Male and female inheritance patterns in tetraploid ‘Moncada’ mandarin

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Abstract

Key message Tetraploid ‘Moncada’ mandarin, used as male and female in interploidy hybridizations, displays mainly tetrasomic inheritance for most LGs, with slight variations according to the direction of the crossing.

Abstract Triploid-breeding programs in citrus are key tool to develop seedless cultivars. Obtaining triploid citrus hybrids may be achieved through different strategies, such as the exploitation of female unreduced gamete in crosses between diploid parents and diploid by tetraploid sexual hybridizations, in which tetraploid genotypes can be used as male or female parents. Genetic configuration of triploid populations from interploid crosses greatly depends on the chromosomal segregation mode of the tetraploid parent used. Here, we have analyzed the inheritance of the tetraploid ‘Moncada’ mandarin and compared the genetic structures of the resulting gametes when used as male and as female parent. The preferential chromosome pairing rate is calculated from the parental heterozygosity restitution (PHR) of codominant molecular markers, indicating the proportion between disomic and tetrasomic segregation. Tetraploid ‘Moncada’ both as female and male parent largely exhibited tetrasomic segregation. However, as female parent, one linkage group (LG8) showed intermediate segregation with tendency towards tetrasomic inheritance, while another linkage group (LG4) evidenced a clear intermediate segregation. On the other hand, when used as male parent two linkage groups (LG5 and LG6) showed values that fit an intermediate inheritance model with tetrasomic tendency. Significant doubled reduction (DR) rates were observed in five linkage groups as female parent, and in six linkage groups as male parent. The new knowledge generated here will serve to define crossing strategies in citrus improvement programs to efficiently obtain new varieties of interest in the global fresh consumption market.

Keywords Citrus · Triploid · Tetraploid · SSR and SNP markers · Disomic inheritance · Tetrasomic inheritance

Introduction

Polyploids are plants with somatic cells that contain three or more complete sets of chromosomes (Ramsey and Schmekel 1998). Ancient whole-genome duplications have been reported in most evolutionary lineages and may represent a crucial mode of speciation and eukaryotic genome evolution (Cai et al. 2019; Van de Peer et al. 2017). In fact, all the angiosperm genomes sequenced to date exhibit evidence of ancient polyploidization events (Cai et al. 2019; Soltis et al. 2014; Van de Peer et al. 2017) and polyploidy is one of the major forces of evolution for plant species, leading to their diversification and differentiation (Gallais 2003; Otto and Whitton 2000; Van de Peer et al. 2017).

Basically, polyploids differ from the diploid counterparts in their ecological, morphological, and physiological characteristics (Dewitte et al. 2009; Guerra et al. 2014;
Polyplodization offers many opportunities as a valuable tool in citrus-breeding programs (Aleza et al. 2016; Cuenca et al. 2015; Grosser and Gmitter 2011; Ollitrault et al. 2008). In Citrus and related genera, diploid genotypes are the most common, with a basic chromosome number $x = 9$ (Krug 1943). However, euploids and aneuploids have been induced or found occasionally, with triploids and tetraploids being the most common euploid variations (Lee 1988). Citrus triploid genotypes are generally seedless, a demanded characteristic for fresh fruit marketing (Aleza et al. 2012a, b, 2016). However, a few seedy triploid lime varieties have been described (Curk et al. 2016). Triploid genotypes in citrus are routinely obtained by sexual hybridization, through unreduced female gametes (Aleza et al. 2016). Triploid genotypes in citrus are obtained by induced triploidization or by colchicine treatment (Aleza et al. 2009; Garcia-Lor et al. 2013b; Nicolosi et al. 2000) and these results were confirmed by sequencing data (Wu et al. 2014, 2018; Xu et al. 2013). Commonly, the tetraploid parents used in interploid hybridizations for triploid breeding result from somatic chromosome doubling occurring spontaneously in nucellar cells or induced by treatment using antimotic agents such as colchicine and oryzaline (Aleza et al. 2009, 2011). In relation with the phylogenetic origin of the parental diploid such somatic tetraploids can be autotetraploid for monospecific varieties, allotetraploids when parental diploid resulted from direct interspecific hybridization or segmental allotetraploid when parental diploid had a more complex admixture genome. These complex genomes may, therefore, impact the observed segregations in breeding programs.

Here, we analyze the segregation pattern of the tetraploid ‘Moncada’ mandarin used both as male and as female parent in interploid crosses by genotyping triploid progenies with Simple Sequence Repeat (SSR) and Single-Nucleotide Polymorphism (SNP) molecular markers.

Diploid ‘Moncada’ mandarin was obtained from after 1980 in a breeding program held at Instituto Valenciano de Investigaciones Agrarias (IVIA) from a handmade pollination between ‘Oroval’ clementine (Citrus clementina Hort. Ex Tan.) and ‘Kara’ mandarin (C. unshiu (Mak) Marc. × C. nobilis Lour.) (Bermejo et al. 2011). Later, tetraploid ‘Moncada’ mandarin was obtained by colchicine treatment of shoot tips grafted in vitro (Aleza et al. 2009). This mandarin hybrid is characterized by its excellent fruit quality, very easy to peel, very late maturity period and also is a non-apomictic variety which makes a very interesting parent in citrus-breeding programs based on sexual hybridizations aimed to recover large populations of triploid hybrids. The breeding implications of the use of the tetraploid ‘Moncada’ mandarin as male or female parent in the recovery of large populations of triploid hybrids are further discussed.

Materials and methods

Plant material

Triploid hybrid progenies were obtained from $4 \times 2x$ and $2x \times 4x$ sexual hybridizations using tetraploid ‘Moncada’
mandarin as female and male parent, respectively. Tetraploid ‘Moncada’ mandarin was obtained directly from shoot tip grafting combined with colchicine treatment (Aleza et al. 2009). In $4 \times \times 2 \times$ sexual hybridization, 72 triploid hybrids were recovered using diploid ‘Anana’ mandarin ($C. reticulata$) as male parent (from here on referred as MA hybridization), whereas in the $2 \times \times 4 \times$ sexual hybridization, 88 triploid hybrids were obtained with the non-apomictic diploid ‘Clemenules’ clementine female parent (from here on referred as CM hybridization). Ploidy-level analysis by flow cytometry and triploid hybrid recovery was performed following the methodology described by Aleza et al. (2012a, b).

