage and also in tracing their identity in the grown up orchards.

Estimating genetic diversity and genetic relatedness

The NJ tree analysis grouped the citrus rootstock accessions into three distinct clusters (Figure 2). The four acidic mandarins, namely 'Cleopatra' mandarin, 'Pectinifera', 'Shekwasha-1' and 'Shekwasha-2', grouped together in the cluster I. The cluster II was the largest cluster with 23 accessions that was further divided into two sub clusters, showing variability within the cluster. The sub-cluster II-1 contained the two trifoliate oranges (Rubidoux trifoliate and Rich 16-6), their hybrids and 'Etrog' citron (*C. medica*). In this sub cluster, citremon was most distantly placed compared to other hybrids (Figure 2). The different sour orange related accessions: 'Standard' sour orange, 'Karun jamir', 'Chinotto', 'Gou tou', 'Kinkoji', 'Aspal' orange, including 'Taiwanica' and 'Karna khatta' were present in the second sub-cluster 'II-2', (Figure 2). The third cluster (cluster-III) was represented by 14 genotypes that included different rough lemon accessions, 'Rangpur' lime, 'Volkamer' lemon, 'Nasnaran', 'Adajamir', 'Box orange', 'Macroptera' and 'Gajanima' along with 'Box orange' (Figure 2). The 'Box orange' (*Severinia buxifolia*) is a distant citrus relative. Its association with citrus was doubtful. We performed hierarchical cluster analysis, which confirmed its distant relationships with the different accessions (supplementary data, Figure S1 at https://doi.org/10.17660/th2017/72.6.3).

The association of acidic mandarins in NJ tree is in agreement with the taxonomic grouping (Tanaka, 1954), nuclear SSR, indel and SNP markers based grouping (Garcia-Lor *et al.*, 2015) of citrus accessions. These mandarins are known to share the same chlorotype (Yamamoto *et al.*, 2013) and mitotype (Froelicher *et al.*, 2011).

Table 4. Differentiation scheme of citrus rootstock accessions with the use of 8 SSR markers. Shaded cells indicate the gap between two amplified alleles.

Groups and accessions	Primers and alleles																																							
	CS41			DY287851			TAA15				CCSM70						CCSM95						CCSM156							mCrCIR01F04a								CS02		
Allele No.	2	3	4	1	5	4	5	8	9	10	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	7	1	3	4	5	6	7	8	9	1	2
Size (bp)	276	266	200	434	162	216	203	182	176	174	139	126	121	117	111	103	142	135	131	123	114	104	128	114	107	98	94	85	82	222	200	198	196	190	182	180	179	241	206
Trifoliate orange and its hybrids	The markers CS41- to differentiate trifoliate oranges from its hybrids and all of them from other citrus accessions; TAA15- to discriminate 'Savage' citrange, 'C35' citrange, 'X-639', 'Swingle' citrumelo and citremon from other trifoliate hybrids; CCSM70- classifies remaining trifoliate hybrids into two groups: 1- ('Carrizo' and 'Troyer' citranges, 'C-32' citrange and 'Kuharske'), 2- ('Yuma' citrange, 'Benton' citrange and 'Sacaton' citrumelo); CCSM95-separates 'Yuma' citrange, 'Benton' citrange and 'Sacaton' citrumelo from one another, also distinguishes 'Carrizo-Troyer' pair from 'C-32' citrange; mCrCIR01F04a- differentiates 'Carrizo-Troyer' pair from 'Kuharske', also able to separate 'Rubidoux' trifoliate from 'Rich16-6'; 'Carrizo' and 'Troyer' citranges were not distinguishable in this study.																																							
Rubidoux trifoliate			4					8		10						6	1																5			8				
Rich 16-6			4					8		10						6	1																	6		8				
Carrizo citrange	2		4			4				10			3			6			3														5				9			
Troyer citrange	2		4			4				10			3			6			3														5				9			
Savage citrange	2		4			4		8																											7					
Yuma citrange	2		4			4				10	1					6			3																7					
Benton citrange	2		4			4				10	1					6						6											5							
C-35 citrange	2		4							10																						4			8				
C-32 citrange	2		4			4				10			3			6						6													7					
Kuharske	2		4			4				10			3			6			3													4								
X639	2		4				5																									4			8				
Sacaton citrumelo	2		4			4				10	1					6		2																7					
Swingle citrumelo	2		4					8																							3			6					
Citremon	2		4						9																											8				
Sour orange related accessions	CCSM70 and CCSM95 can distinguish sour orange and related accessions from the rest of the accessions and can also differentiate different individuals of this group except 'Standard' sour orange and 'Karun jamir'.																																							
Taiwanica	2										1		3						3																					
Chinotto	2										1		3					2	3																					
Karna khatta	2										1									4																				
Aspal orange	2										1								3		5																			
Karun jamir	2										1		3					2																						
Standard sour orange	2										1		3					2																						
Kinkoji	2												3	4																										
Gou tou	2										1						1				5																			

Table 4. Continued.

