Citrus (Rutaceae) SNP Markers Based on Competitive Allele-Specific PCR; Transferability Across the Aurantioideae Subfamily

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Primer Note

Citrus (Rutaceae) SNP markers based on Competitive Allele-Specific PCR; transferability across the Aurantioideae subfamily[1]

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- *Premise of the study:* Single nucleotide polymorphism (SNP) markers based on Competitive Allele-Specific PCR (KASPar) were developed from sequences of three *Citrus* species. Their transferability was tested in 63 *Citrus* genotypes and 19 relative genera of the subfamily Aurantioideae to estimate the potential of SNP markers, selected from a limited intrageneric discovery panel, for ongoing broader diversity analysis at the intra- and intergeneric levels and systematic germplasm bank characterization.
- *Methods and Results:* Forty-two SNP markers were developed using KASPar technology. Forty-one were successfully genotyped in all of the *Citrus* germplasm, where intra- and interspecific polymorphisms were observed. The transferability and diversity decreased with increasing taxonomic distance.
- *Conclusions:* SNP markers based on the KASPar method developed from sequence data of a limited intrageneric discovery panel provide a valuable molecular resource for genetic diversity analysis of germplasm within a genus and should be useful for germplasm fingerprinting at a much broader diversity level.

Key words: Competitive Allele-Specific PCR; genetic diversity; Rutaceae; single-nucleotide polymorphisms (SNPs).

Single nucleotide polymorphisms (SNPs) are the most frequent type of DNA sequence polymorphism. Their abundance and uniform distribution in genomes make them very powerful genetic markers. Several SNP genotyping methods have been developed. For low-to-medium throughput genotyping, the KBioscience Competitive Allele-Specific PCR genotyping system (KASPar; KBioscience Ltd., Hoddesdon, United Kingdom) appears to be an interesting approach (Cuppen, 2007) that has been successfully applied in animals and plants (Nijman et al., 2008; Bauer et al., 2009; Cortes et al., 2011). For genetic diversity studies with SNP markers, it is very important to determine the representativeness of the discovery panel (Albrechtsen et al., 2010). Ascertainment bias of the SNP markers affects the evaluation of genetic parameters, as was observed for the *Citrus* L. genus using SNP markers mined in a single Clementine cultivar (Ollitrault et al., 2012). Recently, Garcia-Lor et al. (2013) sequenced 27 amplified nuclear gene fragments for 45 genotypes of *Citrus*, which resulted in the identification of 1097 SNPs. Taking advantage of these previously obtained SNP data, the objective of this work was to implement a set of polymorphic SNP markers for systematic germplasm bank characterization within the *Citrus* genus and to investigate their transferability across the Aurantioideae [Engler] subfamily. More generally, the objective was to estimate the usefulness of SNP markers developed using KASPar technology, which were selected from a limited intrageneric discovery panel, for broader diversity analysis at the intra- and intergeneric levels.

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METHODS AND RESULTS

The 42 SNP markers used in this study were selected from SNPs identified by Garcia-Lor et al. (2013) in 27 nuclear genes. Most cultivated citrus (except for *C. aurantifolia* (Christm.) Swingle) arose from interspecific hybridization of three ancestral taxa: *C. medica* L., *C. reticulata* Blanco, and *C. maxima* (Burm.) Merr. (Nicolosi et al., 2000; Barkley et al., 2006; Garcia-Lor et al., 2012). Therefore, we selected SNPs between and within these three taxa (based on seven *C. reticulata*, five *C. maxima*, and five *C. medica* accessions). Primers were defined by KBioscience (http://www.kbioscience.co.uk/) from each SNP-locus flanking sequence (Appendix S1). Two allele-specific oligonucleotides and one common oligonucleotide were defined for each locus (Table 1). The KASPar system uses two Förster resonance energy transfer (FRET) cassettes, where fluorometric dye is conjugated to the primer but quenched via resonance energy transfer. In this system, sample DNA is amplified in a thermal cycler using allele-specific primers, leading to the separation of fluorometric dye and quencher when the FRET cassette primer is hybridized with DNA (Cuppen, 2007). Normalized signals of each SNP allele (x and y) were provided by KBioscience. Automatic allele calls provided by KlusterCaller software were visually checked with two-dimensional plot representations using SNPViewer software (KBioscience Ltd.).

Eighty-four accessions (Appendix 1) were genotyped for the 42 SNP markers. The sample set included representatives of the two tribes of the Aurantioideae (Clausenae and Citreae). In Clausenae, the subtribe Clauseniae was represented by four genotypes (three genera). Within the Citreae, three subtribes were represented: Triphasilinae (one genus was included), Balsamocitrinae (represented by six genera), and Citrinae (11 genera represented). For the Citrinae, we adopted the subdivision of this tribe into three groups (as proposed by Swingle and Reece, 1967), namely the primitive citrus fruit group (four accessions of four genera), the near citrus fruit group (three accessions of two

Table 1. Characteristics of 41 SNP primers used for genotyping of the Aurantioideae subfamily.