**Genotyping of the triploid progenies**

To study the genetic structure of the diploid gametes produced by the tetraploid ‘Moncada’ mandarin, progenies along with the parents were genotyped using SSR and SNP markers distributed homogeneously in the nine linkage groups (LGs) of the clementine reference genetic map (Ollitrault et al. 2012a). These markers were heterozygous for ‘Moncada’ mandarin and displayed polymorphism between ‘Moncada’ mandarin and ‘Clemenules’ or ‘Anana’ mandarins. Since ‘Moncada’ is a direct hybrid between clementine and ‘Kara’ mandarin, it was difficult to find heterozygous markers for ‘Moncada’ mandarin with polymorphism with clementine. Finally, 24 SSRs and 19 SNPs markers previously developed were analyzed for both populations. In addition, 11 new SNP markers were developed (Table 1) from a Genotyping-by-Sequencing (GBS) diversity analysis (unpublished data). Detailed information about SSR and SNP markers used in this study is given in Table 2. Given the genetic proximity between the tetraploid ‘Moncada’ and clementines, the exact same set of molecular markers could not be used in both families (CM and MA). Even so, 13 molecular markers were used in common for both families, distributed in eight out of the nine LGs.

PCR amplifications using SSR markers were performed using a thermocycler rep gradient S (Eppendorf®) in 15 μL containing 0.5 μl 1U/μl of Taq DNA polymerase (Fermentas®), 3 μL citrus DNA, 1.5 μl of 2 mM welled (Sigma®) dye-labeled forward primer, 1.5 μl of 2 mM non-dye-labeled reverse primer, 0.2 mM of each dNTP, 1.5 μl 10× PCR buffer, and 0.45 μl 50 mM MgCl2. The PCR protocol was as follows: denaturation at 94 °C for 5 min followed by 40 cycles of 30 s at 94 °C, 30 s at 50 or 55 °C, and 30 s at 72 °C; and a final elongation step of 8 min at 72 °C. Capillary electrophoresis was carried out using a Genetic Analysis System 8000 (Beckman Coulter Inc.). The PCR products were initially denatured at 90 °C for 2 min, loaded at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were

| Table 1 | Primer sequences of the new SNP markers developed in this paper for use in KASPar™ assay |
|---------|---------------------------------------------------------------|
| Markers name | SNP-specific primer | Common primer |
| C1P26815936 | Allele X: ATGATTGTCCTCAGATACTGTGTTAGACG | AAAGCTGAGCTAGTGTCTCCACATTCATA |
| C2_23768463 | Allele X: CAAAGAACCCCTTCCTGAGGCTG | CGTGCTTATACCTCCTCCATGTT |
| C3_11509117 | Allele X: CAAAGAACCCCTTCCTGAGGCTG | GTGCTTATACCTCCTCCATGTT |
| C4P229604 | Allele X: AGGATCTAATGTCACTGGAGGACTG | GTCGCCCTTCTAGGTTGATTAGAATTTGTT |
| C4P25377913 | Allele X: AGTGGTTTTTCTATGTCCCCTTTGGA | CACAAAAGGACTGCAAAATAGGATAA |
| C4P5278891 | Allele X: GTGTTTTTCTATGTCCCCTTTGGA | CACAAAAGGACTGCAAAATAGGATAA |
| C6_15847634 | Allele X: CAGTCAGGTGCACTGGATG | GCGAACAGCTCAAGAATGCTAGAA |
| C6_310721 | Allele X: GGAATATTTTCCCCAAAAAGAAAAGTACCT | GGGTTTGACGGCCGCTTCGCA |
| C8P19129409 | Allele X: CCCAAAGCTAATACAG | GCTATTTTATGTTAGGTTAAAGGCTGTT |
| C9_12216080 | Allele X: CTGCTTTGTATATGGTTGTCAGAT | CGTTTCAGCAGCTTTCTCAAACATTT |
| C9P27534079 | Allele X: GCGAGGAGGTTCAGGCGG | CTCAGAGTTTCAGGTTGAAAGGCTGTT |
Table 2  Information about molecular markers used for genotyping diploid gametes originated by tetraploid ‘Moncada’ mandarin as male and female parent, indicating accession number in Gene Bank or Phytomeze, position in the reference clementine genetic map, noted alleles in ‘Moncada’ and reference

