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 intragenic discovery panel, for broader diversity analysis at the intra- and intergeneric levels.

### 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. aurantiifolia (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 ClusterCaller 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 both tribes of the Auran-
tioideae (Clausenae and Citreae). In Clausenae, the subtribe Clausenae was represented by four genotypes (three genera). Within the Citreae, three sub-tribes were represented: Triphasalinae (one genus was included), Balsamocitri-
nae (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 accesses-
tions of four genera), the near citrus fruit group (three accesses of two
| ID  | Gene                                | SNP-specific primers\(^a\) AlleleX | Common primer\(^b\) | AlleleX | AlleleY | GenBank accession\(^a\) no. |
|-----|-------------------------------------|-----------------------------------|---------------------|---------|---------|---------------------------|
| EMA-M30 | Malic enzyme (EMA)                   | G GCCATATCTTAATATTTTTAGTGACAGGAA  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630064                  |
| ACO-P353 | Aconitase (ACO)                      | T GCTCTGTAGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | C       | T       | JX630065                  |
| ACO-C601 | Aconitase (ACO)                      | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630065                  |
| F3'H-P30 | Flavonoid 3'-hydroxylase (F3'H)    | C CCACATTTGCTCAGACAGGCTT   | T CTGAGCTATATTTGCAAAAGGACTAGTT   | T       | C       | JX630066                  |
| F3'H-M309 | Flavonoid 3'-hydroxylase (F3'H)    | T GCTGTTCTTGAAGCTATATGCCTACTCA   | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | A       | JX630066                  |
| F3'H-C341 | Flavonoid 3'-hydroxylase (F3'H)    | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630066                  |
| PEPC-M316 | Phosphoenolpyruvate carboxylase (PEPC) | T GCTGTTCTTGAAGCTATATGCCTACTCA   | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630066                  |
| PEPC-C328 | Phosphoenolpyruvate carboxylase (PEPC) | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630066                  |
| SOS1-M50 | Salt overly sensitive 1 (SOS1)       | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630068                  |
| CCC1-M85 | Cation chloride cotransporter (CCC1) | T GCTGTTCTTGAAGCTATATGCCTACTCA   | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630069                  |
| CCC1-P727 | Cation chloride cotransporter (CCC1) | T GCTGTTCTTGAAGCTATATGCCTACTCA   | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630069                  |
| TRPA-M593 | Vacular citrate/H\(^+\) symporter (TRPA) | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | G       | A       | JX630070                  |
| INVA-M437 | Acid invertase (INVA)                | G GCCATATCTTAATATTTTTTAGTGACAGGAA  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| INVA-P855 | Acid invertase (INVA)                | G GCCATATCTTAATATTTTTTAGTGACAGGAA  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| MDH-MP69 | Malate dehydrogenase (MDH)          | G GCCATATCTTAATATTTTTTAGTGACAGGAA  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| MDH-M519 | Malate dehydrogenase (MDH)          | G GCCATATCTTAATATTTTTTAGTGACAGGAA  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| ATM-M372 | Malate dehydrogenase (MDH)          | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| PHF-M240 | Phosphofructokinase (PHF)           | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |
| LAPX-M238 | Ascorbate peroxidase (LAPX)         | A AATTTGAGGAGAAACCACTGAATAGTTAGGAG  | G TCTGTTCTTGAAGCTATATGCCTACTCA   | T       | C       | JX630071                  |