ID[a]	Gene	SNP-specific primers[b]	Common primer[c]	AlleleX	AlleleY	GenBank accession[d] no.
EMA-M30	Malic enzyme (EMA)	AlleleX: GCCTATTCATATAATTTAGATGTCAGGAAA AlleleY: CCTATTCATATAATTTAGATGTCAGGAAG	GTTTAGCCCGCACTTTCTTTCTCTTT	T	C	JX630064
ACO-P353	Aconitase (ACO)	AlleleX: ATGTCTGCAGAGAAAACCAGTAAAATG AlleleY: CAATGTCTGCAGAGAAAACCAGTAAAATA	TCTCTGTTTTGAAGCTAATTCCCACTCAA	C	T	JX630065
ACO-C601	Aconitase (ACO)	AlleleX: ATAAAGGCTTATGAAAGAAAGTTTCAACTC AlleleY: CATAAAGGCTTATGAAAGAAAGTTTCAACTT	CTGAAGCTAATTTGCAGACATGGAACATT	G	A	JX630065
F3′H-P30	Flavonoid 3′-hydroxylase (F3′H)	AlleleX: CCCACTTGGCCTACGACGCT AlleleY: CCACTTGGCCTACGACGCC	CTCGGACCATAATCAGCAAAGACCAT	T	C	JX630066
F3′H-M309	Flavonoid 3′-hydroxylase (F3′H)	AlleleX: ACGTCATGAGCTCTACCACCATA AlleleY: CGTCATGAGCTCTACCACCATG	GACCAAAGGGACAGAATCTAATGAGTTTA	T	C	JX630066
F3′H-C341	Flavonoid 3′-hydroxylase (F3′H)	AlleleX: GAGCTCATGACGTCAGCTGGATT AlleleY: GAGCTCATGACGTCAGCTGGATA	GCAATCGAGGGTATAAAATCACCAATGTT	T	A	JX630066
PEPC-M316	Phosphoenolpyruvate carboxylase (PEPC)	AlleleX: TAAAGAGCAATGAATTTCTTCAAACCTAA AlleleY: AAAGAGCAATGAATTTCTTCAAACCTAG	GTGCATTTAAGAACTGAGAAGGCATAGAA	T	C	JX630067
PEPC-C328	Phosphoenolpyruvate carboxylase (PEPC)	AlleleX: TAAAGCTGACTTAAAGAGCAATGAATTC AlleleY: CTTAAAGCTGACTTAAAGAGCAATGAATTT	GAAGGCATAGAATATTCCAYTAGGTTTGAA	G	A	JX630067
SOS1-M50	Salt overly sensitive 1 (SOS1)	AlleleX: GGTTTAGTACTGAGTAAGTTACTTGC AlleleY: AAATGGTTTAGTACTGAGTAAGTTACTTGT	GGACTTTTTCAGGTTTTGCATGTTGTCAA	G	A	JX630068
CCC1-M85	Cation chloride cotransporter (CCC1)	AlleleX: CATTGTGGTTATGAGGTATCCAGAG AlleleY: AACATTGTGGTTATGAGGTATCCAGAA	CAGTAAGGTTTTCACGGCGCCATAT	G	A	JX630069
CCC1-P727	Cation chloride cotransporter (CCC1)	AlleleX: ATCAACCACCCAGCTTACTGCTAT AlleleY: CAACCACCCAGCTTACTGCTAC	GGCACATTCTCTACTAACAAATCCATGTA	T	C	JX630069
TRPA-M593	Vacuolar citrate/H^+ symporter (TRPA)	AlleleX: AACGTGGCAGCAGCAGTGATG AlleleY: AACGTGGCAGCAGCAGTGATC	TCCCAGTGGCCACTGGCATCAT	C	G	JX630070
