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Article

Genomic Instability in Somatic Hybridization between *Poncirus* and *Citrus* Species Aiming to Create New Rootstocks

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Abstract: Rootstocks are an important component for citrus adaptation to increasing biotic and abiotic stresses resulting from global climate change. There is a strong complementarity between *Citrus* species, which adapt to abiotic stresses, and *Poncirus trifoliata* and its intergeneric hybrids, which exhibit resistances or tolerances to major diseases and pests. Thus, symmetrical somatic hybridization between complementary diploid rootstocks of these two genera appears to be an efficient way to develop new tetraploid rootstocks in order to address the new challenges of the citrus industry. New intergeneric somatic hybrids were obtained by electrofusion between protoplasts of *Citrus* and *P. trifoliata* hybrids. Extensive characterization of the nuclear and cytoplasmic genomes was performed by genotyping-by-sequencing (GBS) analysis. This revealed diploid cybrids and nuclear somatic hybrids. Mitochondrial genomes were mostly inherited from the callus parent, but homologous recombination events were observed for one parental combination. Chloroplasts exhibited random uniparental inheritance. GBS revealed local chromosomal instabilities for all nuclear somatic hybrids and whole chromosome eliminations for two hybrids. However, at the whole genome level, symmetrical addition of the nuclear genomes of both parents was predominant and all somatic hybrids displayed at least one trifoliolate orange haplotype throughout the genome.

Keywords: protoplast fusion; somatic embryogenesis; genotyping by sequencing; somaclonal variation; *Citrus sinensis*; *Citrus reticulata*; *Poncirus trifoliata*; citrange; citrumelo; citrandarin



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1. Introduction

Global climate change and environmental instability over seasons and years are significantly affecting agricultural production, particularly of woody crops [1]. Water deficits and salinity have become increasingly frequent in the main citrus production areas. In addition, tropical and subtropical pests, diseases, and vectors have become major threats to regions such as the Mediterranean Basin. This is particularly the case for huanglongbing disease (HLB), the main constraint in the citrus industry worldwide caused by three species of *Candidatus Liberibacter* transmitted by psyllid and creating strong physiological disorder in the canopy and roots [2–4]. The African vector *Trypoxys erythrae* is now present in the Iberian Peninsula.

Genetic resistance and adaptation are crucial components in sustainable cropping systems that can cope with changing environments and increasing stresses. For citrus, rootstock is a critical element for environmental adaptation and disease resistance. Phytophthora, citrus tristeza virus (CTV), and nematodes, which are present worldwide, are

among the disease and pest problems that can be solved with rootstock. The widespread use of citrus grafting was prompted by the world phytophthora crisis in the late 19th century [5]. Sour orange (*C. × aurantium* L.) was the most widely used rootstock until the emergence of a second major disease outbreak caused by CTV that killed millions of trees grafted onto sour orange worldwide in the mid-20th century. This second crisis drove a major diversification of the genetic base of citrus rootstocks, particularly the use of trifoliolate orange (*Poncirus trifoliata* (L.) Raf.), due to its immunity to CTV [6] and its good levels of resistance to phytophthora and nematodes. Furthermore, some authors have described various levels of resistance to HLB [7–10] and its vector *Diaphorina citri* [11] in *P. trifoliata*. Seven quantitative trait loci (QTLs) of tolerance to HLB were recently identified [12] and located in the *P. trifoliata* genome [13]. However, the determinism and consistence of the resistance/tolerance of trifoliolate orange to HLB is still debated [14]. Trifoliolate oranges are sensitive to iron chlorosis in alkaline soils as well as to salinity, which limits their use in some areas, especially in the Mediterranean Basin [15], while some rootstocks of the *Citrus* genus, including Rangpur lime, Cleopatra mandarin, and Alemow, are well known for their better adaptation to abiotic stresses [16]. Intergeneric hybridization between *Citrus* and *Poncirus* species has enabled significant progress in rootstock selection using hybrids such as Swingle citrumelo (*C. × paradisi* Macfad. × *P. trifoliata*); Carrizo, Troyer, and C35 citranges (*C. × sinensis* (L.) Osbeck × *P. trifoliata*); and several citrandarins (*C. reticulata* Blanco × *P. trifoliata*).

In addition to adaptation and disease resistance, rootstock also drives tree vigor and yield, early fruiting, and some quality attributes. Rootstock breeding therefore requires combining a large number of useful traits, while the cumbersome nature of evaluating the different traits severely limits the number of hybrids that can be evaluated. Under these circumstances, symmetrical somatic hybridization is considered a good breeding strategy. Indeed, it is expected to allow the addition of a tetraploid hybrid without sexual recombination, with the dominant favorable traits selected at the parental level. Several research teams throughout the world have successfully applied somatic hybridization in citrus [17–27]. Interestingly, in citrus, polyploidy has been reported to improve adaptation to different types of stress and to promote resilience [28–36].

Protoplast fusion can result in different combinations of nuclear, chloroplastic, and mitochondrial genomes. In citrus, chromosome counts, flow cytometry, and several kinds of molecular markers have been used to characterize the genomes of somatic hybrids. In the case of leaf and callus protoplast fusion, most publications report inheritance of the callus mitochondria and a random uniparental inheritance for the chloroplast (Ollitrault et al. [37] for review). However, a few works have reported potential recombination of the mitochondrial genome [38–41]. For the nuclear genome, only a few markers have generally been used to confirm the hybridity of tetraploid plants. Most of the published works report that protoplast fusion between diploid parents resulted in tetraploid hybrids or diploid cybrids, combining the nuclear genome of one parent with chloroplastic and/or mitochondrial genomes of the second parent. However, some regenerated plants with an unexpected ploidy level have been reported, particularly in very wide hybridization [42]. Chromosome instability was also revealed in products of somatic hybridization between *C. macrophylla* Wester and *P. trifoliata*, in which five tetraploid plants, one pentaploid plant, one mixoploid (triploid–hexaploid) plant, and one heptaploid plant were recovered [43]. In this last study, loss of parental alleles of nuclear markers was observed in most of the regenerated plants. In tetraploids, it mainly affected *C. macrophylla* alleles, whereas *P. trifoliata* alleles were mostly lost in pentaploid and heptaploid.

Segregation of organelles [44–46] and chromosome instability after protoplast fusion have also been described in other plant species. These instabilities involved whole and partial chromosome elimination or recombination [47–50], as well as unexpected ploidy levels [51–53]. They were interpreted as the consequence of a genomic shock resulting from the merger of two highly differentiated nuclear genomes, leading to rapid and extensive alterations at the genetic and epigenetic levels [53–56]. In order to improve the efficiency of

breeding programs based on somatic hybridization at a wide taxonomic level, it is therefore essential to perform in-depth molecular characterizations of the regenerated plants and, particularly, to analyze the inheritance of the genomic regions implied in the expression of selected traits.

In this paper, we focus on somatic hybridization between *Citrus* species and *P. trifoliata* or F1 sexual intergeneric hybrids between *Citrus* and *Poncirus*, such as citranges, citrumelos, and citrandarins. The results of three new combinations of somatic hybridization are presented. These are two combinations with Cleopatra mandarin as one parent and Winter Haven citrumelo or Carrizo citrange as the other parent, and a combination between Shamouti sweet orange and a diploid cybrid (citrumello 4475 nuclear and chloroplast genomes with the mitochondrial genome of Chios mandarin [18]). Extensive genomic characterization of plants regenerated from these three combinations, as well as plants regenerated from eight other intergeneric combinations presented by Dambier et al. [18], was performed by genotyping by sequencing (GBS) using *P. trifoliata* as a reference genome [13]. The genomic regions of *P. trifoliata* implicated in resistance to pests and diseases or adaptation to abiotic stresses were located in this reference genome [13].

2. Materials and Methods

2.1. Somatic Hybridization and Plant Regeneration

Three new intergeneric combinations of somatic hybridization were performed with plant material and callus derived from the INRAE-CIRAD citrus collection (certified as Biological Resource Center (BRC) citrus NF96-600) located in San Ghjulianu, Corsica (France).

Leaf protoplasts were isolated for the Winter Haven citrumelo, the Carrizo citrange, and a citrumelo 4475 cybrid with Chios mandarin mitochondria [18], while embryogenic callus protoplasts were obtained for Cleopatra mandarin and Shamouti sweet orange. Isolation of mesophyll and callus protoplasts was done according to Grosser and Gmitter [57]. Callus protoplasts of Cleopatra mandarin were then combined with Winter Haven citrumelo and Carrizo citrange leaf protoplasts, while callus protoplast of Shamouti sweet orange were combined with leaf protoplasts of citrumelo 4475 cybrid.

For electrofusion, leaf protoplast and callus protoplast density were adjusted to 6×10^5 and 5×10^5 protoplasts/mL, respectively, in 0.8M mannitol + 0.5 mM CaCl₂ solution. Equal volumes of protoplast suspensions from both parents were mixed, and 1 mL of this mixture was poured into 55 × 10 mm Petri dishes for electrofusion. Protoplast suspensions were subjected to an AC electric field for 10 to 20s for optimal protoplast alignment. Then, three pulses (35 μs) of 220 V (DC) were emitted to induce protoplast fusion.

A six-step protocol facilitates early embryo differentiation and plant development.

Step 1: After electrofusion, 1 volume of protoplast suspension was mixed with 4.5 volumes of BH3 0.6 M medium [58] modified by Grosser and Gmitter [57]. Afterwards, 5.5 mL of this new protoplast suspension was poured into a protoplast culture medium (MT medium [59] supplemented with 0.15 M sucrose and 0.5 g malt extract and solidified by 0.22% Gelrite TM, Duchefa, Netherlands) in 90 × 15 mm Petri dishes. Protoplasts were cultivated under dark conditions for 2 weeks at 28 °C. Then, Petri dishes were transferred to a culture room with a 12 h light/day period at 28 °C.