\(^a\) SNP-specific primers and common primers are given for each primer. \(^b\) GenBank accession numbers for each primer.
| ID       | Gene                        | SNP-specific primers | Common primer | AlleleX | AlleleY | GenBank accession no. |
|----------|-----------------------------|----------------------|---------------|---------|---------|----------------------|
| PSY-M30  | Phytoene synthase (PSY)     | AlleleX: GTCCATTGGAATGCTGAAGCATTGGAACGTTG | CGACAGGAATTTGTTGATCATTCAATCTGAT | G       | C       | JX630080             |
| PSY-C461 | Phytoene synthase (PSY)     | AlleleX: CGACAGGCTTAACTAATCTTGTGAATGCTGAACGTTG | AAGTTCTGATGCTACCCTCTCCTCAATTT | T       | A       | JX630080             |
| AOC-M290 | Ascorbate oxydase (AOC)     | AlleleX: AAGGGGTGCATCTGAACCCGGAAGCTTG | CTGCGTTGAAAACCTATGTACGGTACCTT | C       | T       | JX630081             |
| AOC-C593 | Ascorbate oxydase (AOC)     | AlleleX: AAGGGGTGCATCTGAACCCGGAAGCTTG | GTGGTTGAAAACCTATGTACGGTACCTT | G       | A       | JX630082             |
| DXS-C545 | 1-deoxyxylulose 5-phosphate synthase (DXS) | AlleleX: CGCAGGCCTATTAAACTCTTGTCAATGCTGAACGTTG | GGCTTGGAGGAGGCTTCCATT | T       | A       | JX630081             |
| DXS-M618 | 1-deoxyxylulose 5-phosphate synthase (DXS) | AlleleX: CGCAGGCCTATTAAACTCTTGTCAATGCTGAACGTTG | GGCTTGGAGGAGGCTTCCATT | T       | A       | JX630081             |
| FLS-P129 | Flavonol synthase (FLS)     | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX630082             |
| FLS-M400 | Flavonol synthase (FLS)     | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX630082             |
| LCY2-M379| Lycopene β-cyclase (LCY2)   | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| LCYB-M480| Lycopene β-cyclase (LCYB)   | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| LCYB-P736| Lycopene β-cyclase (LCYB)   | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| HYB-M62  | β-Carotene hydroxylase (HYB) | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| HYB-C433 | β-Carotene hydroxylase (HYB) | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| TSC-C80  | Trehalose-6-phosphate synthase (TSC) | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |
| NCED3-M535| 9-cis-epoxy hydroxy carotenoid dioxygenase 3 (NCED3) | AlleleX: CGGTCCTGGAGGAGGCTTCCATT | TCTACATTTTCCTTAGATGACGAAAGGAA | G       | A       | JX567161             |