Group and accessions	Primers and alleles*																																							
	CS41			DY287851			TAA15				CCSM70						CCSM95						CCSM156							mCrCIR01F04a										CS02	
Allele No.	2	3	4	1	5	4	5	8	9	10	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	7	1	3	4	5	6	7	8	9	1	2
Size (bp)	276	266	200	434	162	216	203	182	176	174	139	126	121	117	111	103	142	135	131	123	114	104	128	114	107	98	94	85	82	222	200	198	196	190	182	180	179	241	206
Rough lemon and related accessions	CCSM70 can differentiate the individuals of this group from rest of the accessions, while the use of additional markers DY287851, CCSM95, CCSM156, mCrCIR01F04a and CS02 to separate the different individuals of this group.																																							
Grambhir	2			1							1				5		1				5						5														
Florida rough lemon	2			1							1				5		1				5			2			5													
Italian rough lemon	2			1										4	5																									
'Rangpur' lime	2				5						1				5					4				2		4														
Jalandhari khatti	2			1							1				5		1				5		1	2																
'Nasnaran'		3		1							1				5				3		5				3															
Sohmyndong	2			1							1				5		1				5		1			4														
Jatti khatti	2			1							1				5		1				5					4													1	
Estes Rough Lemon	2			1							1				5		1				5					4													1	2
'Volkamer' lemon	2			1							1				5					4						4		7											
'Ada jamir'	2			1							1				5					4			1				5												
Etrog citron	2				5								3		5									2			5												
Acidic mandarins	mCrCIR01F04a and CS02 can distinguish acidic mandarins from rest of the accessions and also differentiate the individuals of this group except the two 'Shekwasha' accessions																																						
'Cleopatra' mandarin	2																													1			5						
'Pectinifera'	2																															4					9	1	2
'Shekwasha' -1	2																															4					9	1	
'Shekwasha' -2	2																															4					9	1	
Other accessions	CS41, CCCSM70 and CCSM156 can distinguish the different individuals in this group																																						
Box orange													3																										
Macroptera		3										2			5													6							7				
Gajanima		3										2			5										3		5								8				

The NJ based grouping of members of cluster II was probably due to the pedigree bonding of the trifoliate hybrids namely citranges, citrumelos, and citremon with sour orange and related accessions. The citranges are the artificial hybrids of trifoliate orange with sweet orange while citrumelos and citremon, respectively are the hybrids of trifoliate orange with grapefruit and lemon, respectively (Hodgson, 1967). Both sour and sweet oranges are known to have derived from hybridizations between pummelo and mandarin (Wu *et al.*, 2014; Curk *et al.*, 2015) while the grapefruit, one of the parents of the citrumelo, are the result of a back cross event between sweet orange and pummelo (Nicolosi *et al.*, 2000). Therefore, the affinity of sour orange accessions with the citranges could be due to the mandarin or pummelo ancestry, while their closeness with citrumelo reflects the pummelo ancestry.

The factorial analysis is considered as a complementary approach in deciphering the genetic relationships. The first two axis explained 42.2 and 18.4% variance, respectively (Figure 3). The affinities between different accessions observed through NJ tree analysis was also maintained in the factorial analysis, but the factorial analysis helped in arranging the accessions according to their phylogenetic relationships. For instance, the acidic mandarins of cluster I of NJ