Step 2: One month after fusion, proembryos, embryos, and cell lines were transferred to Petri dishes containing 25 mL of regeneration medium (MT medium supplemented with 0.15 M sucrose and 0.5 g malt extract and solidified by 0.3% Gelrite TM) sealed with plastic wrap.

Step 3: One month later, embryos were transferred to a maturation medium (MT galactose 0.15 M and malt extract 0.5 g/L) for further development.

Step 4: When cotyledonary embryos were obtained, they were subcultured on germinating medium (0.1 M MT sorbitol, 0.1 M galactose, 0.5 g/L malt extract, 1 μM GA₃, and 0.3% Gelrite TM).

Step 5: Germinated embryos were transferred to 90 × 15 mm Petri dishes on caulogenesis medium (MT/2, 0.1 M sorbitol, 0.1 M galactose, and 0.3% Gelrite TM).

Step 6: To enhance development, before grafting and greenhouse acclimatization, small in vitro plantlets were transferred to individual containers, with the same medium, 2 months later.

2.2. Ploidy Analysis by Flow Cytometry

Ploidy analysis was carried out using a 'Sysmex Cyflow Space' flow cytometer. Approximately 1 cm² of young leaves of each sample was mixed with the same quantity of a triploid control (Tahiti lime) in a plastic Petri dish containing 0.8 mL Cystain UV ploidy staining solution (Sysmex Partec GmbH Ref 05-5001). After being filtered (30 µm), nuclei were incubated at room temperature for 5 min. Each histogram was generated by the analysis of at least 1000 nuclei.

2.3. GBS

GBS was performed for 5 cybrids and 15 nuclear somatic hybrids resulting from 11 combinations. Their parents and five additional representative accessions of *C. reticulata*, *C. maxima* (Burm.) Merr., and *P. trifoliata* were also included in the analysis (Table 1). In cases where multiple cybrids or nuclear somatic hybrids were regenerated from the same combination of parental line, the samples were differentiated by adding a letter (a to d) at the end of the plant code.

Genomic DNA was isolated using the Plant DNAeasy kit (Qiagen) according to the manufacturer's instructions. The genomic DNA concentration of each sample was adjusted to 20 ng/µL, and ApeKI GBS libraries were prepared following the protocol described by Eslhire et al. [60]. 10 µL of each DNA sample (200 ng) was digested with the ApeKI enzyme (New England Biolabs, Hitchin, UK). Digestion took place at 75 °C for 2 h and then at 65 °C for 20 min for enzyme inactivation. The ligation reaction was completed in the same plate as the digestion, using T4 DNA ligase enzyme (New England Biolabs, Hitchin, UK) at 22 °C for 1 h. Then, the ligase was inactivated prior to pooling the samples by holding it at 65 °C for 20 min. For each library, ligated samples were pooled and PCR-amplified in a single tube. Genome complexity was reduced using PCR primers with one selective base (A) as described by Sonah et al. [61]. Single-end sequencing was performed on a single lane of an Illumina HiSeq4000 at the Genoscope facilities (Paris, France).

RAW sequencing data were cleaned with cutadapt [62] and demultiplexed with GBSX [63]. SNP genotype calling was then performed with the VCF-Hunter 2.1.0 pipeline (<https://github.com/SouthGreenPlatform/VcfHunter>; accessed on 28 July 2021) as described in Baurens et al. [64], using the *Poncirus trifoliata* v1.3.1. genome assembly (https://phytozome-next.jgi.doe.gov/info/Ptrifoliata_v1_3_1; accessed on 28 July 2021) as nuclear reference genome. NC_037463 (<https://www.ncbi.nlm.nih.gov/nuccore/1381390543>; accessed on 28 July 2021) and NC_008334 (https://www.ncbi.nlm.nih.gov/nuccore/NC_008334.1; accessed on 28 July 2021) sequences, respectively, were used as mitochondrial [65] and chloroplast [66] reference genomes. Positions with less than 10 reads were considered to be missing data. Polymorphic positions were filtered for diallelic SNPs and minor allele frequency greater than 0.05.

Clean demultiplexed sequencing data are available in the NCBI SRA (Sequence Read Archive), under accession number PRJNA648274 for Citrumello 4475 already published in Calvez et al. [67], and PRJNA794128 for the 34 others genotypes newly sequenced in the present study.

Table 1. Cybrids, nuclear somatic hybrids, parents, and ancestral taxa representatives analyzed by GBS.

| | Callus Parent | Leaf Parent | Code | Ref. |
|----------------------|-----------------------------|--------------------------------------|-----------------------------------|-------------|
| Parents | | Citrandarin | Cin_0157 | ICVN1100157 |
| | | Winter Haven Citrumelo | Clo_WiHa | ICVN0110147 |
| | | 4475 Citrumelo | Clo_4475 | SRA1112 |
| | | C35 Citrange | Cra_C35 | SRA731 |
| | | Carrizo Citrange | Cra_Carr | SRA989 |
| | | Shamouti Sweet orange | Swo_Sham | SRA299 |
| | | Cleopatra Mandarin | Man_Cleo | SRA991 |
| | | Willow leaf Mandarin | Man_Will | SRA133 |
| | | Chios Mandarin | Man_Chio | SRA598 |
| | | Pomeroy Trifoliolate orange | Pon_Pom1 | SRA1040 |
| Cybrids | Chios mandarin | 4475 Citrumelo | Cy_ManChio_Clo4475a | [18] |
| | Chios mandarin | 4475 Citrumelo | Cy_ManChio_Clo4475b | [18] |
| | Chios mandarin | 0157 Citrandarin | Cy_ManChio_Crin | [18] |
| | Chios mandarin | 4475 Citrumelo | Cy_ManChio_Clo4475c | [18] |
| | Cleopatra mandarin | Winter Haven Citrumelo | Cy_ManCleo_CloWiHa | New |
| Nuclear Hybrids | Cleopatra mandarin | C35 Citrange | HS_ManCleo_CraC35a | [18] |
| | Cleopatra mandarin | C35 Citrange | HS_ManCleo_CraC35b | [18] |
| | Cleopatra mandarin | C35 Citrange | HS_ManCleo_CraC35c | [18] |
| | Cleopatra mandarin | Winter Haven Citrumelo | HS_ManCleo_CloWiHa | New |
| | Cleopatra mandarin | Carrizo Citrange | HS_ManCleo_CraCarb | New |
| | Shamouti Sweet orange | C35 Citrange | HS_SwoSham_CraC35a | [18] |
| | Cleopatra mandarin | C35 Citrange | HS_ManCleo_CraC35d | [18] |
| | Shamouti Sweet orange | Cybrid Chios mandarin/4475 Citrumelo | HS_SwoSham_(Cy_ManChio_Clo4475) a | New |
| | Shamouti Sweet orange | Cybrid Chios mandarin/4475 Citrumelo | HS_SwoSham_(Cy_ManChio_Clo4475) b | New |
| | Shamouti Sweet orange | Cybrid Chios mandarin/4475 Citrumelo | HS_SwoSham_(Cy_ManChio_Clo4475) c | New |
| | Shamouti Sweet orange | Pomeroy Trifoliolate orange | HS_SwoSham_TriPoma | [18] |
| | Shamouti Sweet orange | Pomeroy Trifoliolate orange | HS_SwoSham_TriPomb | [18] |
| | Shamouti Sweet orange | Pomeroy Trifoliolate orange | HS_SwoSham_TriPomc | [18] |
| | Cleopatra mandarin | Pomeroy Trifoliolate orange | HS_ManCleo_TriPome | [18] |
| Willow leaf Mandarin | Pomeroy Trifoliolate orange | HS_ManWill_TriPome | [20] | |
| Additional Ancestors | Sunki Mandarin | | Man_Sunk | SRA970 |
| | Chandler Pummelo | | Pam_Chan | SRA608 |
| | Sunshine Pummelo | | Pam_Suns | SRA324 |
| | Indian Pummelo | | Pam_Inde | ICVN0101133 |
| | Pomeroy trifoliolate orange | | Pon_Pom2 | SRA996 |

2.4. Genetic Analysis

Cytoplasmic genomes of parental lines and somatic hybrids were compared by neighbor-joining analysis on a dissimilarity matrix using DARwin software version 6.0 (<https://darwin.cirad.fr/>; accessed on 28 July 2021). A factorial analysis on the dissimilarity matrix was performed for the nuclear genome with the same software.

Both analyses were based on the Manhattan dissimilarity index:

$$D_{ij} = \frac{1}{K} \sum_{k=1}^K |x_{ik} - x_{jk}|$$

where i and j are the two individuals, k is the locus, K is the total number of loci, and x_{ik} is the frequency of the alternative allele at locus k for the individual i .

For the factorial analysis of the nuclear genome, polyploid plants were treated as the diploid ones by differentiating homozygous for reference or alternative alleles and heterozygous, but without taking into account the allelic doses at heterozygous sites.

This analyses therefore provided only an approximation of the organization of diversity. However, it did clearly distinguish plants regenerated with the contribution of a single parental genome from plants with contributions of both parental nuclear genomes.

2.5. Interspecific Phylogenomic Mosaic Structures along the Nuclear Genome

Ancestral dose analysis along the genome was performed based on the number of allelic reads of ancestral diagnostic SNPs (DSNPs). According to the known phylogenomic structures of the parents, three ancestral species were considered: *P. trifoliata*, *C. maxima*, and *C. reticulata*. Nine accessions were used to select diagnostic SNPs for the ancestral species of our material: three for *C. maxima* (Sunshine, Chandler, and Indian pummelos), four for *C. reticulata* (Willowleaf, Chios, Cleopatra, and Sunki mandarins), and two for *P. trifoliata* (Pomeroy 1 and Pomeroy 2 trifoliolate oranges). For each ancestor, DSNPs that fully differentiated the considered ancestor from the others two (i.e., all accessions of the considered ancestor were homozygous for the same allele and all others accessions homozygous for the second allele) were filtered in Excel. For accession with known introgressed areas (particularly mandarins introgressed by fragments of the *C. maxima* genome and Chandler pummelo by a small *C. reticulata* fragment [68,69]), we removed the data of the considered region/variety from the analysis, as described by Oueslati et al. [69] and Ahmed et al. [70]. TraceAncestor software was then used to analyze the contribution of the three species all along the nuclear genome of the 35 accessions analyzed, following the method described by Ahmed et al. [70].