INVA-M437	Acid invertase (INVA)	AlleleX: GTTCAGCAGATCCTTCGCTGGAA AlleleY: CAGCAGATCCTTCGCTGGAG	ACAGCGGAGTCCAATGTGGAGTTTA	T	C	JX630071
INVA-P855	Acid invertase (INVA)	AlleleX: GGCACTGTCAATAGAATCCTCACAAT AlleleY: GCACTGTCAATAGAATCCTCACAAC	CCTGCAAATATACATACACAATGTTCCAAA	T	C	JX630071
MDH-MP69	Malate dehydrogenase (MDH)	AlleleX: AGGCCACTGAAACTCACAAGTGAT AlleleY: GGCCACTGAAACTCACAAGTGAG	CTGGTGTGAGGTTCAACTCCAAGAA	A	C	JX630072
MDH-M519	Malate dehydrogenase (MDH)	AlleleX: CAGCCTCAACCAAGGTCTTTACTATA AlleleY: AGCCTCAACCAAGGTCTTTACTATG	GATGACCTCTTCAACATCAACGCCAA	T	C	JX630072
ATMR-C372	MRP-like ABC transporter (ATMR)	AlleleX: GAATCATTATTGATGGAATCGACATTTCG AlleleY: AGAATCATTATTGATGGAATCGACATTTCA	ACCTTAGGTCATGAAGCCCCAACAA	G	A	JX630073
ATMR-M728	MRP-like ABC transporter (ATMR)	AlleleX: GTTTGATTTAATGGAAGTCATATGTATCTTTTT AlleleY: TGATTTAATGGAAGTCATATGTATCTTTTG	AAAGTTCAACATTTTGGCATGTTTTAGCTT	T	G	JX630073
CHS-P57	Chalcone synthase (CHS)	AlleleX: CAAGTATGGTAGTTTCAGAAGTGGTA AlleleY: CAAGTATGGTAGTTTCAGAAGTGGTT	AAAACAACCCTGGAAGCCGCGTTTT	T	A	JX630074
CHS-M183	Chalcone synthase (CHS)	AlleleX: GTTGGAGCTGACCCATTCCTG AlleleY: GTTGGAGCTGACCCATTCCTC	GTTAAGTTCCATGAAAGGAGAAGACTCTT	G	C	JX630074
CHI-M598	Chalcone isomerase (CHI)	AlleleX: CGTCACTTTCACGCCGTCCG AlleleY: CGTCACTTTCACGCCGTCCC	TGCGACTTTGTTGATCCTGGAGGTT	C	G	JX630075
PKF-C64	Phosphofructokinase (PKF)	AlleleX: ACTCCCTCTCCCTTCTGTTCTC AlleleY: CACTCCCTCTCCCTTCTGTTCTA	GGCCATCGACGATTTTGAAAGGGTT	C	A	JX630076
PKF-M186	Phosphofructokinase (PKF)	AlleleX: CGTCCGTAACATTACAGATTCAAGAT AlleleY: CGTCCGTAACATTACAGATTCAAGAC	CCGAACAGATTTGGAAACAATTTCGCAAT	T	C	JX630076
NADK2-M285	NADH kinase (NADK2)	AlleleX: CATCTTCTCTTGGTGATACAAGAAAGAA AlleleY: ATCTTCTCTTGGTGATACAAGAAAGAG	AACTCATTTCTAGATCTGATGAGCAGGTT	T	C	JX630077
DFR-M240	Dihydroflavonol 4-reductase (DFR)	AlleleX: CCGAAGAGGGAAACTTTGATGAAG AlleleY: CCGAAGAGGGAAACTTTGATGAAC	GAAAAACTCCAGTGCAGCCTCGAAT	G	C	JX630078
LAPX-M238	Ascorbate peroxidase (LAPX)	AlleleX: GAATTGACCATGGTTTGTGTTTTATTTTC AlleleY: GAATTGACCATGGTTTGTGTTTTATTTTG	GGCAACAACTCCAGCCAACTTCAA	C	G	JX630079

