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Genetic Structure of Diploid Gametes for the Production of Triploid Citrus Hybrids

PhD THESIS PRESENTED BY

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“If we knew what it was we were doing, it would not be called research, would it?”
— Albert Einstein

CONTENTS

Abstract

The citrus industry is an important source of incomes for both individual growers and producing countries. Therefore, breeding for quality especially seedlessness has a pivotal role for the market, since consumers demand seedless fruits. Recovery of triploid hybrids through ploidy manipulation is a very valuable methodology to recover seedless citrus varieties.

Citrus triploid hybrids can be recovered through 2x x 2x taking advantage of the unreduced (2n) gametes formation. 2x x 4x and 4x x 2x sexual hybridizations are also widely exploited. Underlying the production mechanisms and genetic structures of diploid gametes is a key for optimizing polyploid breeding strategies. Two main mechanisms have been found in angiosperm for production of unreduced gametes: First Division Restitution (FDR) and Second Division Restitution (SDR). On the other hand, although tetraploid rootstocks display promising agronomic traits, their meiotic behavior and their segregation analysis is still mostly unknown in citrus. Disomic and tetrasomic models were defined as extreme models for tetraploid segregation, however, an intermediate inheritance model has been described for several crops.

In this framework, this thesis aimed to study three main aspects: (i) the mechanisms underlying unreduced pollen gamete formation in the diploid `CSO´ tangor hybrid used as male parent in 4x x 2x triploid breeding programs, (ii) the frequencies and mechanisms involved in the unreduced 2n female gametes production for `Eureka Frost´ and `Fino´ lemon genotypes, and (iii) the interspecific recombination and the resulting diploid gamete structures of doubled-diploid `Mexican´ lime to evaluate the possibility that natural interploid hybridization maybe the origin of *C. latifolia* (`Tahiti´ lime type) and *C. aurantifolia* (`Tanepao´ lime type) triploid varieties.

The production of 54 tetraploid hybrids from 4x x 2x sexual hybridizations allowed the analysis of the mechanisms underlying unreduced pollen gamete formation. SSR and SNP molecular markers revealed that the majority of these plants were obtained from unreduced 2n pollen of the diploid tangor parent. Then, the maximum-likelihood method based on parental heterozygosity restitution (PHR) of centromeric loci revealed that both FDR with predominant occurrence and to a lower extend SDR were the mechanisms leading to unreduced male gamete formation in the tangor studied. These observations were confirmed by the analysis of PHR pattern along the linkage group (LG) 2. To our knowledge, this is the first report of tetraploid citrus progenies arising from unreduced pollen and the first description of the coexistence of two meiotic restitution mechanisms (SDR and FDR) producing unreduced pollen in citrus.

In order to study the frequencies and the mechanisms involved in the unreduced 2n female gametes production in two different genotypes of lemon, we produced 43 triploid and tetraploid hybrids from 2x x 2x and 2x x 4x sexual hybridizations using `Eureka Frost´ and `Fino´ as female parents. The frequencies of 2n gamete production were respectively 4.9% and 8.3%. The maximum-likelihood analysis and pattern of PHR along LG1 revealed that SDR is the main mechanism of unreduced female lemon gametes (88%), followed by FDR or pre-meiotic doubling (PRD) (7%) and post-meiotic genome doubling (PMD) mechanisms (5%).

This is the first report of the production of a large number of lemon progenies from 2n gametes and the first identification of a new mechanism, PMD that has never been observed in citrus and has rarely been described in other herbaceous or woody species.

Across both studies, we demonstrated at the methodological level the effectiveness of using two complementary approaches, the analysis of the PHR pattern in one LG and the maximum-likelihood method based on centromeric loci for distinguishing between the different mechanisms of unreduced gamete production.

We analyzed the meiotic mechanisms of a doubled diploid `Mexican´ lime, the interspecific recombination and the resulting diploid gamete structures combining a segregation analysis of SSR and SNPs markers, a cytogenetic study and pollen viability evaluation. We concluded that the doubled-diploid `Mexican´ lime had a predominantly disomic segregation for three LGs, intermediate inheritance with disomic tendency was found for five LGs and intermediate models for one LG. The resulting interspecific diploid gamete structures displayed high *C. medica* / *C. micrantha* heterozygosity. The revealed genetic structures of the diploid gametes produced by the doubled–diploid `Mexican´ lime are compatible with the hypothesis that `Tahiti´ and `Tanepao´ triploid varieties results from interploid hybridization involving a doubled-diploid `Mexican´ like lime. This disomic tendency limits the recombination and the diversity of the diploid gamete population; however the observed pollen viability restoration at tetraploid level could be advantageous for intensive breeding projects.

The implications for triploid breeding projects of the meiotic behavior leading to unreduced pollen in `CSO´ tangor, unreduced ovules in lemons and diploid gametes of the DD `Mexican´ lime are discussed.