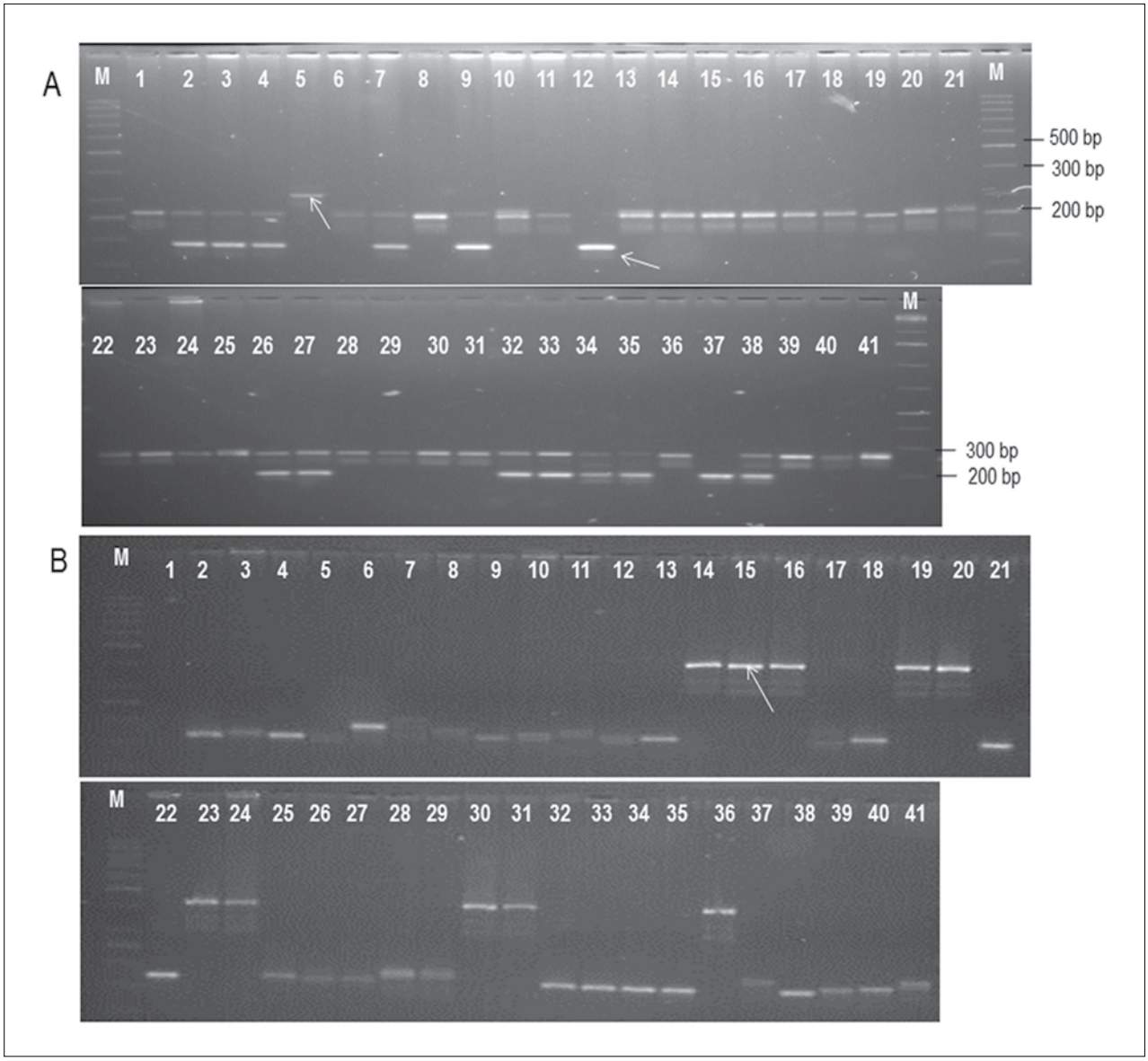

Figure 1. Amplification profile of SSR markers CS41 (panel A) and DY287851 (panel B) for 41 rootstock accessions. **Sample nomenclature 1–21:** (1) 'Cleopatra' mandarin; (2) 'Carrizo' citrange; (3) 'Sacaton' citrumelo; (4) 'Troyer' citrange; (5) 'Box orange'; (6) 'Taiwanica'; (7) 'Savage' citrange; (8) 'Macroptera'; (9) 'Swingle' citrumelo; (10) 'Gajanima'; (11) 'Pectinifera'; (12) 'Rubidoux' trifoliate; (13) 'Chinotto'; (14) 'Grambhir'; (15) 'Florida' rough lemon; (16) 'Italian' rough lemon; (17) 'Karna khatta'; (18) 'Rangpur' lime; (19) 'Nasnaran'; (20) 'Jalandhari khatti'; (21) 'Aspal' orange. **22–41:** (22) 'Karun jamir'; (23) 'Sohmyndong'; (24) 'Jatti khatti'; (25) 'Standard' sour orange; (26) 'Citremon'; (27) 'Yuma' citrange; (28) 'Shekwasha-1'; (29) 'Shekwasha-2'; (30) 'Estes' rough lemon; (31) 'Volkamer' lemon; (32) 'X-639'; (33) 'Benton' citrange; (34) 'C-35' citrange; (35) 'C-32' citrange; (36) 'Adajamir'; (37) 'Rich 16-6'; (38) 'Kuharske'; (39) 'Etrog' citron; (40) 'Kinkoji'; (41) 'Gou tou'. The M codes for size markers. The arrows in upper lane of panel A denotes unique allele for 'Box orange' (5) and the allele specific for the trifoliates (12). In the panel B, the arrow indicates the 434 bp allele present in different rough lemon accessions (14, 15, 16, 20, 23, 24 and 31), 'Nasnaran' (19), 'Volkamer' lemon (30) and 'Adajamir' (36).