TABLE 1. Continued.

ID[a]	Gene	SNP-specific primers[b]	Common primer[c]	AlleleX	AlleleY	GenBank accession[d] no.
PSY-M30	Phytoene synthase (PSY)	AlleleX: GTCCATTTGATATGCTTGATGCTGG AlleleY: GTCCATTTGATATGCTTGATGCTGC	CGACAGGAAATTTGGTTACTGTATCTGAT	G	C	JX630080
PSY-C461	Phytoene synthase (PSY)	AlleleX: CGCAGGCCTATTAAACTCTTGTCA AlleleY: CGCAGGCCTATTAAACTCTTGTCT	AAGTTCTGCATGCTACCCTTCTCAATATT	T	A	JX630080
AOC-M290	Ascorbate oxydase (AOC)	AlleleX: AAGGGGTGCATCTGAGCCAAAG AlleleY: AAAGGGGTGCATCTGAGCCAAAA	CTGCGTTGAAAACTAATGGTACTGTACTT	C	T	JX630081
AOC-C593	Ascorbate oxydase (AOC)	AlleleX: GCCATACCCATGGAATTCGGCT AlleleY: GCCATACCCATGGAATTCGGCA	GGGGTAACTGGAGGGCTCCATT	T	A	JX630081
DXS-C545	1-deoxyxylulose 5-phosphate synthase (DXS)	AlleleX: ACCAAATGCATCATGAACGCTTTCC AlleleY: ACCAAATGCATCATGAACGCTTTCG	GGGGCTTGCAGGATTCCCCAAA	G	C	JX630082
DXS-M618	1-deoxyxylulose 5-phosphate synthase (DXS)	AlleleX: GGTCTTGGTATGTACTTCG AlleleY: CTGCTGGTCTTGGTATGTACTTCA	CCTACAATTTCTCTAGATTGATGAAAGGAA	G	A	JX630082
FLS-P129	Flavonol synthase (FLS)	AlleleX: GGCTTCCGCGATGGAACGTA AlleleY: GGCTTCCGCGATGGAACGTG	CGATCTCGACGACCCCGTTCAA	T	C	JX630083
FLS-M400	Flavonol synthase (FLS)	AlleleX: CCGTCTTCTATCAACTACCGCTTT AlleleY: CGTCTTCTATCAACTACCGCTTC	TTCACCGGTAAGAAGGAGGGTTGTT	T	C	JX630083
LCY2-M379	Lycopene β-cyclase 2 (LCY2)	AlleleX: TGATGAGTTTGAAGACATAGGACTTG AlleleY: GTTGATGAGTTTGAAGACATAGGACTTA	CGGCCAAGTTTTGTCCAAACAGTCTA	G	A	JX566716
LCYB-M480	Lycopene β-cyclase (LCYB)	AlleleX: GAATAACCTTAATAACTTTAGCTTGGTGG AlleleY: GAATAACCTTAATAACTTTAGCTTGGTGA	GCTGCAAAAATGCATAACCAATGGTGTTA	C	T	JX630084
LCYB-P736	Lycopene β-cyclase (LCYB)	AlleleX: GATTCGCATCTGAACAACAATTCGG AlleleY: CGCATCTGAACAACAATTCGC	GAAAAGTAGGAATTTTGCTATTTGCCTCTT	G	C	JX630084
HYB-M62	β-Carotene hydroxylase (HYB)	AlleleX: AAAACAAAACATACGGTGAAAGAGTTGAT AlleleY: AACAAAACATACGGTGAAAGAGTTGAG	GGCTTCTTTAATGGCAAAAACCGAAGAAA	A	C	AF315289
HYB-C433	β-Carotene hydroxylase (HYB)	AlleleX: GAGCAAATGTGCCAAACATTTCAGC AlleleY: AGAGCAAATGTGCCAAACATTTCAGT	GTACAGGGTGGAGAGGTGCCTT	G	A	JX630087
TSC-C80	Trehalose-6-phosphate synthase (TSC)	AlleleX: TCTTGACCACTTGGAAAATGTTCTTT AlleleY: CTTGACCACTTGGAAAATGTTCTTG	GCCTCTTTTGACAACAACAGGCTCAT	T	G	JX630084
NCED3-M535	9-cis-epoxy hydroxy carotenoid dyoxygenase 3 (NCED3)	AlleleX: GACACCTTGTTCTTGTCATAAATCACA AlleleY: ACACCTTGTTCTTGTCATAAATCACC	CAAGTGGTGTTCAAGTTGAATGAGATGAT	T	G	JX630086

[a] ID = SNP locus name.
[b] Allele X and Y forward primers.
[c] Reverse primer.
[d] GenBank accession numbers for the genomic fragment gene sequences of *C. reshni* (corresponding sequences with identification of each SNP marker are also given in Appendix S1).

TABLE 2. Results of initial primer screening in different *Citrus* species and subtribes of the Aurantioideae subfamily.