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).
| Marker       | C. reticulata (N = 12) | C. maxima (N = 11) | C. medica (N = 6) | Citrus (N = 32) | Balsamocitrinae (N = 16) | Near Citrus (N = 6) | Primitive Citrus (N = 4) | Triphalinae (N = 1) | Clausenae (N = 4) | Aurantioideae (N = 84) |
|-------------|------------------------|--------------------|-------------------|-----------------|------------------------|---------------------|------------------------|-------------------|-----------------|---------------------|
| FMA-M30     | 2                      | 0.73               | 0.37              | 1               | 0.00                   | 0.00                | 0.00                    | 0.00              | 1               | 0.00                |
| ACO-P353    | 1                      | 0.00               | 0.00              | 2               | 0.55                   | 0.37                | 1                       | 0.00              | 2               | 0.00                |
| ACO-C601    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| F3'H-P30    | 2                      | 0.00               | 0.00              | 1               | 0.33                   | 0.30                | 1                       | 0.00              | 2               | 0.17                |
| F3'H-M309   | 2                      | 0.00               | 0.00              | 2               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| F3'H-C341   | 2                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| FEP-M316    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| FEP-C328    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| SOSI-M50    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| CCC1-M85    | 2                      | 0.67               | 0.37              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.55                |
| CCC1-P727   | 2                      | 0.58               | 0.37              | 1               | 0.00                   | 0.00                | 2                       | 0.00              | 2               | 0.00                |
| TRPA-M593   | 2                      | 0.58               | 0.33              | 1               | 0.00                   | 0.00                | 2                       | 0.00              | 2               | 0.58                |
| INVA-M437   | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 1               | 0.00                |
| INVA-P855   | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 1               | 0.00                |
| MDH-M696    | 1                      | 0.00               | 0.00              | 2               | 0.33                   | 0.24                | 2                       | 0.00              | 2               | 1.00                |
| MDH-M519    | 2                      | 0.42               | 0.33              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 2               | 0.50                |
| ATMR-C372   | 1                      | 0.00               | 0.00              | 2               | 0.50                   | 0.37                | 1                       | 0.00              | 2               | 0.00                |
| ATMR-M728   | 1                      | 0.00               | 0.00              | 2               | 0.00                   | 0.00                | 1                       | 0.00              | 2               | 0.00                |
| CHS-P57     | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 0               | 0.00                |
| CHS-M183    | 2                      | 0.09               | 0.08              | 1               | 0.00                   | 0.00                | 2                       | 0.17              | 0               | 0.14                |
| CHI-M598    | 2                      | 0.09               | 0.08              | 1               | 0.00                   | 0.00                | 2                       | 0.17              | 2               | 0.31                |
| PKF-C64     | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 1               | 0.00                |
| PKF-M186    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 1                       | 0.00              | 1               | 0.00                |
| NAD2-M285   | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 2                       | 0.33              | 0               | 0.00                |
| DMR-M240    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 0.00                | 2                       | 0.33              | 2               | 0.33                |
| LAPX-M238   | 2                      | 0.50               | 0.35              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 1               | 0.00                |
| PSY-M30     | 1                      | 0.67               | 0.35              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| PSY-C461    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| AOC-M290    | 2                      | 0.45               | 0.29              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| AOC-C593    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| DXS-C545    | 1                      | 0.00               | 0.00              | 2               | 0.33                   | 0.35                | 2                       | 0.00              | 1               | 0.33                |
| DXS-M618    | 2                      | 0.50               | 0.30              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.33                |
| FLS-P129    | 1                      | 0.00               | 0.00              | 2               | 0.27                   | 0.34                | 1                       | 0.00              | 2               | 0.00                |
| FLS-M400    | 2                      | 0.50               | 0.30              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| LCY2-M379   | 1                      | 0.67               | 0.35              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| LCYB-M480   | 2                      | 0.33               | 0.30              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| LCYB-P376   | 1                      | 0.00               | 0.00              | 2               | 0.00                   | 0.00                | 1                       | 0.00              | 1               | 0.00                |
| HYB-M62     | 2                      | 0.42               | 0.33              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| HYB-C433    | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 2               | 0.00                |
| TSC-C60     | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 1               | 0.00                |
| NCEC3-M535  | 1                      | 0.00               | 0.00              | 1               | 0.00                   | 2                   | 0                       | 0.00              | 1               | 0.00                |

**Note:** A = number of alleles; \( H_o \) = expected heterozygosity; \( H_e \) = observed heterozygosity; MD = missing data (%); \( N \) = sample size.

*True citrus excluding the Citrus genus.*
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 Citraceae tribe, the Triphasilinae and the Balsamocitrinae, respectively. Missing data reached 26.8% in the Clauseniaceae tribe. These results indicate an increasing loss of transferability with increasing taxonomic distance. As expected due to 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 (Nicoli 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 phylogenetic studies within the “true citrus fruit trees” group. Therefore, SNP markers based on the KASPar method developed from sequence data of a limited intragenic 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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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. hispidii Swingle: 281, IVIA; F. japonica (Tanaka) 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. australis: 378, IVIA; Australian Wild Lime, 314, IVIA; New Guinea Wild Lime, 315, IVIA.

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

Near citrus fruit: Atalantia ceyslanica (Atr.) Oliiv.: 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; Pleiospermum sp.: 380, IVIA; Severinia buxifolia (Poir.) Ten.: 147, IVIA; Severinia disticha (Blanco) Swingle: 418, IVIA.

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

2. Clauseniaceae

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

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