| Marker     | Gene bank/phytomeze accession | Male–female parent | Marker type | Linkage group | Genetic position (cM) | Distance to centromere (cM) | Alleles | References |
|------------|-------------------------------|-------------------|-------------|---------------|-----------------------|-----------------------------|---------|------------|
| mCrCIR02G08 | FR692362                              | M/F                | SSR         | 1             | 16.73                 | 43.93                       | 244–246 | Ollitrault et al. (2012a) |
| CIBE5720   | ET082224                              | M                  | SSR         | 1             | 57.76                 | 2.9                         | 329–337 | Ollitrault et al. (2010)  |
| CIC2810-01 | ET103213                                | F                  | SNP         | 1             | 63.40                 | 2.74                        | AC      | Ollitrault et al. (2012b) |
| EMA-M30    | JX600646                               | F                  | SNP         | 1             | 69.72                 | 9.06                        | CT      | Garcia-Lor et al. (2013a) |
| CIC5950-02 | ET083949                                | F                  | SNP         | 1             | 91.36                 | 30.7                        | GA      | Ollitrault et al. (2012b) |
| C1P26815936 | FR677569                            | M/F                | SSR         | 2             | 13.37                 | 43.53                       | 236–238 | Cuenca et al. (2011)      |
| mCrCIR02D09 | JK-CAC15                                | –                  | SSR         | 2             | 52.56                 | 4.34                        | 150–160 | Kijas et al. (1997)       |
| C2_23768463 | CIBE5720                                | M                  | SNP         | 3             | 90.41                 | 33.51                       | 185–189 | Froelicher et al. (2008)  |
| mCrCIR07D05 | CIC2810-01                              | F                  | SNP         | 1             | 93.92                 | 37.02                       | CA      | Ollitrault et al. (2012b) |
| JK-TAA41   | –                                      | M                  | SSR         | 2             | 160.74                | 103.84                      | 154–163 | Kijas et al. (1997)       |
| MEST256    | DY290355                                | F                  | SNP         | 3             | 17.02                 | 73.58                       | 209–225 | Garcia-Lor et al. (2012)  |
| INVA-P855  | JX630071                                | M                  | SNP         | 3             | 30.21                 | 60.39                       | CT      | Garcia-Lor et al. (2013a) |
| CIC4681-02 | ET109640                                | F                  | SNP         | 3             | 92.78                 | 2.18                        | TA      | Ollitrault et al. (2012b) |
| C3_11509117 | –                                      | M                  | SNP         | 3             | 89.58                 | 1.02                        | GC      | New                     |
| CX0124     | CN187496                                | M                  | SNP         | 3             | 110.27                | 19.67                       | 164–170 | In preparation            |
| ATMR-M728  | JX630073                                | F                  | SNP         | 3             | 141.92                | 51.32                       | GT      | Garcia-Lor et al. (2013a) |
| CHS-M183   | JX630074                                | M                  | SNP         | 3             | 167.33                | 76.73                       | GC      | Garcia-Lor et al. (2013a) |
| C4P229604  | M                                      | SNP                | 4            | 0.802         | 15.29                 | GA                          | New     |
| MEST070    | M                                      | SNP                | 4            | 4.23          | 11.87                 | 217–229                     | In preparation            |
| CHI-M598   | JX630074                                | F                  | SNP         | 4             | 11.37                 | 4.73                        | GC      | Garcia-Lor et al. (2013a) |
| C4P5278891 | M                                      | SNP                | 4            | 18.45         | 2.35                  | AG                          | New     |
| mCrCIR06A02 | AM489738                                | F                  | SSR         | 4             | 62.42                 | 46.32                       | 222–225 | Froelicher et al. (2008)  |
| C4P25377913 | –                                      | M                  | SNP         | 4             | 88.72                 | 72.62                       | AG      | New                     |
| CIC0446-01 | ET091387                                | F                  | SNP         | 4             | 77.78                 | 61.68                       | AT      | Ollitrault et al. (2012b) |
| CI03D12a   | M                                      | SNP                | 4            | 90.06         | 73.96                 | 261–281                     | Aleza et al. (2011)        |
| MEST015    | FC912829                                | M                  | SNP         | 5             | 16.21                 | 6.89                        | 174–186 | Garcia-Lor et al. (2012)  |
| CMS30      | M                                      | SNP                | 5            | 31.35         | 8.25                  | 150–152                     | Ahmad et al. (2003)        |
| MEST104    | DY273697                                | F                  | SNP         | 5             | 34.95                 | 11.85                       | 236–238 | Garcia-Lor et al. (2012)  |
| CIC5842-02 | ET083106                                | F                  | SNP         | 5             | 71.8                  | 48.7                        | AC      | Ollitrault et al. (2012b) |
| mCrCIR07E12 | AM489750                                | M                  | SNP         | 5             | 95.43                 | 72.33                       | 138–142 | Froelicher et al. (2008)  |
| CIC2417-04 | ET101382                                | F                  | SNP         | 5             | 103.36                | 80.26                       | TA      | Ollitrault et al. (2012b) |
| C6_310721  | ET01372                                 | F                  | SNP         | 6             | 0.32                  | 5.88                        | TC      | New                     |
| CIC2414-01 | ET01372                                 | F                  | SNP         | 6             | 8.11                  | 1.91                        | AG      | Garcia-Lor et al. (2013a) |
| C6_15847634 | M                                      | SNP                | 6            | 15.38         | 9.18                  | GT                          | New     |
| LAPX-M238  | JX630079                                | M/F                 | SNP         | 6             | 19.16                 | 12.96                       | GC      | Garcia-Lor et al. (2013a) |
| CI02F12    | FR677570                                | F                  | SNP         | 6             | 60.84                 | 54.64                       | 122–130 | Cuenca et al. (2011)      |
| AOC-M290   | JX630081                                | F                  | SNP         | 6             | 85.88                 | 79.68                       | TC      | Garcia-Lor et al. (2013a) |
| MEST123    | DY276100                                | M                  | SNP         | 6             | 91.87                 | 85.67                       | 252–260 | Aleza et al. (2011)       |
| MEST107    | DY274062                                | F                  | SNP         | 7             | 8.89                  | 87.51                       | 176–184 | Cuenca et al. (2011)      |
| FLS-M400   | JX630083                                | M                  | SNP         | 7             | 45.99                 | 50.41                       | CT      | Garcia-Lor et al. (2013a) |
| mCrCIR03B07 | FR677573                                | M/F                 | SSR         | 7             | 83.39                 | 13.01                       | 261–265 | Cuenca et al. (2011)      |
| CI07C07    | AJ567409                                | M/F                 | SSR         | 7             | 98.01                 | 1.61                        | 227–234 | Froelicher et al. (2008)  |
| mCrCIR01F04a | AM489736                               | M/F                 | SSR         | 8             | 5.91                  | 48.29                       | 188–210 | Froelicher et al. (2008)  |
| CIC1208-01 | ET070547                                | F                  | SNP         | 8             | 33.17                 | 21.03                       | AG      | Ollitrault et al. (2012b) |
sized based on a DNA size standard (400 bp). GenomeLab™ v.10.0 (Beckman Coulter Inc.) genetic analysis software was used for data collection.

SNP markers were genotyped using KASPar™ technology by LGC Genomics (Hoddesdon, UK). The KASPar™ genotyping system is a competitive, allele-specific dual Förster resonance energy transfer (FRET)-based assay for SNP genotyping. Primers were directly designed by LGC Genomics based on the SNP locus flanking sequence. Detailed explanation of the specific conditions and reagents used in KASPar™ technique can be found in Cuppen (2007). The allelic dose estimation in the heterozygous triploid hybrids was performed as described by Cuenca et al. (2013).