	C. reticulata (*N* = 12)			*C. maxima* (*N* = 11)			*C. medica* (*N* = 6)			*Citrus* (*N* = 32)		True citrus* (*N* = 16)		Balsamocitrinae (*N* = 6)		Near Citrus (*N* = 3)		Primitive Citrus (*N* = 4)		Triphasilinae (*N* = 1)		Clauseniae (*N* = 4)		Aurantioideae (*N* = 84)			
Marker	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	MD	*A*	MD	*A*	MD	*A*	H_o	*A*	MD	*A*	MD	*A*	MD	*A*	H_o	H_e	MD
EMA-M30	2	0.73	0.37	1	0.00	0.00	1	0.00	0.00	2	3.13	1	0.00	1	66.67	0	100.00	0	100.00	1	0.00	1	75.00	2	0.29	0.26	17.86
ACO-P353	1	0.00	0.00	2	0.55	0.37	1	0.00	0.00	2	0.00	1	0.00	2	33.33	1	0.00	2	0.00	1	0.00	1	25.00	2	0.16	0.27	3.57
ACO-C601	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	33.33	1	0.00	1	0.00	1	0.00	1	25.00	2	0.06	0.19	3.57
F3′H-P30	1	0.00	0.00	2	0.10	0.09	1	0.00	0.00	2	6.25	1	0.00	1	0.00	1	0.00	1	0.00	0	100.00	1	0.00	2	0.10	0.24	4.76
F3′H-M309	2	0.33	0.30	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	16.67	1	0.00	1	0.00	1	0.00	1	0.00	2	0.07	0.09	1.19
F3′H-C341	1	0.00	0.00	1	0.00	0.00	2	0.17	0.37	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.04	0.10	1.19
PEPC-M316	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	3.13	1	0.00	1	16.67	1	0.00	1	25.00	1	0.00	1	50.00	2	0.11	0.29	5.95
PEPC-C328	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	16.67	1	0.00	2	0.00	1	0.00	1	0.00	2	0.08	0.18	1.19
SOS1-M50	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	75.00	2	0.12	0.35	3.57
CCC1-M85	2	0.67	0.37	1	0.00	0.00	1	0.00	0.00	2	0.00	2	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	50.00	2	0.24	0.31	2.38
CCC1-P727	2	0.58	0.37	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	25.00	2	0.16	0.34	1.19
TRPA-M593	2	0.58	0.33	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	25.00	2	0.20	0.37	1.19
INVA-M437	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	50.00	1	0.00	1	0.00	1	0.00	1	50.00	2	0.13	0.29	5.95
INVA-P855	1	0.00	0.00	1	0.00	0.00	2	0.33	0.24	2	0.00	2	0.00	2	16.67	2	0.00	2	0.00	2	1.00	2	0.00	2	0.22	0.37	2.38
MDH-MP69	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	2	6.25	1	33.33	1	0.00	1	0.00	1	0.00	1	50.00	2	0.18	0.37	5.95
MDH-M519	2	0.42	0.33	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	16.67	1	0.00	1	25.00	1	0.00	1	50.00	2	0.18	0.24	4.76
ATMR-C372	1	0.00	0.00	1	0.00	0.00	2	0.50	0.37	2	0.00	1	0.00	1	16.67	1	0.00	1	0.00	1	0.00	1	25.00	2	0.09	0.12	2.38
ATMR-M728	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	3.13	2	6.25	1	83.33	1	33.33	1	0.00	1	0.00	0	100.00	2	0.15	0.37	14.29
CHS-P57	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.08	0.26	0.00
CHS-M183	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	66.67	1	0.00	1	0.00	1	0.00	1	25.00	2	0.06	0.31	5.95
CHI-M598	2	0.09	0.08	1	0.00	0.00	2	0.17	0.14	2	3.13	2	6.25	2	33.33	1	0.00	2	0.00	1	0.00	1	50.00	2	0.22	0.37	7.14
PKF-C64	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	3.13	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.05	0.14	1.19
PKF-M186	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	0	100.00	1	33.33	1	75.00	0	100.00	1	25.00	2	0.13	0.37	14.29
NADK2-M285	1	0.00	0.00	1	0.00	0.00	2	0.33	0.24	2	0.00	2	6.25	1	50.00	1	33.33	1	25.00	0	100.00	1	25.00	2	0.20	0.34	9.52
DFR-M240	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	3.13	2	0.00	1	83.33	2	33.33	2	0.00	0	100.00	1	0.00	2	0.17	0.37	10.71
LAPX-M238	2	0.50	0.35	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	75.00	2	0.22	0.26	3.57
PSY-M30	2	0.67	0.35	1	0.00	0.00	1	0.00	0.00	2	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	25.00	2	0.27	0.37	1.19
PSY-C461	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	33.33	1	0.00	1	0.00	1	0.00	1	25.00	2	0.06	0.17	3.57
AOC-M290	2	0.45	0.29	1	0.00	0.00	1	0.00	0.00	2	6.25	1	0.00	2	16.67	2	0.00	2	0.00	1	0.00	2	0.00	2	0.28	0.30	3.57
AOC-C593	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	25.00	2	0.06	0.17	1.19
DXS-C545	1	0.00	0.00	1	0.00	0.00	2	0.33	0.35	2	0.00	1	0.00	1	16.67	1	0.00	1	0.00	1	0.00	1	0.00	2	0.07	0.13	1.19
DXS-M618	2	0.50	0.30	1	0.00	0.00	1	0.00	0.00	2	0.00	2	0.00	2	16.67	2	0.00	1	0.00	1	0.00	1	50.00	2	0.21	0.27	3.57
FLS-P129	1	0.00	0.00	2	0.27	0.34	1	0.00	0.00	2	0.00	2	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.18	0.26	0.00
FLS-M400	2	0.50	0.30	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	2	16.67	1	0.00	1	0.00	1	0.00	2	0.00	2	0.18	0.27	1.19
LCY2-M379	2	0.67	0.35	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.27	0.26	1.19
LCYB-M480	2	0.33	0.24	1	0.00	0.00	1	0.00	0.00	2	0.00	2	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	25.00	2	0.23	0.34	1.19
LCYB-P736	1	0.00	0.00	2	0.18	0.37	1	0.00	0.00	2	0.00	1	0.00	1	66.67	1	0.00	1	0.00	1	0.00	1	25.00	2	0.06	0.16	5.95
HYB-M62	2	0.42	0.33	1	0.00	0.00	1	0.00	0.00	2	3.13	2	0.00	1	0.00	1	0.00	1	25.00	1	0.00	1	75.00	2	0.27	0.37	5.95
HYB-C433	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	2	0.00	1	33.33	1	0.00	1	0.00	1	0.00	2	0.10	0.19	1.19
TSC-C80	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	3.13	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	1	0.00	2	0.07	0.16	1.19
NCED3-M535	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	2	0.00	1	0.00	1	16.67	1	0.00	1	0.00	1	0.00	1	25.00	2	0.13	0.29	2.38
Mean	1.37	0.18	0.11	1.10	0.03	0.03	1.15	0.04	0.04	2	0.91	1.32	0.61	1.22	22.36	1.07	6.50	1.12	6.71	0.93	9.78	1.07	26.83	2	0.15	0.26	4.15