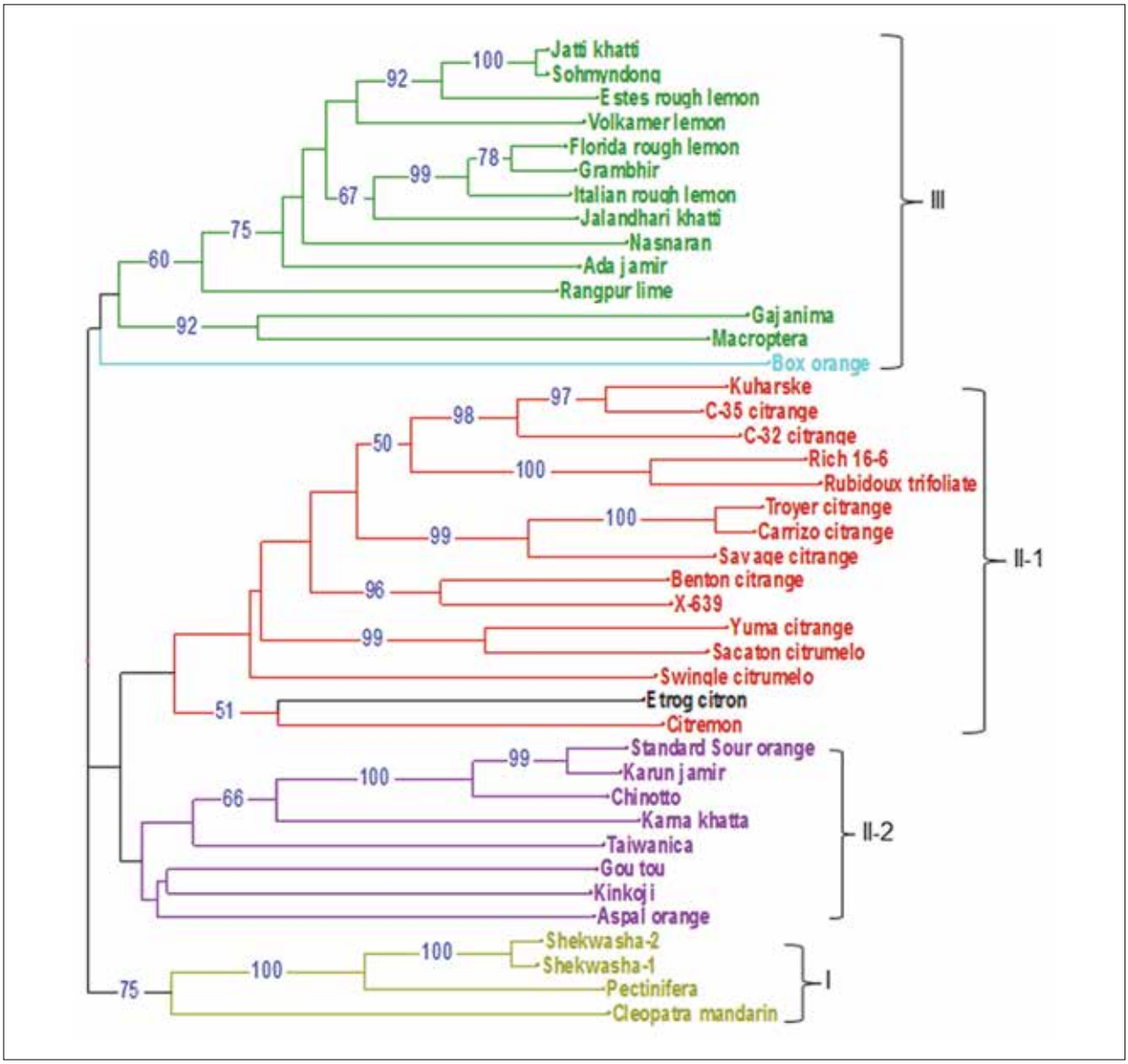

Figure 2. Unweighted neighbour joining (NJ) tree analysis of 41 *Citrus* accessions based on 49 SSR markers data. The bootstrap support values of ≥ 50% are indicated on the nodes.

tree were present together with members of cluster III *i.e.* different accessions of rough lemon, 'Rangpur' lime, 'Volkamer' lemon, 'Nasnaran' and Macroptera on the same axis in the factorial analysis (Figure 3). This corroborates the previously published phylogenetic relationships (Nicolosi *et al.*, 2000; Barkley *et al.*, 2006; Froelicher *et al.*, 2011). Similarly, 'Nasnaran' has been confirmed to be an interspecific hybrid between a member of *Papeda* sub-genus and acidic mandarin (Ollitrault *et al.*, 2012; Curk *et al.*, 2015). We had 'Macroptera' as representative of the *Papeda* group in our study. The 'Nasnaran' displayed intermediate position between 'Pectinifera'/'Shekwasha' accessions and 'Macroptera'. The 'Ada jamir' is a relatively less studied accession so far, it showed closeness with different accessions of rough lemon and 'Nasnaran' in the factorial analysis.

The factorial analysis not only proved effective in differentiating the clusters II-1 and II-2 of NJ tree but was also helpful in resolving the genetic differences between the members of cluster II-2. The affinity between members of cluster II-1 observed in NJ tree was maintained in factorial analysis too. Among the members of cluster II-2, the 'Standard' sour orange, 'Karun jamir' and 'Chinotto' appeared on one side of the axis while 'Karna khatta', 'Aspal' orange, 'Kinkoji', 'Taiwanica', 'Etrog' citron, 'Gou tou' were present on the other side of the axis (Figure 3). 'Chinotto' is morphologically distinct from sour orange in terms of foliage and fruit, but it has been shown to be closely related to sour orange (Polat *et al.*, 2012), that was also verified in our study. Among the other members of this subgroup, 'Karna khatta' was the most distant accession. The fruit characters of this accession shows resemblances with sour orange, citron and pummelo (Uchoi *et al.*, 2016). Based on nuclear and cytoplasmic data, it has been proposed to be a hybrid between acidic mandarin and citron (Curk *et al.*, 2016) while *rbcL* sequence data relates its close association with both mandarin (*C. reticulata*) and pummelo (*C. maxima*) (Uchoi *et al.*, 2016). Its position in the factorial analysis alluded to its proximity with sour orange and citron. A look at the tree genetic distance values revealed