### Data analysis

#### Inferring the diploid gamete genetic configuration

In interploid crosses leading to triploid progenies, diploid gametes are transmitted from the tetraploid parent (Aleza et al. 2012a, b). For loci with completely different parental allelic configurations (A₁A₂ × A₃A₄), the genotype of the 2x gamete can be read directly from the configuration of triallelic triploid hybrids. When the female and male parents share one allele (A₁A₂ × A₂A₃ or A₁A₂ × A₃A₄), we inferred the structure of the 2x gamete forming biallelic triploid hybrids from the allelic dose, as described by Cuenca et al. (2011, 2013). We confirmed that all triploid hybrids were formed through the fusion of a diploid gamete from the tetraploid parent and a haploid gamete from the diploid parent by observing triallelic configuration in the hybrids for at least one marker or from dosage estimation.

### Parental heterozygosity restitution (PHR)

The PHR was calculated for each locus as the percentage of triploid individuals with the heterozygous allelic configuration inherited from tetraploid ‘Moncada’ mandarin transmitted through diploid gametes. Similarly, PHR was calculated for each individual as the percentage of loci with the same heterozygous allelic configuration as tetraploid ‘Moncada’ mandarin.

### Estimation of preferential association frequency and maximum double reduction rate

For citrus, Stift et al. (2008) proposed a segregation model for allotetraploids, which was simplified by Aleza et al. (2016) for tetraploid resulting from somatic chromosome doubling. It is considered that in such tetraploid, for centromeric loci, the expected frequencies of each type of gamete depend only on the ‘tetrasomic’ parameter (τ), corresponding to the proportion of gametes formed by random associations of meiotic chromosomes (i.e., random bivalent or tetravalent pairing). The estimation of τ was performed using a maximum likelihood approach from the analysis of the marker closest to the centromere for each LG, as proposed by Aleza et al. (2016). This value ranges from 0 for completely disomic to 1 for complete tetrasomic inheritance. Confidence intervals (CIs) were estimated following a similar approach to the LOD drop-off method (Lander and Botstein 1989), by finding the values at either side of the estimated τ that corresponded to a tenfold decrease in probability. Then, preferential pairing (PP) was calculated as 1 − τ.

The double reduction rate (DR) and its confidence interval (CI) for each LG were estimated as proposed by Aleza et al. (2016). Briefly, DR is estimated from τ values for each LG for the markers furthest from the centromere applying a maximum likelihood approach, and the

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**Table 2 (continued)**

| Marker | Gene bank/phytozome accession | Male–female parent | Marker type | Linkage group | Genetic position (cM) | Distance to centromere (cM) | Alleles | References |
|--------|-----------------------------|-------------------|-------------|---------------|----------------------|-----------------------------|--------|-----------|
| mCrCIRO7B05 | AM489747 | F | SSR | 8 | 57.78 | 3.58 | 203–209 | Froelicher et al. (2008) |
| C8P19129409 | M | SNP | 8 | 77.07 | 22.87 | CG | New |
| mCrCIRO2C09 | FR692359 | F | SSR | 8 | 95.32 | 41.12 | 248–255 | Ollitrault et al. (2012b) |
| mCrCIRO2A09 | FR677568 | M | SSR | 8 | 98.18 | 43.98 | 152–162 | Cuenca et al. (2011) |
| CIC5087-01 | ET111514 | F | SNP | 9 | 15.88 | 36.32 | TA | Ollitrault et al. (2012b) |
| C9_12216080 | M/F | SNP | 9 | 23.58 | 28.62 | AG | New |
| mCrCIRO7F11 | FR677567 | M/F | SSR | 9 | 49.47 | 2.73 | 146–160 | Kamiri et al. (2011) |
| C9P27534079 | M | SNP | 9 | 59.04 | 5.84 | AG | New |

SSR simple sequence repeat, SNP single nucleotide polymorphism, M male parent, F female parent
CI corresponds to the values on each side with a tenfold decrease in the probability.

Population diversity organization

Genetic differences between individuals were estimated using the DARwin6 software (Perrier and Jacquemound-Collet 2018) and analyzed with a neighbor-joining analysis using the simple matching dissimilarity index:

\[ d_{i,j} = 1 - \frac{1}{L} \sum_{l=1}^{L} \frac{m_{i,l}}{\pi}, \]

where \( d_{i,j} \) is the dissimilarity between units \( i \) and \( j \), \( L \) is the number of \( loci \), \( m_{i,l} \) is the number of matching alleles for \( locus \ l \), and \( \pi \) is the ploidy. From the dissimilarity matrix obtained, a weighted neighbor-joining tree (Saitou and Nei 1987) was computed.

The potential distortion in allelic segregation was analyzed using Chi-square test (\( \chi^2 \)) with the Bonferroni correction for multiple testing applied (Bonferroni 1936; Goeman and Solari 2014; Holm 1979).

For group differentiation between the analyzed triploid hybrids of each progeny, the \( G/N \) relation was used, where \( G \) is the number of groups differentiated by the molecular markers used within each LG, and \( N \) is the total number of genotypes. The groups were obtained with the DARwin6 software (Perrier and Jacquemound-Collet 2018).

Results and discussion

Triploid genotyping

The genotyping of the triploid progenies was performed with 36 markers for MA and 31 for CM hybridizations, which allowed the unequivocal allelic differentiation between both parents and the determination of the origin of the diploid gametes that gave rise to each triploid hybrid.

Triallelic configurations with two alleles arising from triploid ‘Moncada’ were observed for all hybrids from MA for at least one SSR marker, directly confirming that the \( 2\times \) gametes came from the triploid ‘Moncada’ progenitor. However, for CM hybrids, all molecular markers showed biallelic configurations, and the allele dosages were estimated as proposed by Cuenca et al. (2015). Finally, all triploid hybrids in both families were confirmed to arise from the fusion of a diploid gamete from triploid ‘Moncada’ and a haploid gamete from the diploid genitor (Fig. 1). Once the origin of the \( 2\times \) gametes was confirmed, their genetic configurations were inferred for all marker-gamete combinations (Supplementary Table 1). An example for assessing genetic configuration from the direct observation of triallelic hybrids and the dosage estimation the peak ratio from a triallelic hybrid for the CI01F04a SSR marker is given in Fig. 1. In this case, tetraploid ‘Moncada’ shows 186/210 alleles (Fig. 1a) and ‘Anana’ 199/201 alleles (Fig. 1b). Hybrid ‘MA14’ shows 186/199/210 allele configuration (Fig. 1c), thus allows directly inferring 186/210 configuration for the \( 2\times \) gamete from tetraploid ‘Moncada’ (heterozygosity restitution). In contrast, the hybrid ‘MA50’ for the same marker shows 199/210 allelic configuration (Fig. 1d), and therefore, the allelic dose estimation was done considering the relationship between the alleles 199/210 of the triallelic triploid hybrid as a baseline. It was concluded a 199/210:210 genotype for ‘MA50’ and consequently 210/210 genotypes for the \( 2\times \) gamete from tetraploid ‘Moncada’ (no heterozygosity restitution).