3. Results

3.1. Plant Regeneration from Protoplast Fusion

One to two months after fusion, chlorophyllian embryos were observed for the three new combinations (Figure 1). The higher regeneration rates were obtained for sweet orange combined with the Cleopatra mandarin/4475 citrumelo cybrid. Embryos and microcalli were replicated in the same solid medium for 1 month, and thus a few additional embryos were obtained. All the embryos were then transferred to the maturation medium, where some of them successfully changed to cotyledonary embryos. The two further steps (germination and caulogenesis) were successful for a few embryos. The grafting of in vitro plantlets and greenhouse acclimatization were then successful, with a total of five, four, and 14 grafted plants obtained for Cleopatra mandarin + Winter Haven citrumelo, Cleopatra mandarin + Carrizo citrange, and Shamouti sweet orange + 4475 citrumelo-Chios cybrid, respectively. All regenerated plants displayed trifoliolate leaves, suggesting they were all cybrids or nuclear somatic hybrids.

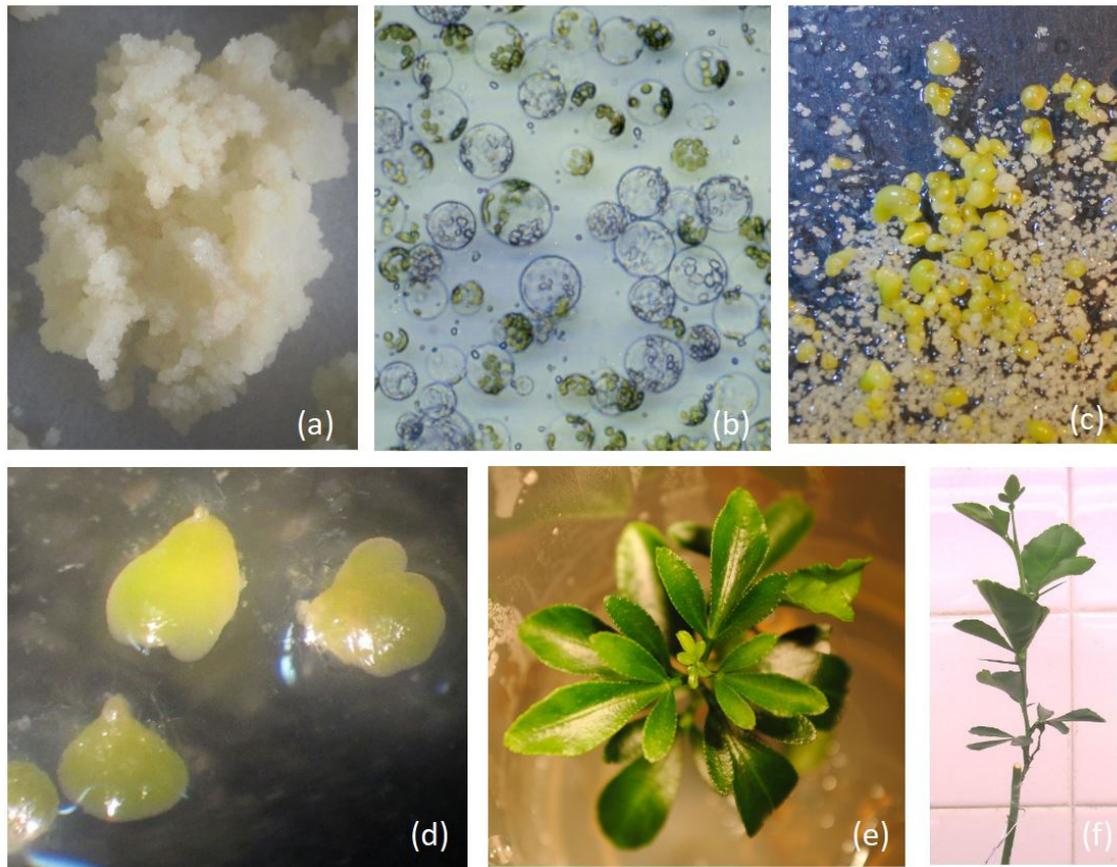


Figure 1. Process of somatic hybridization and plant regeneration: (a) embryogenic callus line of Shamouti sweet orange; (b) protoplast fused after three pulses (35 μ s) of 220 V (DC); (c) pro-embryo 1 month after fusion; (d) heart-shaped embryos two months after fusion; (e) in vitro plantlet of Cleopatra + Winter Haven tetraploid somatic hybrid; (f) grafted plant of Shamouti sweet orange + 4475 citrumelo-Chios cybrid tetraploid somatic hybrid.

3.2. Ploidy Estimation by Flow Cytometry

All plants regenerated from Shamouti sweet orange + 4475 citrumelo-Chios cybrid and Cleopatra mandarin/Carrizo citrange were estimated to be tetraploid, whereas one diploid and four tetraploid plants were found from the Cleopatra mandarin + Winter Haven citrumelo combination (Figure 2).

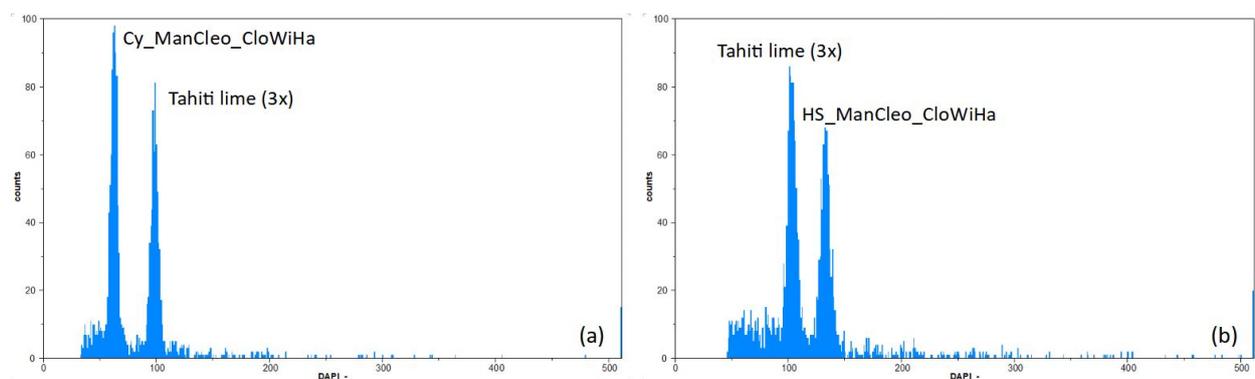


Figure 2. Identification of near diploid and tetraploid plants by flow cytometry in the Cleopatra mandarin + Winter Haven combination using the triploid Tahiti lime as internal control: (a) diploid like plant, (b) tetraploid like plant.

3.3. Inheritance of Mitochondrial Genome

Two hundred and ten diallelic SNPs were identified for the mitochondrial genomes. After filtering for the complete absence of missing data, we selected 24 SNPs, allowing the three ancestors involved to be fully distinguished (Figure 3). Two subclades were observed for *C. reticulata*: one corresponding to Sunki and Cleopatra mandarin and the other to Willowleaf and Chios mandarins. Except for the Shamouti sweet orange/C35 Citrange and Cleopatra mandarin/Citrindarin combinations, the analysis differentiated the two parents for all combinations. When the two parents were distinguished, most cybrids and somatic hybrids inherited the mitochondrial genome of the callus parent. However, the four somatic hybrids between Cleopatra mandarin and C35 citrange had different mitochondrial haplotypes than the parental lines. Eleven SNPs differentiated the mitochondria of Cleopatra mandarin from that of C35 citrange (i.e., sweet orange mitochondria). According to the order of the SNPs on the mitochondrial reference genome, for three somatic hybrids, the mitochondrial haplotypes combined two C35 citrange-specific alleles, then five consecutive Cleopatra-specific alleles, and finished with four C35 citrange-specific alleles. The last somatic hybrid (HS_ManCleo_CraC35c) displayed two sweet orange, two Cleopatra, and then seven sweet orange-specific alleles (Supplementary Materials Table S1).