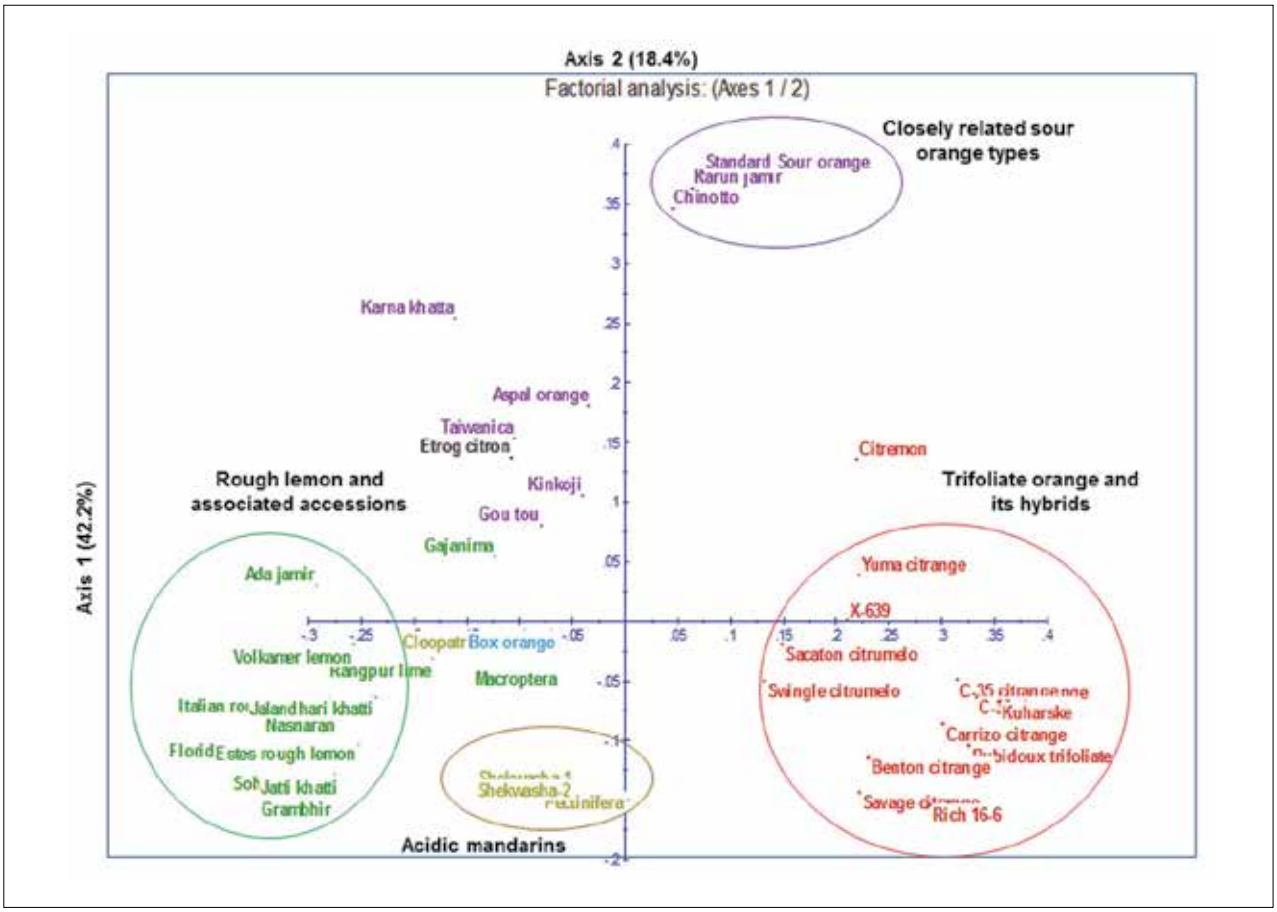

Figure 3. Factorial analysis of SSR data using DARwin 6.0 to depict inter-genetic relationships among 41 *Citrus* rootstock accessions.

that 'Karna khatta' had maximum similarity (60%) with sour orange and about 40% similarity with 'Etrog' citron. The other members like 'Taiwanica' and 'Kinkoji' were previously proposed to be hybrids of pummelo with other *Citrus* types (Penjor *et al.*, 2013), what could not be ascertained since no pummelo accession was included in the present study.

The citremon, a hybrid between lemon and trifoliate orange, was present in the triangular centre of sour orange, 'Etrog' citron and trifoliate oranges, which probably states its developmental history. The lemons, the one of the parents of citremon are known to have been produced from the crossing between sour orange and citron. This was proposed by Nicolosi *et al.* (2000) based on RAPD, SCAR and chloroplast based markers data and has been verified through nuclear SSR (Garcia-Lor *et al.*, 2012) and SNP markers (Ollitrault *et al.*, 2012). Similarly, the hybrid of known pedigree, *i.e.*, 'X-639' occupied intermediate position between their parents in the factorial analysis.