The potential distortion in allelic segregation for the two types of homozygous gametes was analyzed using Chi-square test (\( \chi^2 \)) with the Bonferroni correction for multiple testing applied. Only the marker MEST256 in LG3 for the MA population (Table 3) and the markers CHS-M183, MEST123 and FLS-M400 in LG3, LG6, and LG7, respectively, for the CM population presented distortion in allelic segregations (Table 4).

Other citrus studies showed segregation distortions. Bernet et al. (2010) analyzed reciprocal crosses between ‘Fortune’ mandarin and ‘Chandler’ pummelo, obtaining progenies with allelic frequencies distorted in both populations. In the same way, Ollitrault et al. (2012a) observed segregation distortions in male and female gametes of ‘Clemenules’ clementine. In both studies, distortions were higher for the male gametes and the authors suggested that general factors such as mechanisms of gamete abortion, pollen competition, or gametophytic incompatibility could be related with them (Bernet et al. 2010; Ollitrault et al. 2012a).

Genetic structure of diploid gamete populations arising from tetraploid ‘Moncada’ mandarin as female and male parent

Variability of PHR

The PHR obtained from tetraploid ‘Moncada’ as male and female parent was calculated at gamete and marker level. At the gamete level, PHR presented a unimodal distribution when tetraploid ‘Moncada’ was used as female parent (Fig. 2), with a PHR average of 0.654 ± 0.093. The unimodal distribution observed in tetraploid ‘Moncada’ as female parent was similarly observed for tetraploid ‘Clemenules’ clementine analyzed by Aleza et al. (2016). In contrast, a more heterogeneous distribution was observed when used as male parent, displaying 14 diploid gametes (‘CM19’, ‘CM21’, ‘CM25’, ‘CM48’, ‘CM54’, ‘CM55’, ‘CM60’, ‘CM73’, ‘CM74’, ‘CM75’, ‘CM78’, ‘CM83’, ‘CM85’, and
‘CM86’) with very low PHR values, ranging from 0.10 to 0.40. Therefore, the average of PHR was a little bit lower (0.599 ± 0.085) (Fig. 2). At marker level, both populations displayed a unimodal distribution of PHR, although the diploid male gamete population showed lower PHR values, probably originated by the diploid male gametes with low PHR values (Fig. 3).

MA produced 2× gametes with PHR values ranging from 0.528 for the CIC5842-02 SNP locus in LG5 to 0.833 for the CHI-M598 SNP locus in LG4 (Table 3). For the remaining LGs, PHR values remain mostly constant along the chromosome. On the other hand, CM produced 2× gametes with PHR values ranging from 0.432 for the TAA41 SSR locus in LG2 to 0.761 for the C6_1584763 SNP locus in LG6 (Table 4).

Comparing tetraploid ‘Moncada’ as female and male parent, the largest differences are found in LG 4 and 8. As female parent, PHR values were 0.794 ± 0.31 for LG4 and 0.74 ± 0.06 for LG 8; as male parent, PHR values were 0.614 ± 0.091 and 0.614 ± 0.041 for LG4 and 8, respectively (Table 5).

Genotypic variability

The genetic structure of these two populations was calculated by a neighbor-joining analysis (Fig. 4), allowing the differentiation of hybrid groups within each family and determine their genetic distance. The molecular markers used in this work made possible the differentiation of all triploid hybrids within each progeny (G/N = 1) (Table 5). The average genetic distance between gametes was slightly higher for CM (0.308 ± 0.0029) than for MA (0.278 ± 0.0027). In addition, the genetic structure of the MA population gametes is more homogeneous and compact than that obtained for the CM population. Comparing the genetic distances of both population gametes in relation to the tetraploid ‘Moncada’, CM displayed a genetic distance of 0.200 ± 0.093, whereas for MA, this distance was 0.173 ± 0.054. The results found for tetraploid ‘Moncada’, as male and female parent are consistent with those described by Aleza et al. (2016), which found a genetic distance value to tetraploid ‘Clemenules’ clementine of 0.176 ± 0.012 for the population of triploid hybrids obtained with this genotype as female parent. Nevertheless, in the CM gamete population, a group with higher genetic distance to the tetraploid ‘Moncada’ (0.362 ± 0.043) was observed (Fig. 4). This subpopulation is constituted by the same 14 diploid gametes described above with very low PHR. The genetic analysis performed in these
hybrids reveals the same allelic homozygosity configuration in nine (CIBE5720, C2_23768463, TAA41, CHSM183, C4P5278891, C4P25377913, Ci03D12a, Ci03B07, and C8P19129409) over the 31 molecular markers used, and also with two other SSR markers (MEST123 and Ci07D05) with the same homozygosity configuration except for only one diploid gamete. These molecular markers are located in all LGs, with the exception of LG9, and in the LG2 and LG6, three over the four markers analyzed in each LG, displayed the same allelic configuration in homozygosity.