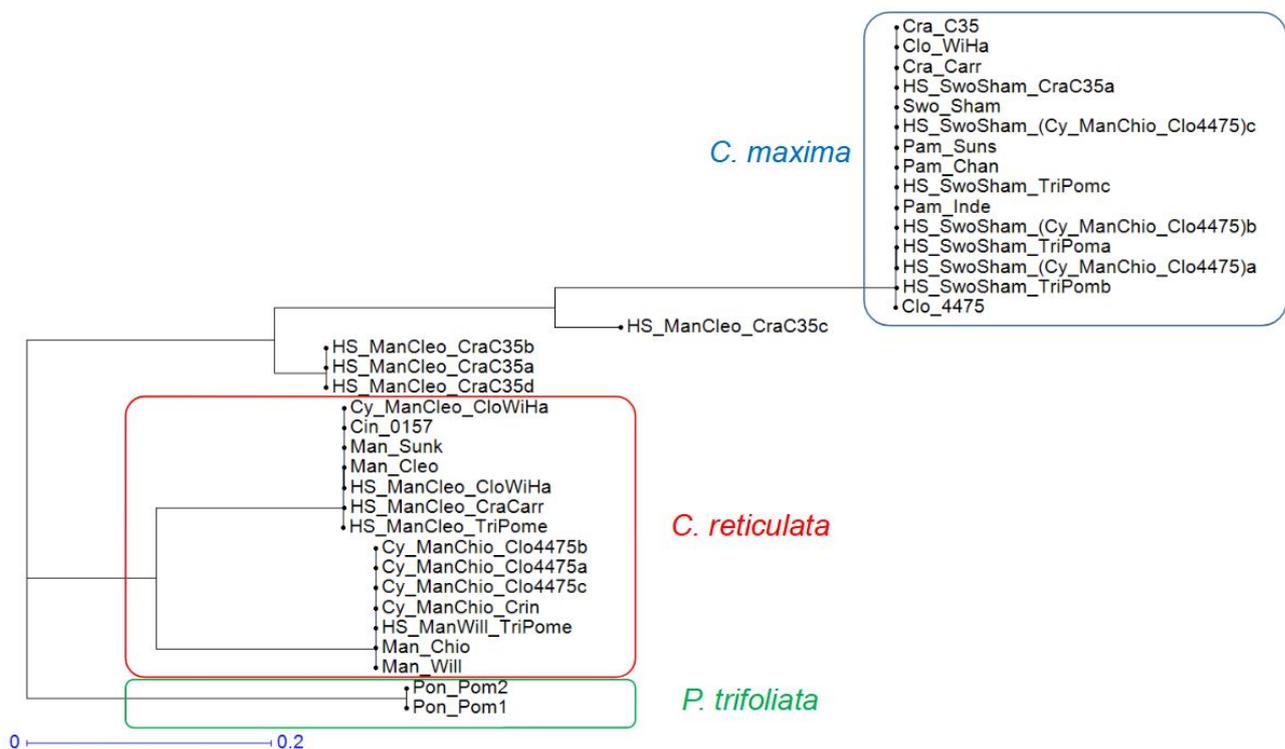


Figure 3. Inheritance of mitochondrial genomes: NJ tree inferred from 24 diallelic SNPs.

3.4. Inheritance of Chloroplastic Genome

Only four diallelic SNPs were identified and used for the analysis. Despite this low number, three haplotypes were identified corresponding to the three ancestor species of the parental lines, cybrids, and somatic hybrids (Figure 4). They differentiated the parental chloroplasts for eight of the 11 combinations. Three combinations had the same chlorotype for their two parents. Thus, both parents of Shamouti sweet orange + 4475 citrumelo cybrid and Shamouti sweet orange + C35 citrange share the *C. maxima* chlorotype, while the *C. reticulata* chlorotype is shared by the parents of the Chios mandarin + citrandarin combination. For the combination with differentiated parental chlorotypes, four somatic hybrids with Pomeroy trifoliolate orange (one with Willowleaf mandarin, one with Cleopatra

mandarin, and two with sweet orange) inherited the *P. trifoliata* chloroplast. The *C. reticulata* chloroplast was found for the Cleopatra mandarin + Winter Haven somatic hybrid. All other cybrids and nuclear somatic hybrids displayed the *C. maxima* chloroplast inherited from the callus parent (one Shamouti sweet orange + trifoliolate orange somatic hybrid) or the leaf parents (the three Chios mandarin/4475 Citrumelo cybrids, the Cleopatra mandarin/Winter Haven cybrid, the four Cleopatra mandarin/C35 citrange somatic hybrids, and the Cleopatra mandarin + Carrizo citrange somatic hybrid).

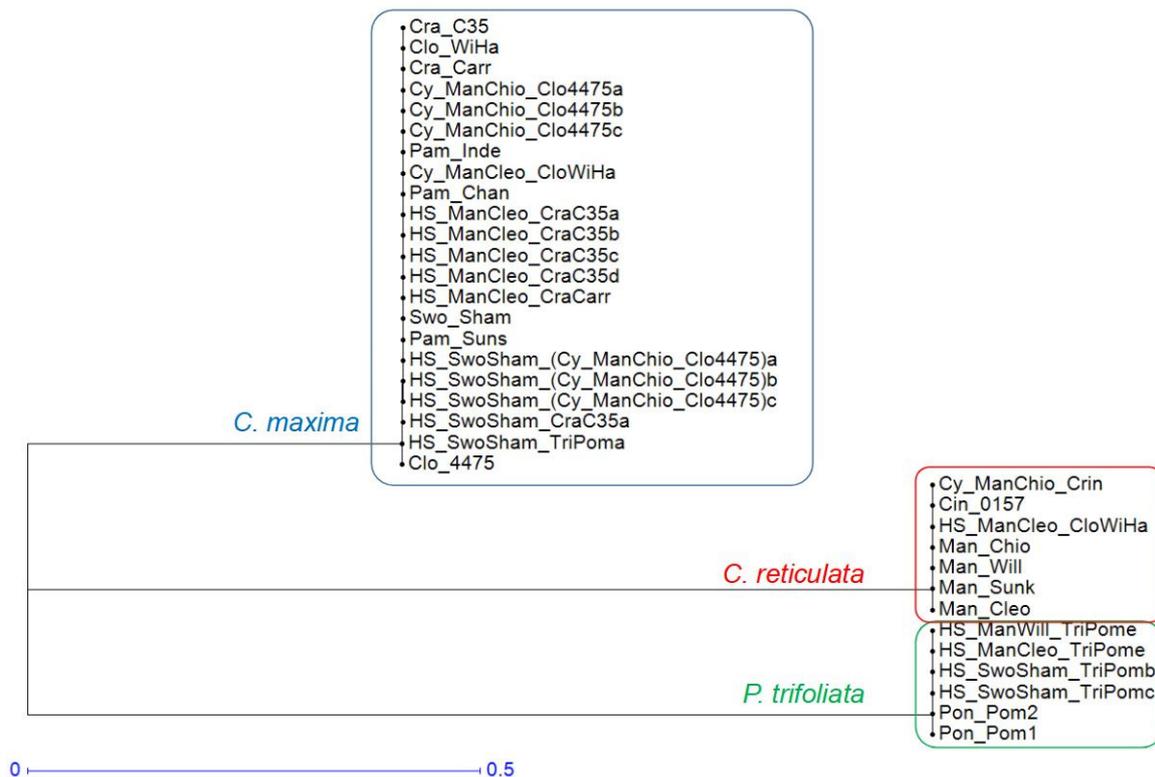


Figure 4. Inheritance of chloroplastic genomes: NJ tree inferred from four diallelic SNPs.

3.5. Inheritance of Nuclear Genomes

3.5.1. SNP Mining

After genotype calling on the *P. trifoliata* reference genome and filtering for diallelic SNPs with less than 25% of missing data and minimum allele frequency of 0.05, we obtained 109,406 SNPs. These were used to search for diagnostic SNPs from the three ancestral genomes involved in our material. An additional filtering for no missing data was performed for the analysis of genetic diversity organization by factorial analysis and delivered 46,826 SNPs.

3.5.2. First Approximation of the Nuclear Inheritance by Factorial Analysis of SNP Data

According to the factorial analysis, all diploid plants (codified as Cy_**) regenerated from citrus callus protoplast + trifoliolate orange or trifoliolate hybrids displayed genetic profiles very close to the trifoliolate parents (Figure 5). The plants considered tetraploid from flow cytometry analysis (codified as HS_**) were distant from both parents, reflecting nuclear hybridity.

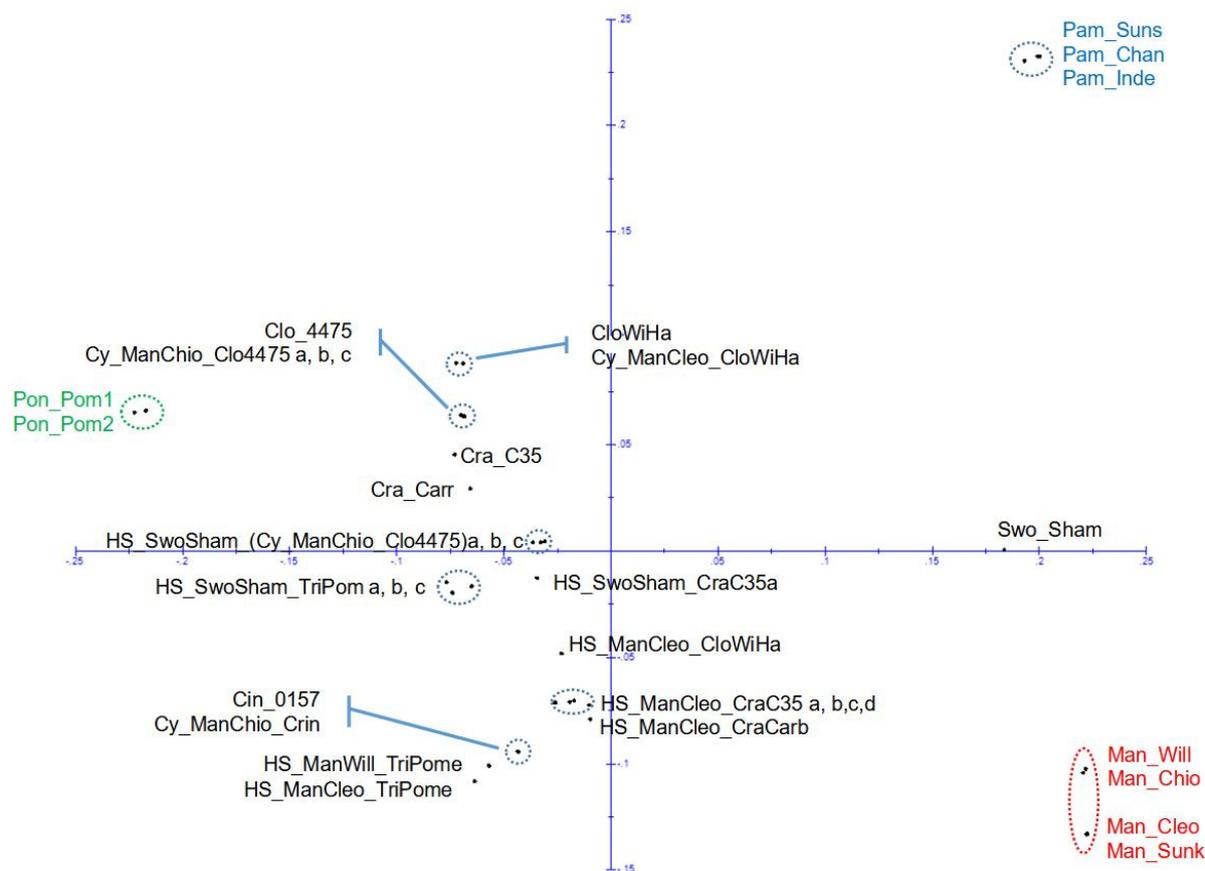


Figure 5. Factorial analysis on a dissimilarity matrix based on 46,826 nuclear SNPs.