The factorial analysis also displayed wide genetic distances between accessions of rough lemon, 'Rangpur' lime and the *Phytophthora* donors like sour orange and trifoliate orange. Therefore, the *Phytophthora* tolerance of rough lemons and 'Rangpur' lime can be improved by using sour orange and trifoliate orange as breeding parents.

Conclusion

The study shows the importance of SSR markers in fingerprinting and determining the genetic relationships of important citrus rootstocks. The subset of 8 SSR markers holds utility in rootstock identification and certification program. The marker CS41 found to be an important marker to differentiate trifoliate and its hybrids from other *Citrus* accessions while DY287851 could differentiate rough lemons from 'Rangpur' lime. The phylogenetic information will prove useful in selection of diverse parents for rootstock breeding.

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References

Anonymous (2015). Citrus area and production in India. In Final area and production estimates for horticultural crops for 2014–15. Available at http:// nhb.gov.in.

Barkley, N.A., Roose, M.L., Krueger, R.R., and Federici, C.T. (2006). Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theor. Appl. Genet. *112*, 1519–1531. https://doi.org/10.1007/s00122-006-0255-9.

Benson, H.P., Lee, D., and Mcclure, J.P. (1997). A multiple objective linear programming model for the citrus rootstock selection problem in Florida. J. Multi Crit. Dec. Anal. *6*, 283–295. https://doi.org/10.1002/(SICI)1099-1360(199709)6:5<283::AID-MCDA160>3.0.CO;2-R.

Carvalho, R., Soares Filho, W.S., Brasileiro-Vidal, A.C., and Guerra, M. (2005). The relationships among lemons, limes and citron: a chromosomal comparison. Cytogenet. Genome Res. *109*, 276–282. https://doi.org/10.1159/000082410.

Castle, W.S. (1995). Rootstock as a fruit quality factor in citrus and deciduous tree crops. N. Z. J. Crop Hortic. Sci. *23*, 383–394. https://doi.org/10.1080/01140671.1995.9513914.

Castle, W.S. (2010). A career perspective on citrus rootstocks, their development and commercialization. HortScience *45*, 11–15.

Chen, C., Zhou, P., Choi, Y.A., Huang, S., and Gmitter, F.G. (2006). Mining and characterizing microsatellites from citrus ESTs. Theor. Appl. Genet. *112*, 1248–1257. https://doi.org/10.1007/s00122-006-0226-1.

Cristofani-Yaly, M., Novelli, V.M., Bastianel, M., and Machado, M.A. (2011). Transferability and level of heterozygosity of microsatellite markers in *Citrus* species. Plant Mol. Biol. Rep. *29*, 418–423. https://doi.org/10.1007/s11105-010-0241-x.

Cuenca, J., Froelicher, Y., Aleza, P., Juarez, J., Navarro, L., and Ollitrault, P. (2011). Multilocus half-tetrad analysis and centromere mapping in citrus: evidence of SDR mechanism for 2*n* megagametophyte production and partial chiasma interference in mandarin 'Fortune'. Heredity *107*, 462–470. https://doi.org/10.1038/hdy.2011.33.

Curk, F., Ancillo, G., Garcia-Lor, A., Luro, F., Perrier, X., Jacquemoud-Collet, J.P., Navarro, L., and Ollitrault, P. (2014). Next generation haplotyping to decipher nuclear genomic interspecific admixture in *Citrus* species: analysis of chromosome 2. BMC Genet. *15*, 152. https://doi.org/10.1186/s12863-014-0152-1.

Curk, F., Ancillo, G., Ollitrault, F., Perrier, X., Jacquemoud-Collet, J.P., Garcia-Lor, A., Navarro, L., and Ollitrault, P. (2015). Nuclear species-diagnostic SNP markers mined from 454 amplicon sequencing reveal admixture genomic structure of modern citrus varieties. PLoS ONE *10*, e0125628. https://doi.org/10.1371/journal.pone.0125628.

Curk, F., Ollitrault, F., Garcia-Lor, A., Luro, F., Navarro, L., and Ollitrault, P. (2016). Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. Ann. Bot. *117*, 565–583. https://doi.org/10.1093/aob/mcw005.

Deng, Z.N., Gentile, A., Nicolosi, E., Continella, G., and Tribulato, E. (1996). Parentage determination of some citrus hybrids by molecular markers. Proc. Int. Soc. Citricul. *2*, 849–854.

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. *19*, 11–15.

Fan, H., and Chu, J.Y. (2007). A brief review of short tandem repeat mutation. Genom., Proteom. and Bioinform. *5*, 7–14. https://doi.org/10.1016/S1672-0229(07)60009-6.

Fang, D., Krueger, R.R., and Roose, M.L. (1998). Phylogenetic relationships among selected citrus germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. J. Am. Soc. Hortic. Sci. *123*, 612–617.

Froelicher, Y., Mouhaya, W., Bassene, J.-B., Costantino, G., Kamiri, M., Luro, F., Morillon, R., and Ollitrault, P. (2011). New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. Tree Genet. Genom. *7*, 49–61. https://doi.org/10.1007/s11295-010-0314-x.