Preferential pairing (PP) and maximum double reduction (DR)

The genome of many cultivated citrus is composed of mosaics of the ancestral species (Curk et al. 2014, 2015; Wu et al. 2014, 2018). The works carried out on citrus phylogeny (Oueslati et al. 2017; Wu et al. 2014, 2018) have shown that the genomes of the progenitors that gave rise to ‘Moncada’ mandarin (‘Oroval’ clementine (C. deliciosa × C. sinensis) and ‘Kara’ mandarin (C. unshiu × C. nobilis) are constituted by an interspecific mandarin/pummelo mosaic structure; therefore, ‘Moncada’ mandarin also has an interspecific structure in its chromosomes.

and PP were calculated for each LG from the segregation data of the markers closest to the centromere using

### Table 3

| Locus     | LG | Location | PHR  | Chi square | P value |
|-----------|----|----------|------|------------|---------|
| CI02G08   | 1  | 16.73    | 0.611| 1.2857     | 0.257   |
| CIC2810-01| 1  | 63.40    | 0.569| 0.0323     | 0.857   |
| EMA-M30   | 1  | 69.72    | 0.569| 0.0323     | 0.857   |
| CIC5950   | 1  | 91.36    | 0.556| 0.5000     | 0.480   |
| Ci02D09   | 2  | 13.37    | 0.542| 0.7576     | 0.384   |
| CAC15     | 2  | 52.56    | 0.694| 0.1818     | 0.760   |
| C2_23768463| 2 | 81.04    | 0.681| 0.0435     | 0.835   |
| CIC3712-01| 2  | 93.92    | 0.542| 0.7576     | 0.384   |

### Table 4

| Locus     | LG | Location | PHR  | Chi square | P value |
|-----------|----|----------|------|------------|---------|
| CI02G08   | 1  | 16.73    | 0.614| 2.941      | 0.086   |
| CIBE5720  | 1  | 63.40    | 0.576| 9.524      | 0.000   |
| CI02D09   | 2  | 13.37    | 0.557| 3.930      | 0.047   |
| MEST123   | 2  | 90.41    | 0.511| 3.930      | 0.047   |
| TAA41     | 2  | 83.39    | 0.586| 1.000      | 0.317   |

Preferential pairing (PP) and maximum double reduction (DR)

The genome of many cultivated citrus is composed of mosaics of the ancestral species (Curk et al. 2014, 2015; Wu et al. 2014, 2018). The works carried out on citrus phylogeny (Oueslati et al. 2017; Wu et al. 2014, 2018) have shown that the genomes of the progenitors that gave rise to ‘Moncada’ mandarin (‘Oroval’ clementine (C. deliciosa × C. sinensis) and ‘Kara’ mandarin (C. unshiu × C. nobilis) are constituted by an interspecific mandarin/pummelo mosaic structure; therefore, ‘Moncada’ mandarin also has an interspecific structure in its chromosomes.

and PP were calculated for each LG from the segregation data of the markers closest to the centromere using
the probability models (Aleza et al. 2016). These markers were located between 1.0 and 24.1 cM from the centromere. For tetraploid ‘Moncada’ as female parent (Table 6), complete tetrasomic inheritance was the best model for seven out of the nine LGs (LG1, LG2, LG3, LG5, LG6, LG7, and LG9). For LG8, an intermediate inheritance with tendency towards a tetrasomic inheritance (PP = 0.375) was estimated, while the LG4 evidenced a clear intermediate inheritance (PP = 0.5). For tetraploid ‘Moncada’ as male parent (Table 7), most of the chromosomes fit the tetrasomic inheritance model with the markers used, with PP = 0 for LG1, LG2, LG3, LG4, LG7, LG8, and LG9, while LG5 and LG6 showed values that fit an intermediate inheritance model with tetrasomic tendency (PP = 0.215 and 0.115, respectively).

Likewise, clementines also present an interspecific mandarin/pummelo structure (Wu et al. 2018) Aleza et al. (2016) studied the segregation model in tetraploid ‘Clemenules’ clementine as female parent, obtaining very similar results, as we report for the tetraploid ‘Moncada’ mandarin, generally fitting the tetrasomic inheritance model except for LG4, which fitted the intermediate inheritance model. However, they also reported that the LG6 and LG8 showed values that fit the intermediate inheritance model, with high tetrasomic tendency. Comparatively, we found that for ‘Moncada’ mandarin as female parent, the LG6 shows tetrasomic segregation, while results for the LG8 agree with the after as was reported for the tetraploid ‘Clemenules’ clementine, but with higher PP value. Subsequently, Rouiss et al. (2018) analyzed the segregation
model of the tetraploid ‘Mexican’ lime (C. aurantiifolia), which originated from an interspecific hybridization between C. micrantha (papeda) and C. medica (Citron) (Curk et al. 2016; Nicolosi et al. 2000; Wu et al. 2018). The results showed that tetraploid ‘Mexican’ lime has intermediary inheritance with a preferential disomic trend. In addition, Kamiri et al. (2018) assessed the meiotic behavior of an intergeneric tetraploid somatic hybrid resulting from symmetric protoplast fusion of diploid C. reticulata and diploid Poncirus trifoliata, and observed an intermediate inheritance with a preferential disomic trend. On the other hand, the genotyping of the triploid progeny derived from a cross between diploid pummelo (C. maxima) and an allotetraploid intergeneric somatic hybrid between C. reticulata and C. limon showed a tetrasomic and intermediate inheritance for this citrus interspecific somatic hybrid (Kamiri et al. 2011). Altogether, these studies reveal that the preferential pairing of tetraploid citrus genotypes greatly varies in relation to their constitutive genomes. The differentiation between C. medica and C. micrantha as well as the one between C. reticulata and P. trifoliata seems to have a much more impact in preferential pairing than the one between C. maxima and C. reticulata. Tetraploid ‘Moncada’ differs slightly in the segregation model when used as female or male parent. These sex-specific differences were also observed.
### Table 6: Estimation of Preferential Pairing (PP) and Double Reduction (DR) rate for tetraploid ‘Moncada’ mandarin as female parent for markers located close and far from the centromere within each of the nine LGs