3.5.3. Search for Diagnostic SNPs of Ancestral Genomes

Respectively, 8416, 10,155, and 15,574 DSNPs were selected for *C. maxima*, *C. reticulata* and *P. trifoliata* (Table 2). These 34,145 DSNPs (detailed list in Supplementary Materials Table S2) were then used to analyze the ancestral allelic doses along the genome of parental lines, cybrids, and nuclear somatic hybrids.

Table 2. Number of DSNP for the three ancestors on the nine chromosomes.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | Total |
|----------------------|------|------|------|------|------|------|------|------|------|--------|
| <i>C. maxima</i> | 909 | 961 | 1468 | 861 | 852 | 1057 | 824 | 752 | 732 | 8416 |
| <i>C. reticulata</i> | 962 | 1297 | 1919 | 1040 | 1068 | 1071 | 995 | 909 | 894 | 10,155 |
| <i>P. trifoliata</i> | 1849 | 2051 | 2740 | 1753 | 1587 | 1496 | 1502 | 1255 | 1341 | 15,574 |

3.5.4. Interspecific Mosaic Structure of the Parental Lines, Cybrids, and Nuclear Somatic Hybrids

To identify potential ploidy variation along the genome, the first step in the analysis was to study the ancestral read frequencies for DSNPs all along the genome. For a diploid, the theoretical frequencies are 0, 0.5 and 1; for a triploid, 0, 0.33, 0.66, and 1; for a tetraploid, 0, 0.25, 0.5, 0.75, and 1; and for a pentaploid, 0; 0.2; 0.4; 0.6; 0.8, and 1. Therefore, the coordinated variations in the read frequencies for the three ancestors reveal ploidy variation along the genome.

We found that ploidy variation could affect an entire chromosome, as illustrated by Chr9 of HS_SwoSham_TriPomb and HS_SwoSham_TriPomc (Figure 6), or just a region of chromosome, as illustrated by four chromosomes of the HS_ManCleo_TriPome somatic hybrid (Figure 7). In both figures, each dot is the average of ancestral read frequencies for

45 successive DSNPs of the ancestor considered with sliding windows every 15 DSNPs. For each window, the genomic position is the average of the position of the considered DSNPs.

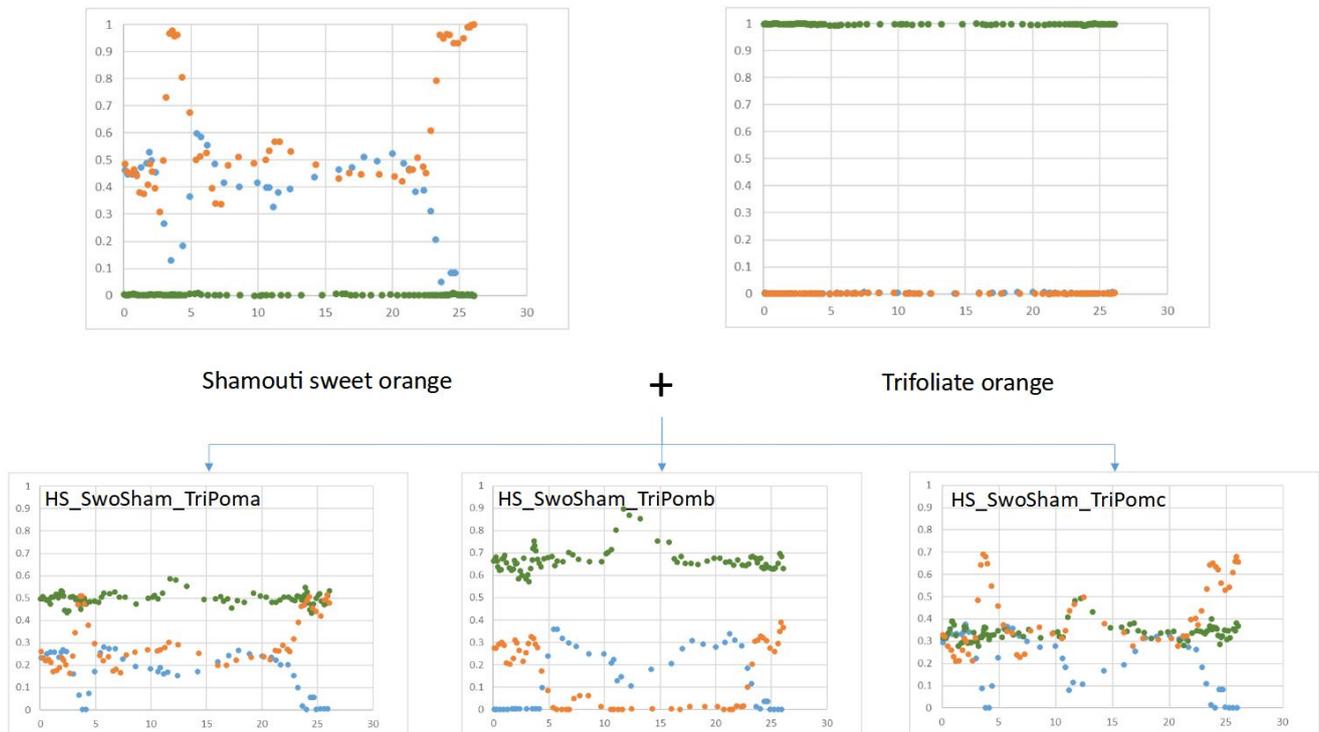


Figure 6. Frequencies of the diagnostic alleles of the three ancestral genomes along Chr9 of three somatic hybrids between *C. sinensis* cv Shamouti and *P. trifoliata* cv Pomeroy. Green: *P. trifoliata*; orange: *C. reticulata*; blue: *C. maxima*. The unit of the x axes is Mb.

We analyzed three plants obtained from the combination between Shamouti sweet orange and Pomeroy trifoliolate orange (Figure 6). For Chr9, HS_SwoSham_TriPoma displayed a logical profile for a tetraploid, resulting from the addition of sweet orange and trifoliolate orange all along the chromosome with a frequency for *P. trifoliata* around 0.5 and an alternation of *C. maxima* and *C. reticulata* together at 0.25 or *C. reticulata* alone at 0.5. For HS_SwoSham_TriPomb, the *P. trifoliata* read dose was greater than 0.66 all along the chromosome, while *C. reticulata* and *C. maxima* alternated around 0.33. The most plausible configuration is triploid with two doses of *P. trifoliata* and the loss of one chromosome from sweet orange (*C. maxima* fragment from 0 to 2 Mb and then *C. reticulata* to the end). For the HS_SwoSham_TriPomc somatic hybrid, the profile was consistent with a triploid configuration with the loss of one chromosome from *P. trifoliata*. For all three plants as well as sweet orange, we observed a decrease for *C. maxima* reads and an increase for the other ancestors around 12 Mb, which may be explained by a loss of pummelo genomic region in the centromeric area of sweet orange Chr9.

Ploidy-like variations were also identified in four genomic regions of the Cleopatra mandarin + Pomeroy trifoliolate orange somatic hybrid (HS_ManCleo_TriPome; Figure 7). This hybrid was expected to display a constant frequency of 0.5 throughout the genome for both *P. trifoliata* and *C. reticulata* ancestors (except for a small region at the beginning of Chr3, where Cleopatra mandarin is heterozygously introgressed by *C. maxima*). We observed significant deviations with proportions close to 0.33/0.66, corresponding to a loss of one dose of *C. reticulata* at the end of Chr3 (37 Mb to end) and Chr8 (27 Mb to end). For the first half of Chr4 (start to 14 Mb) and much of Chr7 (start to 21 Mb), the ratios were close to 0.4 for *P. trifoliata* and 0.6 for *C. reticulata*. This pattern may correspond to a local pentaploidy-like configuration with two doses of *P. trifoliata* and three of *C. reticulata*. However, we cannot totally rule out a local triploidy with the loss of one dose of *P. trifoliata*.