Garcia-Lor, A., Luro, F., Navarro, L., and Ollitrault, P. (2012). Comparative use of indel and SSR markers in deciphering the interspecific structure of cultivated citrus genetic diversity: a perspective for genetic association studies. Mol. Genet. Genom. *287*, 77–94. https://doi.org/10.1007/s00438-011-0658-4.

Garcia-Lor, A., Luro, F., Ollitrault, P., and Navarro, L. (2015). Genetic diversity and population structure analysis of mandarin germplasm by nuclear, chloroplastic and mitochondrial markers. Tree Genet. Genom. *11*, 123. https://doi.org/10.1007/s11295-015-0951-1.

Gulsen, O., and Roose, M.L. (2001). Lemons: Diversity and relationships with selected citrus genotypes as measured with nuclear genome markers. J. Am. Soc. Hortic. Sci. *126*, 309–317.

Hazarika, T.K., Hazarika, B.N., and Shukla, A.C. (2014). Genetic variability and phylogenetic relationships studies of genus *Citrus* L. with the application of molecular markers. Genet. Resour. Crop Evol. *61*, 1441–1454. https://doi.org/10.1007/s10722-014-0188-0.

Hodgson, R.W., 1967. Horticultural Varieties of Citrus (Berkeley: University of California Press).

Kijas, J.M.H., Thomas, M.R., Fowler, J.C.S., and Roose, M.L. (1997). Integration of trinucleotide microsatellites into a linkage map of Citrus. Theor. Appl. Genet. *94*, 701–706. https://doi.org/10.1007/s001220050468.

Kumar, K., Gill, M.I.S., Kaur, H., Choudhary, O.P., and Gosal, S.S. (2010). In vitro mutagenesis and somaclonal variation assisted salt tolerance in 'Rough Lemon' (*Citrus jambhiri* Lush.). Eur. J. Hortic. Sci. *75*, 233–238.

Kyndt, T., Dung, T.N., Goetghebeur, P., Toan, H.T., and Gheysen, G. (2010). Analysis of ITS of the rDNA to infer phylogenetic relationships among Vietnamese citrus accessions. Genet. Resour. Crop Evol. *57*, 183–192. https://doi.org/10.1007/s10722-009-9460-0.

Luro, F.L., Costantino, G., Terol, J., Argout, X., Allario, T., Wincker, P., Talon, M., Ollitrault, P., and Morillon, R. (2008). Transferability of the EST-SSRs developed on Nules clementine (*Citrus clementina* Hort ex Tan) to other *Citrus* species and their effectiveness for genetic mapping. BMC Genomics *9*, 287. https://doi.org/10.1186/1471-2164-9-287.

Mourao Filho, F.d.A.A., Pio, R., Mendes, B.M.J., de Azevedo, F.A., Schinor, E.H., Entelmann, F.A., Alves, A.S.R., and Cantuarias-Avilés, T.E. (2008). Evaluation of citrus somatic hybrids for tolerance to *Phytophthora nicotianae* and citrus tristeza virus. Sci. Hortic. *115*, 301–308. https://doi.org/10.1016/j.scienta.2007.10.004.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics *89*, 583–590.

Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G., and Tribulato, E. (2000). Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. *100*, 1155–1166. https://doi.org/10.1007/s001220051419.

Novelli, V.M., Cristofani, M., Souza, A.A., and Machado, M.A. (2006). Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* L. Osbeck). Genet. Mol. Biol. *29*, 90–96. https://doi.org/10.1590/S1415-47572006000100018.

Ollitrault, P., Terol, J., Garcia-Lor, A., Bérard, A., Chauveau, A., Froelicher, Y., Belzile, C., Morillon, R., Navarro, L., Brunel, D., and Talon, M. (2012). SNP mining in *C. clementina* BAC end sequences; transferability in the *Citrus* genus (*Rutaceae*), phylogenetic inferences and perspectives for genetic mapping. BMC Genomics *13*, 13. https://doi.org/10.1186/1471-2164-13-13.

Palmieri, D.A., Novelli, V.M., Bastianel, M., Cristofani-Yaly, M., Astúa-Monge, G., Carlos, E.F., Oliveira, A.C.d., and Machado, M.A. (2007). Frequency and distribution of microsatellites from ESTs of citrus. Genet. Mol. Biol. *30*, 1009–1018. https://doi.org/10.1590/S1415-47572007000500029.

Penjor, T., Yamamoto, M., Uehara, M., Ide, M., Matsumoto, N., Matsumoto, R., and Nagano, Y. (2013). Phylogenetic relationships of *Citrus* and its relatives based on *matK* gene sequences. PLoS ONE *8*, e62574. https://doi.org/10.1371/journal.pone.0062574.

Perrier, X., and Jacquemoud-Collet, J. (2006). DARwin software (http://darwin. cirad. fr/darwin).