| LG | Locus        | DC (cM) | $A_1A_1$ | $A_1A_2$ | $A_2A_2$ | $\tau$ | CI      | PP       | CI      | DR       | CI      |
|----|--------------|---------|----------|----------|----------|--------|---------|---------|---------|----------|---------|
| 1  | CIC2810-01   | 2.7     | 16       | 41       | 15       | 1      | 1–0.845 | 0       | 0–0.165 | 0.083    | 0–0.273 |
| 1  | CI02G08      | 43.9    | 11       | 44       | 17       | 0      | 0.000   | 0       | 0–0.195 | 0.251    | 0.05–0.458 |
| 2  | CAC15        | 4.3     | 10       | 50       | 12       | 0.915  | 1–0.595 | 0.085   | 0–0.195 | 0.085    | 0.05–0.458 |
| 2  | TAA41        | 103.8   | 24       | 39       | 9        | 0.085  | 0.251   | 0.05–0.458 |
| 3  | C3_11509117  | 1.0     | 16       | 49       | 7        | 0.96   | 1–0.815 | 0.04    | 0–0.185 | 0.216    | 0.025–0.413 |
| 3  | MEST256      | 73.6    | 30       | 39       | 3        | 0.04   | 0.216   | 0.025–0.413 |
| 4  | CHI–M598     | 4.7     | 6        | 60       | 6        | 0.5    | 0.790–0.260 | 0.5      | 0.180–0.740 |
| 4  | CI03D12a     | 74.0    | 8        | 57       | 7        | 0.5    | 0.125   | 0–0.467 |
| 5  | MEST104      | 11.9    | 14       | 44       | 14       | 1      | 1–0.785 | 0       | 0–0.215 | 0.063    | 0–0.251 |
| 5  | CIC2417-04   | 80.3    | 12       | 45       | 15       | 0      | 0.125   | 0–0.467 |
| 6  | CIC2414-01   | 1.9     | 13       | 45       | 14       | 1      | 1–0.805 | 0       | 0–0.195 | 0.021    | 0–0.208 |
| 6  | AOC-M290     | 79.7    | 10       | 47       | 15       | 0      | 0.140   | 0–0.467 |
| 7  | CI07C07      | 1.6     | 14       | 46       | 12       | 1      | 1–0.735 | 0       | 0–0.265 | 0.021    | 0–0.208 |
| 7  | MEST107      | 87.5    | 11       | 43       | 18       | 0      | 0.104   | 0–0.293 |
| 8  | CIC1208-01   | 3.58    | 7        | 57       | 8        | 0.625  | 0.965–0.355 | 0.375   | 0.035–0.645 |
| 8  | CI01F04a     | 48.3    | 8        | 55       | 9        | 0.375  | 0.067   | 0–0.347 |
| 9  | CI07F11      | 2.7     | 13       | 42       | 17       | 1      | 1–0.820 | 0       | 0–0.180 | 0.167    | 0–0.356 |
| 9  | CIC5087-01   | 36.3    | 13       | 40       | 19       | 0      | 0.165   | 0–0.336 |

Allelic configurations for the loci used to estimate DR have been highlighted in italics.

LG linkage group, DC distance to the centromere in cM [derived from reference genetic map data (Ollitrault et al. 2012a) and location of centromere (Aleza et al. 2015)], $A_1A_1$ number of individuals with that allelic configuration, $\tau$ tetrasomic rate, CI confidence interval, PP preferential pairing, DR double reduction rate.

### Table 7: Estimation of Preferential Pairing (PP) and Double Reduction (DR) rate for tetraploid ‘Moncada’ mandarin as male parent for markers located close and far from the centromere within each of the nine LGs

| LG | Locus | DC (cM) | $A_1A_1$ | $A_1A_2$ | $A_2A_2$ | $\tau$ | CI       | PP   | CI       | DR    | CI       |
|----|-------|---------|----------|----------|----------|--------|---------|------|---------|-------|---------|
| 1  | CIBE5720 | 2.9     | 11       | 46       | 31       | 1      | 1–0.895 | 0    | 0–0.105 |       |         |
| 1  | CI02P26815936 | 56.9 | 14 | 56 | 18 | 0 | 0.045 | 0–0.216 |
| 2  | C2_23768463 | 24.1 | 11 | 46 | 31 | 1 | 1–0.895 | 0 | 0–0.105 |       |         |
| 2  | TAA41 | 103.8 | 26 | 38 | 24 | 0 | 0.352 | 0.181–0.518 |
| 3  | CIC1208-01 | 3.58 | 7 | 57 | 8 | 0.625 | 0.965–0.355 | 0.375 | 0.035–0.645 |
| 3  | CI01F04a | 48.3 | 8 | 55 | 9 | 0.375 | 0.067 | 0–0.347 |
| 4  | CI07C07 | 1.6 | 14 | 46 | 12 | 1 | 1–0.735 | 0 | 0–0.265 |       |         |
| 4  | MEST104 | 11.9 | 14 | 44 | 14 | 1 | 1–0.785 | 0 | 0–0.215 |       |         |
| 5  | CIC2417-04 | 80.3 | 12 | 45 | 15 | 0 | 0.063 | 0–0.251 |
| 5  | CIC2414-01 | 1.9 | 13 | 45 | 14 | 1 | 1–0.805 | 0 | 0–0.195 |       |         |
| 6  | AOC-M290 | 79.7 | 10 | 47 | 15 | 0 | 0.140 | 0–0.467 |
| 7  | CI07F11 | 2.7 | 13 | 42 | 17 | 1 | 1–0.820 | 0 | 0–0.180 |       |         |
| 7  | CIC5087-01 | 36.3 | 13 | 40 | 19 | 0 | 0.165 | 0–0.336 |

Allelic configurations for the loci used to estimate DR have been highlighted in italics.

LG linkage group, DC distance to the centromere in cM [derived from reference genetic map data (Ollitrault et al. 2012a) and location of centromere (Aleza et al. 2015)], $A_1A_1$ number of individuals with that allelic configuration, $\tau$ tetrasomic rate, CI confidence interval, PP preferential pairing, DR double reduction rate.
for salmon fish (Allendorf and Danzmann 1997). Disomic segregation was observed in females, while segregation in males was best explained by a mixture of disomic and tetrasomic inheritance.