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; MD = missing data (%); *N* = sample size.
*True citrus excluding the *Citrus* genus.

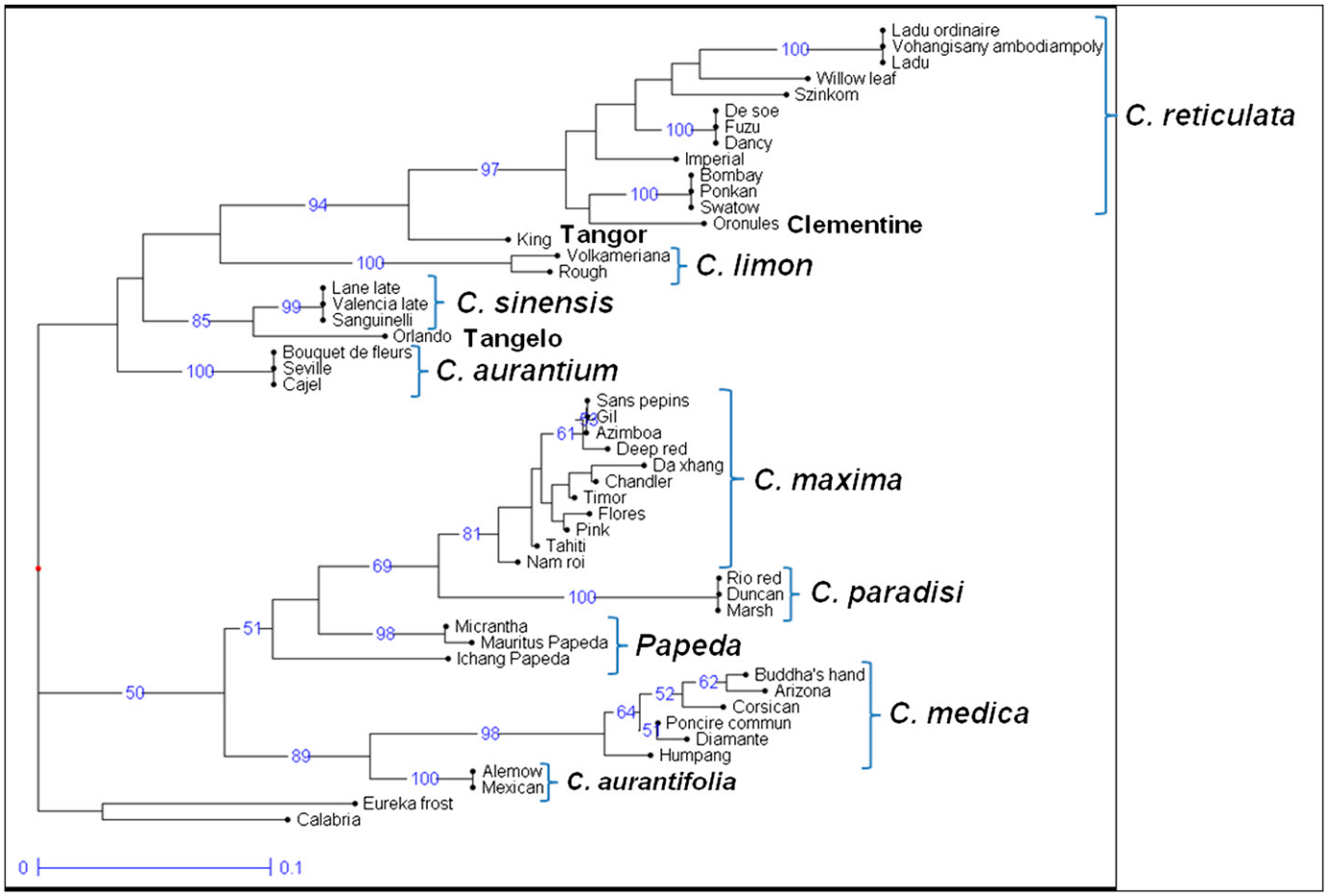

Fig. 1. Neighbor-joining analysis based on simple matching dissimilarities from 41 SNP loci for 50 accessions belonging to the genus *Citrus*, including secondary species and hybrids. Numbers near nodes are bootstrap values based on 1000 resamplings (only values >50% are indicated).

genera), and the "true citrus fruit trees" group (48 accessions of six genera). High-molecular-weight genomic DNA was extracted from leaf samples using a DNeasy Plant Mini Kit (QIAGEN, Madrid, Spain) according to the manufacturer's instructions.