Polat, I., Kacar, Y.A., Yesiloglu, T., Uzun, A., Tuzcu, O., Gulsen, O., Incesu, M., Kafa, G., Turgutoglu, E., and Anil, S. (2012). Molecular characterization of sour orange (*Citrus aurantium*) accessions and their relatives using SSR and SRAP markers. Gen. Mol. Res. *11*, 3267–3276. https://doi.org/10.4238/2012.September.12.10.

Sarao, N.K., Vikal, Y., Singh, K., Joshi, M.A., and Sharma R.C. (2010). SSR marker-based DNA fingerprinting and cultivar identification of rice (*Oryza sativa* L.) in Punjab state of India. Plant Genet. Resour. - C. *8*, 42–44. https://doi.org/10.1017/S1479262109990128.

Scora, R.W. (1975). On the history and origin of *Citrus*. Bull. Torrey Bot. Club *102*, 369–375. https://doi.org/10.2307/2484763.

Shahzadi, K., Naz, S., and Riaz, S. (2014). Assessing genetic diversity of Pakistani citrus varieties using microsatellite markers. J. Anim. Plant Sci. *24*, 1752–1757.

Snoussi, H., Duval, M.-F., Garcia-Lor, A., Belfalah, Z., Froelicher, Y., Risterucci, A.-M., Perrier, X., Jacquemoud-Collet, J.-P., Navarro, L., Harrabi, M., and Ollitrault, P. (2012). Assessment of the genetic diversity of the Tunisian citrus rootstock germplasm. BMC Genet. *13*, 16. https://doi.org/10.1186/1471-2156-13-16.

Sonkar, R., Huchche, A., Ram, L., and Singh, S. (2002). *Citrus* rootstocks scenario with special reference to India – A Review. Agric. Rev. – Agric. Res. Commun. Centre India *23*, 93–109.

Storey, R., and Walker, R. (1999). *Citrus* and salinity: a review. Sci. Hortic. *78*, 39–81. https://doi.org/10.1016/S0304-4238(98)00190-3.

Swingle, W.T., and Reece, P.C. (1967). The botany of *Citrus* and its wild relatives. In The *Citrus* Industry, Vol. 1, W. Reuther, H.J. Webber, and L.D. Batchelor, eds. (Berkeley, Ca., USA: University of California Press).

Tanaka, T. (1954). Species Problem in *Citrus* (Tokyo, Japan: Japanese Society for Promotion of Science).

Tanaka, T. (1977). Fundamental discussion of citrus classification. Stud. Citrologia *14*, 1–6.

Tazima, Z.H., Neves, C.S.V.J., Yada, I.F.U., and Leite Júnior, R.P. (2013). Performance of 'Okitsu' satsuma mandarin on nine rootstocks. Sci. Agric. *70*, 422–427. https://doi.org/10.1590/S0103-90162013000600007.

Uchoi, A., Malik, S.K., Choudhary, R., Kumar, S., Rohini, M.R., Pal, D., Ercisli, S., and Chaudhury, R. (2016). Inferring phylogenetic relationships of Indian citron (*Citrus medica* L.) based on *rbcL* and *matK* Sequences of Chloroplast DNA. Biochem. Genet. *54*, 249–269. https://doi.org/10.1007/s10528-016-9716-2.

Wu, G.A., Prochnik, S., Jenkins, J., Salse, J., Hellsten, U., Murat, F., Perrier, X., Ruiz, M., Scalabrin, S., Terol, J., Takita, M.A., Labadie, K., Poulain, J., Couloux, A., Jabbari, K., Cattonaro, F., Del Fabbro, C., Pinosio, S., Zuccolo, A., Chapman, J., Grimwood, J., Tadeo, F.R., Estornell, L.H., Muñoz-Sanz, J.V., Ibanez, V., Herrero-Ortega, A., Aleza, P., Pérez-Pérez, J., Ramón, D., Brunel, D., Luro, F., Chen, C., Farmerie, W.G., Desany, B., Kodira, C., Mohiuddin, M., Harkins, T., Fredrikson, K., Burns, P., Lomsadze, A., Borodovsky, M., Reforgiato, G., Freitas-Astúa, J., Quetier, F., Navarro, L., Roose, M., Wincker, P., Schmutz, J., Morgante, M., Machado, M.A., Talon, M., Jaillon, O., Ollitrault, P., Gmitter, F., and Rokhsar, D. (2014). Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. Nat. Biotechnol. *32*, 656–662. https://doi.org/10.1038/nbt.2906.

Yamamoto, M., Tsuchimochi, Y., Ninomiya, T., Koga, T., Kitajima, A., Yamasaki, A., Inafuku-Teramoto, S., Yang, X., Yang, X., Zhong, G., Nasir, N., Kubo, T., and Tominaga, S. (2013). Diversity of chloroplast DNA in various mandarins (*Citrus* spp.) and other Citrus demonstrated by CAPS analysis. J. Jpn. Soc. Hortic. Sci. *82*, 106–113. https://doi.org/10.2503/jjshs1.82.106.

Yeh, F., and Boyle, T. (1997). Sample genetic analysis of co-dominant and dominant markers and quantitative traits. Belg. J. Bot. *129*, 157.

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