The tetraploid ‘Moncada’ as female parent showed significant values of DR in LG2, LG3, LG4, LG7, and LG9. For all LGs, the confidence intervals (CI) for DR values include the value of 1/6, considered as the maximum value of DR for tetrasomic segregation and one crossover event occurring between the marker and the corresponding centromere (Haynes and Douches 1993; Mather 1936; Bourke et al. 2015), although LGs 2 and 3 displayed a higher estimation of DR. When tetraploid ‘Moncada’ was used as male parent, significant values of DR were obtained for LG2, LG3, LG4, LG6, and LG7. For LG3, LG4, LG6, and LG7, the confidence intervals (CI) for DR values include the maximum value of DR under the hypothesis described above. In addition, LG2 and LG5 showed higher DR values. Tetraploid ‘Moncada’ shows the same trend as female and male parent in DR values for LG1, LG2, LG3, LG4, LG7, and LG8. The frequency of DR considers maximum values of 0 for random chromosome segregation hypothesis, 1/7 with pure random chromatid segregation hypothesis, and 1/6 with complete equalional segregation (Mather 1935; Muller 1914). Estimated values over 1/6 should be due to the segregation distortion observed for the corresponding markers. Indeed, our model analysis is based on Mendelian segregation hypothesis, while negative sporophytic selection for dominant gene may induce a diminution of heterozygous frequencies (for the gene and linked markers) and results in overestimation of DR. Different works have been performed with the objective to estimate the DR frequency and these values have been ranged from 0 to almost 0.30 (Fisher 1947, 1950; Haynes and Douches 1993; Tai 1982a, b; Welch 1960; Wu et al. 2001). The values of DR rate can differ between loci according the tetrasomic inheritance model. This variability depends on both the chromosome in which the marker is located and the position of the marker within the chromosome. There are chromosomes with a greater tendency to form multivalent that would originate higher values of DR (Butruille and Boiteux 2000). In addition, DR could be better estimated using larger populations (Butruille and Boiteux 2000) and it is more probable to occur in markers located in telomeric rather than in centromeric regions, in which the probability of recombination events is close to zero (Aleza et al. 2015; Butruille and Boiteux 2000; Welch 1960). In addition, Butruille and Boiteux (2000) indicated that DR causes a decrease of the equilibrium frequencies of deleterious alleles, and it has much more influence on genes subjected to gametophytic selection than on genes solely under sporophytic selection. With gametophytic selection, low frequencies of DR are enough to reduce equilibrium frequencies several folds.

**Implications for citrus-breeding programs**

Two strategies are routinely exploited for obtaining citrus triploids, i.e., interploid hybridizations between 2x and 4x parents (Aleza et al. 2012a, b; Starrantino and Recupero 1982) and through female 2n gametes (Aleza et al. 2010; Cuenca et al. 2011, 2015). In interploid hybridizations, the tetraploid parent results usually from somatic chromosome doubling arising spontaneously in nucellar cells or induced by colchicine treatment. The study of the origin of the diploid gametes, which greatly influences the structure of the resulting triploid hybrid populations, is of great interest to select the most appropriate strategies to obtain new hybrids with desired characteristics. Cuenca et al. (2015) demonstrated that SDR mechanism gives rise to the 2n megagametophytes in diploid ‘Moncada’ mandarin. The use of this strategy produces hybrid progenies with large genetic variation, due to the relatively low transmission of the parental heterozygosity to the offspring (about 40% on average), thus resulting in high number of new allelic multilocus combinations. In this paper, we have analyzed the chromosome segregation in the tetraploid ‘Moncada’ mandarin, which showed predominantly tetrasomic segregation, when used both as female and male parent, with an average PHR of 65% when used as female and 60% as male parent. Moreover, PHR is relatively constant along the chromosomes. Therefore, if we compared with SDR-2n female gametes, interploid hybridizations with tetraploid ‘Moncada’ mandarin as tetraploid parent are potentially a more efficient strategy for the development of new varieties that are genotypically more similar to the ‘Moncada’ mandarin.

Furthermore, depending on the LG in which a gene controlling an eventual trait of interest is located, the genetic regulation of the trait and the direction of the crossing, different segregation in the offspring can be obtained. For example, the PHR in LG8 is higher when tetraploid Moncada is used as female than as male parent, and therefore, the progeny will show higher heterogeneity in this LG when using tetraploid Moncada as male parent. Considering a trait of interest controlled by a single dominant allele at a locus in LG8, the probability to obtain triploid hybrids that inherit the trait of interest is higher using tetraploid ‘Moncada’ as female parent.

Tetraploid ‘Moncada’ mandarin displayed significant values of DR as male and female parent. DR results in a decrease of PHR and thus an increase of inbreeding (Haynes and Douches 1993). The production of higher levels of homozygosity could be useful in triploid mandarin breeding for the potential cleaning effect that DR can have by revealing deleterious alleles to selection (Butruille and Boiteux 2000; Bourke et al. 2015). DR also could increase the accumulation of rare but favorable allelic configurations through selection with molecular markers (Bourke et al. 2015).
The knowledge of the difference in segregations according to the crossing strategy (2n gametes or interploid hybridization) to obtain hybrid triploid progenies with the ‘Moncada’ mandarin opens a range of possibilities for designing efficient breeding programs aimed to obtain innovative products to fulfill the market demands.

Conclusions

The analysis of codominant marker segregation over the nine citrus chromosomes allowed to unravel the segregation pattern of the tetraploid ‘Moncada’. Using both as female and male parent, it displayed tetrasomic inheritance for most LGs, with slight variations according to the direction of the crossing. As female parent, LG8 showed intermediate inheritance with tendency towards tetrasomic inheritance, and LG4 evidenced clear intermediate inheritance. As male parent, LG5 and LG6 showed values that fit an intermediate inheritance model with tetrasomic tendency. Significant DR rates were found in LG2, LG3, LG4, LG7, and LG9 when using tetraploid Moncada as female parent and in LG2, LG3, LG4, LG5, LG6, and LG7 as male parent. Likewise, differences in PHR were found between tetraploid ‘Moncada’ as female parent and male parent, with higher values in LG 4 and LG 8 as female parent. The new knowledge generated here will serve to define crossing strategies in citrus improvement programs to efficiently obtain new varieties of interest in the global fresh consumption market.

Author contribution statement

PO and PA conceived and designed the experiments. MG performed the experiments. MG, AGL, NO, JC, and PA analyzed the data. PO provided a statistical method for the estimation of PP and maximum DR and new SNP markers from GBS data analysis. MG, JC, and PA wrote the manuscript with input and review of LN and PO. All authors read and approved the final version of this manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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