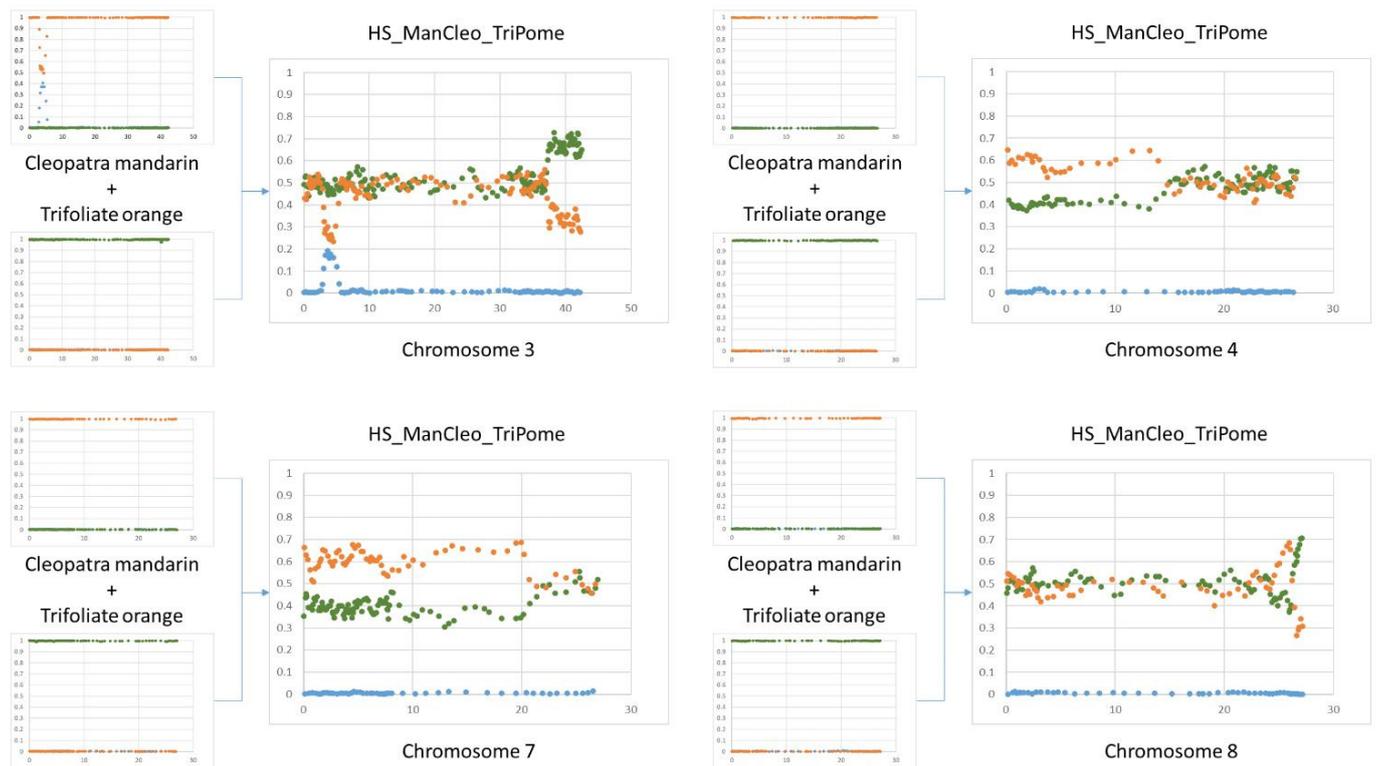


Figure 7. Frequencies of the diagnostic alleles of the three ancestral genomes along four chromosomes of the somatic hybrids between Cleopatra mandarin and *P. trifoliata* cv Pomeroy. Green: *P. trifoliata*; orange: *C. reticulata*; blue: *C. maxima*. The unit of the x axes is Mb.

Ancestor read-frequency curves are given for all plants and chromosomes in Supplementary Materials Figure S1. All profiles for diploid parental lines corresponded to 0, 0.5, or 1 frequencies for all three ancestors. No ploidy variation was observed for the cybrids, which displayed very similar profiles to their leaf parents. For the nuclear somatic hybrids, the ancestor doses were mostly in agreement with the addition of the two parental doses. However, some discrepancies were observed. The phylogenomic karyotypes of the 10 different genomic structures identified are given in Figure 8. Table 3 summarizes the cytoplasmic and nuclear genome constitution for cybrids and nuclear somatic hybrids.

The four somatic hybrids between Cleopatra mandarin and C35 citrange (HS_ManCleo_CraC35) displayed very similar profiles, with potential triploidy at the very beginning (start to 2 Mb) and end (37 Mb to end) of Chr3 and at the beginning of Chr9 (start to 14 Mb), with a loss of one dose of *C. reticulata* in all cases. A potential pentaploidy-like situation with four doses of *C. reticulata* and one for *P. trifoliata* was identified at the beginning of Chr7 (start to 4 Mb).

The Cleopatra mandarin + Winter Haven citrumelo somatic hybrid (HS_ManCleo_CloWiHa) had a triploid profile with a loss of one dose of *C. reticulata* at the end of Chr3 (37 Mb to end). Its profile in the first half of Chr4 (start to 14 Mb) was consistent with a pentaploid-like situation, with one dose of *C. maxima* and *P. trifoliata* and three doses of *C. reticulata*.

For the Cleopatra mandarin + Carrizo citrange (HS_ManCleo_CraCarb), we once again observed a potential triploid situation with the loss of one *C. reticulata* dose at the end of Chr3 (37 Mb to end) and a potential pentaploid-like situation in the first half of Chr4 (start to 14 Mb) with four *C. reticulata* doses and one *P. trifoliata* dose. A similar pentaploid-like situation was also observed at the beginning of Chr7 (1 to 4 Mb).

Table 3. Synthesis of genome characterization for cybrids and nuclear somatic hybrids.

| | Code | Estimated Ploidy | Mitochondria | Chloroplast | Nucleus |
|-----------------|-----------------------------------|------------------|-------------------|---------------------------|---------------------|
| Cybrids | Cy_ManChio_Clo4475a | 2 | Chios Mand. | 4475 Citrumelo | 4475 Citrumelo |
| | Cy_ManChio_Clo4475b | 2 | Chios Mand. | 4476 Citrumelo | 4476 Citrumelo |
| | Cy_ManChio_Clo4475c | 2 | Chios Mand. | 4477 Citrumelo | 4477 Citrumelo |
| | Cy_ManChio_Crin | 2 | Chios Mand. | <i>C. reticulata</i> type | Citrandarin |
| | Cy_ManCleo_CloWiHa | 2 | Cleopatra Mand. | Winter Haven Citru. | Winter Haven Citru. |
| Nuclear Hybrids | HS_ManCleo_CloWiHa | 4 | Cleopatra Mand. | Cleopatra Mand. | Hybrid/Instability |
| | HS_ManCleo_CraC35a | 4 | Chimeric | C35 Citrange | Hybrid/Instability |
| | HS_ManCleo_CraC35b | 4 | Chimeric | C35 Citrange | Hybrid/Instability |
| | HS_ManCleo_CraC35c | 4 | Chimeric | C35 Citrange | Hybrid/Instability |
| | HS_ManCleo_CraC35d | 4 | Chimeric | C35 Citrange | Hybrid/Instability |
| | HS_ManCleo_CraCarb | 4 | Cleopatra Mand. | Carrizo Citrange | Hybrid/Instability |
| | HS_ManCleo_TriPome | 4 | Cleopatra Mand. | Pomeroy Tr. Or. | Hybrid/Instability |
| | HS_ManWill_TriPome | 4 | Willow leaf Mand. | Pomeroy Tr. Or. | Hybrid/Instability |
| | HS_SwoSham_(Cy_ManChio_Clo4475) a | 4 | Shamouti Sw. Or. | <i>C. maxima</i> type | Hybrid/Instability |
| | HS_SwoSham_(Cy_ManChio_Clo4475) b | 4 | Shamouti Sw. Or. | <i>C. maxima</i> type | Hybrid/Instability |
| | HS_SwoSham_(Cy_ManChio_Clo4475) c | 4 | Shamouti Sw. Or. | <i>C. maxima</i> type | Hybrid/Instability |
| | HS_SwoSham_CraC35a | 4 | Shamouti Sw. Or. | <i>C. maxima</i> type | Hybrid/Instability |
| | HS_SwoSham_TriPoma | 4 | Shamouti Sw. Or. | Shamouti Sw. Or. | Hybrid/Instability |
| | HS_SwoSham_TriPomb | 4 | Shamouti Sw. Or. | Pomeroy Tr. Or. | Hybrid/Instability |
| | HS_SwoSham_TriPomc | 4 | Shamouti Sw. Or. | Pomeroy Tr. Or. | Hybrid/Instability |

The HS_SwoSham_CraC35 somatic hybrid between Shamouti orange and C35 citrange displayed a triploid-like area in Chr2 (5 to 11 Mb) with the loss of one dose of *C. maxima*. We also identified probable small local triploid situations with losses of one dose of *C. reticulata* in Chr1 (3 to 4 Mb), Chr3 (3 to 4 Mb), and Chr6 (start to 1 Mb).

All three somatic hybrids between Shamouti sweet orange and the cybrid of citrumello 4475 with Chios mandarin mitochondria (HS_SwoSham_[Cy_ManChio_Clo4475]) displayed identical profiles. As with the somatic hybrids between Shamouti and C. 35 citrange, we observed local triploid configurations with the loss of one *C. maxima* dose in Chr2 (5 to 11 Mb) and small losses of one dose of *C. reticulata* in Chr1 (3 to 4 Mb), Chr3 (3 to 4 Mb), and Chr6 (start to 1 Mb).

In addition to the variation in ploidy and ancestral contribution for Chr9 as a whole between the three Shamouti orange + trifoliolate orange somatic hybrids (HS_SwoSham_TriPom), we observed local ploidy variations at the same locations in all three hybrids. These concerned the losses of one dose of *C. reticulata* in Chr1 (3 to 4 Mb), Chr3 (3 to 4 Mb), and Chr6 (start to 1 Mb), as well as the loss of one dose of *C. maxima* on Chr2 (5 to 11 Mb). In addition, we noted a complete loss of one *P. trifoliata* chromosome for Chr5 of the HS_SwoSham_TriPomc somatic hybrid. Accurate flow cytometry analysis of the three HS_SwoSham_TriPom somatic hybrids with three replicates, using Tahiti lime as internal control, revealed significant genome size variations. These variations were consistent with the losses of two and one chromosomes in HS_SwoSham_TriPomc (ratio somatic hybrid/Tahiti lime peak values: 1.150 +/- 0.005) and HS_SwoSham_TriPomb (1.186 +/- 0.005), respectively, compared with the complete tetraploid HS_SwoSham_TriPoma (1.219 +/- 0.003).

For the Willowleaf mandarin + trifoliolate orange somatic hybrid (HS_ManWill_TriPome), we observed a pentaploid-like region at the beginning of Chr2 (0 to 6 Mb; 3 *C. reticulata* doses, 2 *P. trifoliata* doses) and two triploid-like regions with one dose of *C. reticulata* and two doses of *P. trifoliata* at the end of Chr5 (27 to 32 Mb) and the beginning of Chr6 (0 to 7.5 Mb).