From the 42 SNP primers tested, only one did not produce polymorphisms. To check the accuracy of the allele call for the 41 other markers, we compared the KASPar genotyping data with Sanger sequencing data available for 35 accessions of the "true citrus fruit trees" (Garcia-Lor et al., 2013). The conformity level was 95.41%, while 2.99% did not agree and 1.60% were missing data.

The allele number and the percentage of missing data are presented for each taxon (Table 2). The expected (H_e) and observed heterozygosity (H_o) were evaluated for *C. reticulata*, *C. maxima*, *C. medica*, the *Citrus* genus, and the "true citrus fruit trees" excluding the *Citrus* genus. Data analysis was conducted with PowerMarker version 3.25 (Liu and Muse, 2005) and DARwin (Perrier and Jacquemoud-Collet, 2006) software.

The missing data rate was very low in *Citrus* (0.9%) and, generally, in the "true citrus fruit trees" group (0.6%, excluding the *Citrus* genus). The missing data rate increased to 6.5% and 6.7% in the close citrus and primitive citrus groups of the Citrinae subtribe, respectively, reaching a level of 9.8% and 22.4% for the two other subtribes of the Citreae tribe, the Triphasilinae and the Balsamocitrinae, respectively. Missing data reached 26.8% in the Clauseniae tribe. These results indicate an increasing loss of transferability with increasing taxonomic distance. As expected due to the discovery panel, the *Citrus* genus was the most polymorphic (an average of two alleles per locus; $H_e = 0.30$; $H_o = 0.23$), followed by the "true citrus fruit trees" group excluding the *Citrus* genus (alleles per locus [*A*] = 1.32; $H_e = 0.09$; $H_o = 0.02$). Diversity within and between the other taxa decreased considerably (data not shown). However, despite this important loss of polymorphism, all citrus relatives were differentiated when missing amplification was considered to represent null alleles, providing molecular fingerprinting for traceability in germplasm bank management.

Among the *Citrus* ancestral taxa, *C. reticulata* was the most polymorphic ($A = 1.37$; $H_e = 0.11$), followed by *C. medica* ($A = 1.15$; $H_e = 0.04$), and *C. maxima* ($A = 1.10$; $H_e = 0.03$). Considering as subpopulations the three species used in the discovery panel, the F_{ST} value was very high (0.842). The high level of differentiation between *C. reticulata*, *C. maxima*, and *C. medica* for this SNP panel was well illustrated by neighbor-joining analysis (Fig. 1). The relative position of the accessions of secondary species (*C. aurantium* L., *C. aurantifolia*, *C. limon* (L.) Osbeck, *C. paradisi* Macfad., and *C. sinensis* (L.) Osbeck) and hybrids (Clementine, tangor, and tangelo) agrees with previous molecular studies (Nicolosi et al., 2000; Ollitrault et al., 2012; Garcia-Lor et al., 2012). Therefore, these markers should be useful as phylogenetic tracers of DNA fragments in secondary cultivated citrus species.

CONCLUSIONS

Forty-one SNP markers were successfully developed from SNP loci mined by Sanger sequencing in a discovery panel including 17 genotypes of the three main cultivated *Citrus* ancestral taxa. The genotyping data displayed high conformity with previous sequencing data. Genotyping was highly successful within the *Citrus* genus, and the genetic organization displayed by this SNP marker panel was in agreement with previous studies. The frequency of missing data was higher for the citrus relatives and increased with taxonomic distances within the Aurantioideae subfamily, suggesting incomplete transferability. The polymorphism revealed within the relatives of the "true citrus fruit trees" group remained relatively high but decreased

strongly when considering the other citrus relatives. However, all citrus relative genotypes were differentiated. The markers that were developed appeared to be useful for phylogenic studies within the "true citrus fruit trees" group. Therefore, SNP markers based on the KASPar method developed from sequence data of a limited intrageneric discovery panel provide a valuable molecular resource for genetic diversity analysis of germplasm within a genus and should be useful for germplasm fingerprinting at a much broader diversity level.

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Appendix 1. Accessions analyzed in this study. Information presented: species name, Latin name or common name, accession number, ex-situ germplasm bank. IVIA = Carretera Moncada, Naquera, Km 4.4, Apartado Oficial, 46113 Moncada (Valencia), Spain; INRA/CIRAD = Station INRA, 20230 San Giuliano, France.