4. Discussion

4.1. Obtaining New Nuclear Somatic Hybrids for Three Intergeneric Parental Combinations and One New Cybrid

All three new parental combinations performed in this work produced tetraploid-like plants according to flow cytometry analysis. Their trifoliolate leaves suggested that allotetraploid somatic hybrids were obtained. The three plants analyzed from the combination of Shamouti sweet orange with the cybrid 4475 citrumelo with Chios mandarin mitochondria were identical. They had chloroplast and mitochondria from sweet orange and a nuclear genome resulting mainly from the symmetrical addition of the parental genomes, except for four small local triploid configurations. The first one resulted from the loss of one dose of *C. maxima* in Chr2 between 5 and 10 Mb and the others from losses of small genomic fragments of *C. reticulata* (around 1 Mb each) in Chr1, Chr3, and Chr6. The two plants obtained from the combinations of Cleopatra mandarin with Carrizo citrange and with Winter Haven citrumelo presented the same local chromosome instability. They displayed local triploidy at the end of Chr3, with the loss of one dose of *C. reticulata*, and potential pentaploidy in the first half of Chr4. At this location, we detected an additional dose of *C. reticulata* beyond that expected from parental addition. In the rest of the genome, the phylogenomic karyotypes of the two somatic hybrids were in full agreement, with symmetrical addition of the parental genomes.

The citranges and citrumelo genotypes we used, as well as their trifoliolate orange parents, are resistant to CTV, tolerant to nematodes and *Phytophthora* spp., and promote high fruit quality and good yields. However, they are rather sensitive to salinity, calcareous soils, and *Citrus exocortis* virus (CEV). CEV is only transmitted by budwoods or during horticultural practices if contaminated tools are used. It is therefore not a major problem in countries with sanitary certification systems, but can be a major constraint otherwise. The Cleopatra mandarin presents good complementarity with citranges and citrumelos. Indeed, Cleopatra mandarin is one of the rootstocks with the highest tolerance to salinity. It is also

tolerant to calcareous soils, CEV, and CTV. However, this genotype is susceptible to certain strains of *Phytophthora* and nematodes and promotes relatively low yields compared with other rootstocks, although fruit quality is good. Cleopatra mandarin + Carrizo citrange and Cleopatra mandarin + Winter Haven citrumelo appear to be suitable candidates to combine (a) the CTV resistance and nematode and *Phytophthora* tolerance from trifoliolate hybrids with (b) the salt, calcareous, and CEV tolerance of Cleopatra mandarin.

Sweet oranges are highly susceptible to *Phytophthora*, but tolerant to calcareous soils and CEV; thus, we hope that the somatic hybrid combining Shamouti sweet orange and 4475 citrumelo-Chios cybrids may be tolerant to biotic stresses and better adapted to calcareous soils than the 4475 citrumelo alone.

In addition to the tetraploid-like hybrid, we regenerated a diploid cybrid with the nucleus and chloroplast of Winter Haven citrumelo and the mitochondria of Cleopatra mandarin. We saw no evidence for structural variation in the nuclear genome. Obtaining citrus cybrids as byproducts of standard symmetrical somatic hybridization has been reported in many citrus protoplast fusion works [27,38,71–76]. Their accurate phenotypical characterization should help determine the impact of nucleo-cytoplasmic interaction on agronomic traits, and may lead to improved selections.

These new cybrid and somatic hybrids are in addition to those previously described by Dambier et al. [18] for eight intergeneric combinations and the large number of *Citrus* + *Poncirus* intergeneric hybrids developed in Florida [20,22,25,77], Spain [24], Japan [27,78], and China [26,79–81]. In addition to potential direct interest as rootstocks, intergeneric somatic hybrids have been used in Florida as parents for the “tetrazyg breeding” strategy, which aims to combine favorable traits via sexual recombination at the tetraploid level [22].

4.2. Efficiency of GBS with *ApeK1* Restriction Enzyme to Mark Two Cytoplasmic and Nuclear Genomes

For the first time in citrus spp., GBS was used for simultaneous characterization of the chloroplast, mitochondrial, and nuclear genomes. The integration of a large part of the chloroplast genome on the *C. sinensis* mitochondrial reference genome published by Yu et al. [65] resulted in a very limited number of polymorphic markers for the chloroplast. Indeed, we discarded those reads that mapped different regions of the overall reference genome (i.e., nuclear + cytoplasmic) from the genotype calling analysis. However, the four chloroplast markers were sufficient to trace the three ancestral chloroplast haplotypes and their inheritance in cybrids and nuclear somatic hybrids. The coverage of the mitochondrial genome was good, with 210 diallelic markers identified. After selecting 24 markers with no missing data, we identified four haplotypes for the parents. Two haplotypes within *C. reticulata* distinguished the cultivated mandarins from the acid mandarin group, as previously shown by Froelicher et al. [82] with four mitochondrial InDel markers. At the nuclear level, the TraceAncestor method developed within the *Citrus* genus to estimate ancestral allelic doses [70] was effective to trace the *C. maxima*, *C. reticulata*. And *P. trifoliata* genomes, with 8416, 10,155, and 15574 DSNPs, respectively. The higher number of *P. trifoliata* DSNPs when compared with *C. maxima* and *C. reticulata* makes sense. Indeed, *P. trifoliata* is the most phylogenetically distant species from the other two taxa and therefore its genome has more specific mutations (DSNPs). For *C. maxima* and *C. reticulata*, these results were much larger than the 2087 and 3491 DSNPs found by Ahmed et al. [70]. This is in part due to the evolution of the sequencing read length from 100 bp on the Illumina HiSeq2000 platform for Ahmed et al. [70] to 150 bp on the HiSeq 4000 platform for our study. Moreover, our greatly increased sequencing depth resulted in fewer errors and less missing data. The limited discovery panel in our study, which focused on genotypes used for somatic hybridization, and the absence of representatives of *C. medica* and *C. micrantha* also enhanced the number of discriminant markers. With this set of 34,145 codominant SNP markers, our study represents the most extensive characterization of the nuclear genomes of citrus somatic hybrids ever performed.

4.3. Inheritance of Cytoplasmic Genomes

As previously described by several authors and reviewed by Ollitrault et al. [37], we observed random uniparental inheritance of the chloroplast from leaf or callus parents. However, given the small number of markers and their location on only the short single-copy sequence of the chloroplast, we cannot definitively prove the absence of chloroplast genome recombination. The coverage of the mitochondrial genome was better with the selection of 24 SNPs without missing data. For all five cybrids and 11 of the 15 nuclear somatic hybrids, these markers revealed the systematic inheritance of mitochondrial haplotypes from the callus parent. These results are in agreement with previous mitochondria characterizations of citrus somatic hybrids and cybrids. In these studies, mitochondrial genomes from the callus parent were prevalent in both regenerated cybrids and somatic hybrids, suggesting a critical role for these organelles in somatic embryogenesis [37–39,74,75,83–87]. However, all four plants regenerated for the Cleopatra + C35 combination displayed an admixture of genomic fragments from Cleopatra and C35 mitochondria. The conservation of a single parental fragment suggested homologous recombination. Such rearrangements of the mitochondrial genome have also been reported in some studies following asymmetric and symmetric somatic hybridizations in citrus [38–41,84,88].

More generally, somatic hybridization in plants often results in recombinant mitochondrial genomes, as previously reported for several plant species [89–94]. Characterization of these chimeric mitochondrial genomes has usually been limited to a few parent-specific markers, but recently a few complete mtDNAs sequences from cybrid have been published [92–94]. In these studies, the cybrid mitochondrial genomes were chimeric and larger than each parental mtDNA. Despite their increased size, the cybrid mtDNAs contained single haplotypes for most mitochondrial genes. We observed the latter trait in the chimeric mitochondrial genomes of Cleopatra mandarin + C35 somatic hybrids. Sanchez-Puerta et al. [95] proposed that retaining a single-copy form of each gene could be a selective mechanism to minimize intracellular incompatibilities or a neutral process that preferentially eliminates duplicated regions, while Garcia et al. [94] concluded that cybrid plant mitochondria undergo homologous recombination mainly through a break-induced replication (BIR) pathway, keeping a single allele for each gene. The BIR pathway could also be the source of the chimeric mitochondrial genomes of our four Cleopatra + C35 somatic hybrids.

4.4. Inheritance and Instability of the Nuclear Genome

Most publications on citrus somatic hybridization at the Citrinae subtribe level have concluded successful symmetric addition of nuclear parental genomes, usually based on a limited number of nuclear markers [18,42,76,96,97]. This assumption has been enforced by a few larger genome scan analyses with dominant AFLPs or ISSRs, revealing the complete addition of parental profiles for somatic hybridization between sexually compatible genera [26,80,98]. However, dominant markers do not identify the loss or addition of one dose of an allele in cases where the considered allele is present with more than one copy in the two parents. Therefore, the findings of these studies do not rule out of the loss or addition of genomic regions.

The citrus literature also reports a few cases of unexpected ploidy levels as well as aneuploid plants among somatic hybrids for some intergeneric combinations. Chromosome counts on different flushes of *C. sinensis* + *Fortunella crassifolia* somatic hybrids showed that the plants were chimeras containing non-tetraploid cells in addition to amphidiploid cells [99]. Grosser et al. [100] found that their somatic hybrids between *C. sinensis* and *Severenia buxifolia* were triploids. Flow cytometry analysis revealed that pentaploid plants and hexaploid plants were regenerated from the fusion of tetraploid *Fortunella hindsii* with diploid *P. trifoliata* [101]. All plants regenerated from *C. sinensis* var Bonanza + *Clausena lansium* were hexaploid, while both parents were found to be diploids [102,103]. Another somatic hybridization experiment between *C. sinensis* var Newhal and *Clausena lansium* produced triploid plants in addition to the expected tetraploid ones [104]. Guo and

Deng [42] stated that “The possible explanations for genetic variation of these wide somatic hybrids include: somaclonal variation (possibly induced by plant growth regulators during culture); genetic variability in the embryogenic callus parent; chromosome elimination or polyploidization following wide protoplast fusion.”

Our nuclear genome study with 34,145 genome-wide SNP markers revealed genomic instability for the 15 nuclear somatic hybrids analyzed, but no instability for the five diploid cybrid genomes. For the nuclear somatic hybrids, most of the variation concerned local chromosomal regions with local pentaploid- or triploid-like genomic configurations, corresponding to the addition or loss, respectively, of one dose for the callus parent. We found some of these variations systematically in all somatic hybrids involving the same callus parent. This was the case for all somatic hybrids with Cleopatra as their callus parent. They displayed potential triploidy with the loss of one *C. reticulata* dose at the end of Chr3. For the two new somatic hybrids of Cleopatra mandarin with Winter Haven citrumelo and Carrizo citrange, as well as for the Cleopatra + trifoliolate orange hybrids, we also observed a pentaploidy-like situation in the first half of Chr4. In both cases, this corresponded to the addition of one dose of *C. reticulata*. All nuclear somatic hybrids obtained with the Shamouti sweet orange callus displayed four triploid-like genomic regions. One region in Chr2 between 5 and 10 Mb lost a dose of *C. maxima*, and others had small losses (around 1 Mb each) of one dose of *C. reticulata* in Chr1, Chr3, and Chr6. These observations suggest that these additions or losses were present at the embryogenic callus line level and resulted from in vitro-induced somaclonal variations. The addition of a genomic region likely results from duplication and insertion into parental chromosomes rather than aneuploidy with additional partial chromosomes. Additional cytogenetic studies are necessary to assess the structural variations affecting the plants under consideration.

Early studies stated that adult plants regenerated from citrus embryogenic calluses induced from apomictic citrus ovules generally displayed little or no somaclonal variation [105], even after protoplast isolation [106]. However, interesting somaclonal variants have been selected from citrus embryogenic callus cultures [107], and local ploidy variation has been observed for other plants after in vitro culture.

We also observed complete chromosome losses for two nuclear somatic hybrids from the Shamouti sweet orange + trifoliolate orange combination among the three hybrids analyzed. The HS_SwoSham_TriPomc lost one *P. trifoliata* chromosome, for Chr5 and Chr9. These losses of chromosomes of the leaf parent are evidence for genomic instability after protoplast fusion during the plant regeneration process. Such kinds of chromosome elimination and genomic instabilities after protoplast fusion have been widely described in other plants such as *Brassicaceae* species [44], *Nicotiana* + *Atropa* [47], *Oryza sativa subsp japonica*, *Oryza sativa subsp indica* [54], *Gentiana cruciate*, and *G. tibetica* [53]. These instabilities are generally associated with the in vitro process of obtaining protoplast and regenerating plants, but also with “genome shock” [108] in cases of wide hybridization.

4.5. Implications of Genomic Instability for Rootstock Breeding

As mentioned before, it is generally assumed that citrus somatic hybridization results in the complete addition of the parental genomes. Therefore, this method is considered particularly promising for adding all dominant traits from both parents in rootstock breeding projects [18–20,22,26,80]. The assumption of complete genome addition also leads to the consideration that the different somatic hybrids regenerated from an identical combination have the same genomic constitution, and thus should have the same phenotypic behavior. Therefore, in general, only one somatic hybrid line per parental combination is used for further evaluation of all the traits needed for rootstock selection. Evidence for chromosome instabilities for all putative allotetraploids and the variability between three plants regenerated from Shamouti sweet orange/Pomeroy trifoliolate orange combination definitively rule out these assumptions. With the objective of combining favorable traits of both parents, we therefore recommend that a thorough genomic characterization of all

plants regenerated from a given combination be performed to select the one with the least chromosome instability.

Such an analysis would also allow the selection of plants with different genomic constitutions to explore intra-combination phenotypic diversity and even to select plants that may have lost chromosome fragments carrying unfavorable dominant genes from one of the parents. Somatic hybridization in citrus plants should not be considered only as a tool for symmetric addition of genomes, but also as a method to induce new structural variability with potential elimination of unfavorable genomic areas.

Several QTL analyses have been performed in citrus for tolerances to diverse biotic and abiotic stress such as cold [109–111], salt stress [109,112], alternaria brown spot [113,114], phytophthora [115], CTV [115,116], nematodes [117], and HLB [12]. However, few concrete major loci for adaptation or resistance have been located in the different reference citrus genomes assembled in pseudochromosome. For useful rootstock traits, the most recent survey was published by Peng et al. [13], with the location of major QTLs and resistance genes for several diseases and pests in the *P. trifoliata* reference genome. The single dominant locus (Ctv) identified for CTV resistance in *P. trifoliata* [118] and mapped genetically and physically [119,120] is located in the middle of Chr7 near the Tyr1 region (1 Mb in size) involved in nematode resistance [121,122]. For HLB, Peng et al. [13] identified seven unique genomic regions in chromosomes 6, 7, 8, and 9 for the 14 QTLs published by Huang et al. [12]. These regions include 20 *P. trifoliata* genes that are homologs of previously identified DEGs potentially associated with HLB [13]. Peng et al. [13] concluded that chromosomes 7 and 9 of *P. trifoliata* may play a very important role in the transmission of resistance/tolerance to HLB, CTV, and nematodes.

Despite the chromosome instability, we did not observe complete loss of *P. trifoliata* in any genomic region of our somatic hybrids. For somatic hybridizations with trifoliolate orange sexual hybrids (citranges, citru-melo, citrandarin) as a parent, no loss of *P. trifoliata* genomic fragments was observed. Thus, we can consider that all useful *P. trifoliata* genes for CTV and nematodes present in the trifoliolate orange parental sexual hybrids were transmitted to the allo-tetraploid somatic hybrids. For two somatic hybrids between sweet orange Shamouti and Pomeroy trifoliolate orange, we observed the complete loss of a haplotype on chromosome 9. We believe that these hybrids retained the CTV and nematode resistance gene located on Chr7, but may have lost some genes useful for HLB tolerance (indeed, QTL identification from sweet orange x trifoliolate orange progeny [12] indicates that trifoliolate orange has heterozygosity for the responsible genes). The Shamouti sweet orange x Pomeroy trifoliolate orange somatic hybrid with complete transmission of the parental trifoliolate orange genome (HS_SwoSham_TriPoma) therefore appears to be the most interesting for further phenotypic evaluation and rootstock selection. The other somatic hybrids with Pomeroy trifoliolate orange did not lose any genomic regions of *P. trifoliata*. Therefore, we believe that these somatic hybrids inherited the various disease and pest resistance genes carried by the trifoliolate orange genome.

However, in addition to structural rearrangement, it is known that genome shock in wide hybridization affects gene expression in relation to epigenetic modifications [123–126]. These new epigenetic and gene regulatory patterns also result in phenotypic instability [123]. Therefore, confirmation that our somatic hybrids inherited a complete set of genes of *P. trifoliata* is not sufficient to conclude that all favorable phenotypic traits of trifoliolate orange are transmitted to the somatic hybrids. Phenotypic evaluations demonstrated that CTV resistance of *P. trifoliata* [18], HLB resistance of *M. australasica* [127], and adaptation to calcareous soils of *C. reticulata* [18] were successfully inherited in allotetraploid somatic hybrids. Further extensive phenotypic and genomic studies including transcriptome, small RNA, epigenome, and mobilome should be conducted to understand the implication of inter-generic somatic hybridization in the functional genomic of the somatic hybrids. Such knowledge would help to improve the rationale and the efficiency of somatic hybridization methods for *citrus* rootstock breeding.

5. Conclusions

We regenerated tetraploid-like plants from three new somatic hybridization combinations: Cleopatra mandarin + Winter Haven citrumelo, Cleopatra mandarin + Carrizo citrange, and Shamouti Sweet orange + Cybrid Citrumelo-Chios. One new cybrid with Winter Haven citrumelo nucleus and chloroplast and Cleopatra mandarin mitochondria was also obtained. GBS was powerful for characterizing chloroplast, mitochondrial, and nuclear genomes of the parents, cybrids, and nuclear somatic hybrids. The chloroplast genome displayed random uniparental inheritance. Mitochondria were mostly inherited from the callus parent. However, we noted clear homologous recombinations between the mitochondrial genome of Cleopatra mandarin and C35 citrange among their four somatic hybrids. The nuclear genomes of the five cybrids were identical to their parental ones, with no chromosome instability. Conversely, we observed chromosome instability for all tetraploid-like somatic hybrids with loss or addition of genomic doses for the callus parent. This suggests that instability resulted from the process of embryogenic callus induction and maintenance before protoplast fusion. The loss of whole chromosomes from trifoliolate orange leaf parents in combination with Shamouti sweet orange callus protoplasts demonstrated that chromosome instability could also occur after protoplast fusion. The evidence for chromosome instability among all the tetraploid-like somatic hybrids we studied contradicts the hypothesis of full symmetric addition of the two parental genomes. Our results also demonstrate the need for accurate genomic characterization of regenerated plants in order to identify those that will undergo full phenotypic evaluation for new rootstock selection. All the cybrids and the tetraploid-like somatic hybrid we studied inherited at least one *P. trifoliata* haplotype all along their genomes, and therefore one copy of all useful trifoliolate orange genes. These somatic hybrids will be useful for further studies to analyze the impact of genome shock and local ploidy variations in the neoregulation of gene expression and phenotypic variability in intergenic somatic hybrids. They will be evaluated as rootstock in Mediterranean and tropical conditions under various abiotic and biotic constraints. The indirect evidence for genomic instability at the embryogenic callus level also points to the need to optimize the somatic embryogenetic process in order to eliminate or at least limit such variations. In the future, this will be key to the effectiveness of not only somatic hybridization projects, but also genome editing systems that can take advantage of citrus somatic embryogenesis.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agriculture12020134/s1>, Figure S1: evolution of ancestor read frequencies along the nine chromosomes for the five cybrids, the 15 somatic hybrids, and their parents. Table S1: allelic constitution of the mitochondrial genomes of the 35 accessions analyzed by GBS. Table S2: list and characteristics of the diagnostic SNPs for *C. maxima*, *C. reticulata*, and *P. trifoliata*.

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