1. Citreae

Balsamocitrinae: ***Aegle marmelos*** (L.) Corrêa: 345, IVIA; ***Aeglopsis chevalieri*** Swingle: 308, IVIA; ***Afraegle paniculata*** (Schum. & Thonn.) Engl.: 273, IVIA; ***Balsamocitrus dawei*** Stapf: 372, IVIA; ***Feroniella oblata*** Swingle: 585, IVIA; ***Swinglea glutinosa*** (Blanco) Merr.: 292, IVIA.

Citrinae

True citrus fruit:

Citrus**: *C. maxima*** (Burm.) Merr.: Azimboa, 420, IVIA; Chandler, 207, IVIA; Da xanh, 589, IVIA; Deep red, 277, IVIA; Flores, 673, INRA/CIRAD; Gil, 321, IVIA; Nam roi, 590, IVIA; Pink, 275, IVIA; Sans Pepins, 710, INRA/CIRAD; Tahiti, 727, INRA/CIRAD; Timor, 707, INRA/CIRAD. ***C. medica*** L.: Arizona, 169, IVIA; Buddha hand, 202, IVIA; Corsican, 567, IVIA; Diamante, 560, IVIA; Humpang, 722, INRA/CIRAD; Poncire Commun, 701, INRA/CIRAD. ***C. reticulata*** Blanco: Bombay, 518, INRA/CIRAD; Dancy, 434, IVIA; De soe, 713, INRA/CIRAD; Imperial, 576, IVIA; Fuzhu, 571, IVIA; Ladu, 595, INRA/CIRAD; Ladu ordinaire, 590, INRA/CIRAD; Ponkan, 482, IVIA; Swatow, 175, INRA/CIRAD; Szinkom, 597, INRA/CIRAD; Vohangisany ambodiampoly, 437, SRA; Willow leaf, 154, IVIA. ***Papeda*****: *C. hystrix*** DC.: Combava, 178, IVIA; ***C. ichangensis*** Swingle: Papeda Ichang, 358, IVIA; ***C. micrantha*** Wester: Micrantha, IVIA.

Secondary species: ***C. aurantifolia*** (Christm.) Swingle: Alemow, 288, IVIA; Calabria, 254, IVIA; Mexican, 164, IVIA. ***C. aurantium*** L.: Bouquet de fleurs, 139, IVIA; Cajel, 108, IVIA; Seville, 117, IVIA. ***C. limon*** (L.) Osbeck: Eureka frost, 297, IVIA; Rough lemon, 333, IVIA; Volkamer lemon, 432, IVIA; ***C. paradisi*** Macfad.: Duncan, 274, IVIA; Marsh, 176, IVIA; Rio red, 289, IVIA. ***C. sinensis*** (L.) Osbeck: Lane late, 198, IVIA; Sanguinelli, 34, IVIA; Valencia late, 363, IVIA.

Hybrids: Clementine, Clemenules, 22, IVIA; Tangelo, Orlando, 101, IVIA; Tangor, King, 477, IVIA.

Clymenia: ***C. polyandra*** (Tanaka) Swingle: 584, IVIA.

Eremocitrus: ***E. glauca*** (Lindl.) Swingle: 346, IVIA.

Fortunella**: *F. crassifolia*** Swingle: 280, IVIA; ***F. hindsii*** Swingle: 281, IVIA; ***F. japonica*** (Thunb.) Swingle: 381, IVIA; ***F. margarita*** (Lour.) Swingle: 38, IVIA; ***Fortunella* sp.**: 98, IVIA.

Microcitrus: ***M. australasica*** Swingle: 150, IVIA; ***M. australis*** Swingle: 313, IVIA; ***M. australis* × *M. australasica***: 378, IVIA; Australian Wild Lime, 314, IVIA; New Guinea Wild Lime, 315, IVIA.

Poncirus trifoliata (L.) Raf.: Flying Dragon, 537, IVIA; Pomeroy, 374, IVIA; Rich 75, 236, IVIA; Rubidoux, 217, IVIA.

Near citrus fruit: ***Atalantia ceylanica*** (Arn.) Oliv.: 172, IVIA; ***Atalantia citroides*** Pierre ex Guillaumin, 284, IVIA; ***Citropsis gilletiana*** Swingle & M. Kellerm.: 517, IVIA.

Primitive citrus fruit: ***Hesperethusa crenulata*** (Roxb.) M. Roem.: 580, IVIA; *Pleiospermium* sp., 380, IVIA; ***Severinia buxifolia*** (Poir.) Ten.: 147, IVIA; ***Severinia disticha*** (Blanco) Swingle: 418, IVIA.

Triphasilinae: ***Triphasia trifolia*** (Burm. f.) P. Wilson: 182, IVIA.

2. Clauseneae

Clauseniae: ***Clausena excavata*** Burm. f.: 311, IVIA; ***Clausena lansium*** (Lour.) Skeels: 343, IVIA; ***Glycosmis pentaphylla*** (Retz.) DC.: 148, IVIA; ***Murraya koenigii*** (L.) Spreng.: 377, IVIA.