Resumen

La citricultura es una fuente importante de ingresos tanto para los citricultores como para los países productores. La mejora de la calidad e especialmente la ausencia de semillas en los frutos, es una exigencia del mercado de consumo en fresco ya que los consumidores no aceptan la presencia de semillas en los frutos. La obtención de híbridos triploides mediante la manipulación del nivel de ploidía es una metodología eficaz para la obtención de nuevas variedades de cítricos sin semillas.

En cítricos se pueden obtener híbridos triploides mediante cruzamientos 2x x 2x como consecuencia de la formación de gametos no reducidos (2n) y mediante hibridaciones sexuales entre parentales diploides y tetraploides (2x x 4x y 4x x 2x). La identificación de los mecanismos implicados en la formación de gametos no reducidos y las estructuras genéticas de los gametos diploides originados por los parentales tetraploides es crucial para optimizar las estrategias de mejora a nivel poliploide. En angiospermas se han descrito principalmente dos mecanismos de formación de gametos no reducidos, Restitución de la Primera División meiótica (FDR) y Restitución de la Segunda División meiótica (SDR). Por otro lado, se ha observado que los portainjertos tetraploides de cítricos presentan un comportamiento agronómico muy interesante, pero existe un gran desconocimiento sobre las meiosis y modelos de segregación de este tipo de plantas. Los modelos disómico y tetrasómico son modelos extremos para la segregación de genotipos tetraploides, aunque se han descrito modelos de segregación intermedios para diferentes cultivos.

En este contexto, esta tesis doctoral estudia tres aspectos principales: (i) los mecanismos responsables de la formación de gametos no reducidos de polen originados por un híbrido diploide entre clementina y naranjo (tangor `CSO´) que se ha utilizado como parental masculino en hibridaciones sexuales 4x x 2x, (ii) las frecuencias y los mecanismos implicados en la producción de gametos no reducidos femeninos en dos genotipos de limón, `Eureka Frost´ y `Fino´, y (iii) el análisis de la recombinación interespecífica y las estructuras de los gametos diploides originados por la lima `Mejicana´ doble diploide con el objetivo de evaluar la posibilidad de que las variedades triploides de lima *C. latifolia* (lima tipo `Tahiti´) y *C. aurantifolia* (lima tipo `Tanepao´) se hayan originado a partir de un cruzamiento natural a nivel interploide.

La obtención de 54 híbridos tetraploides a partir de hibridaciones sexuales 4x x 2x permitió analizar los mecanismos responsables de la formación de gametos no reducidos de polen. El análisis de estas plantas con marcadores moleculares SSRs y SNPs reveló que la mayoría de estas plantas se obtuvieron a partir de gametos no reducido de polen del parental masculino diploide tangor `CSO´. A continuación, el análisis mediante la utilización del método de máxima verosimilitud basado en la restitución de la heterocigosidad parental (PHR) en los loci centroméricos indicó que FDR y SDR son los mecanismos implicados con una mayor dominancia de FDR respecto SDR. Estos resultados se confirmaron posteriormente mediante el análisis de la restitución de la heterocigosidad en el grupo de ligamiento (LG) 2. Con los datos publicados hasta la fecha, es la primera vez que se han obtenido progenies tetraploides de cítricos mediante gametos no reducidos de polen y es la primera descripción en cítricos de la coexistencia de dos mecanismos de restitución meiótica, SDR y FDR, implicados en la formación de gametos 2n de polen.

Con el fin de estudiar las frecuencias y los mecanismos implicados en la producción de gametos no reducidos en dos genotipos diferentes de limón, se obtuvieron 43 híbridos triploides y tetraploides a partir de hibridaciones sexuales 2x x 2x y 2x x 4x utilizando los limones diploides `Eureka Frost´ y `Fino´ como parentales femeninos. Las frecuencias de producción de gametos 2n fueron respectivamente 4,9% y 8,3%. El análisis de máxima verosimilitud y el patrón de PHR a lo largo del LG1 reveló que SDR es el mecanismo principal implicado en la formación de gametos no reducidos femeninos (88%), seguido por FDR o duplicación del genoma pre-meiosis (PRD) (7%) y se identificó un nuevo mecanismo originado a partir de la duplicación del genoma post-meiosis (PMD) (5%). En este trabajo se describe por primera vez en cítricos la producción de un elevado número de híbridos de limón a partir de gametos 2n y es la primera vez que se identifica un nuevo mecanismo PMD que nunca se ha observado en cítricos y rara vez se ha descrito en otras especies herbáceas o leñosas. En ambos estudios se demostró a nivel metodológico la efectividad del uso de dos métodos complementarios, el análisis del patrón de PHR a lo largo de un LG y el método de máxima verosimilitud basado en la utilización de loci centroméricos para distinguir entre los diferentes mecanismos implicados en la formación de gametos no reducidos en limón.

También se ha analizado el modelo de segregación cromosómica de la lima `Mejicana´ doble diploide así como la recombinación interespecífica y las estructuras de los gametos diploides resultantes. Este trabajo se ha realizado mediante el análisis de la viabilidad del polen junto con un análisis citogenético y con marcadores SSRs y SNPs. Estos trabajos nos han permitido concluir que la lima `Mejicana´ DD presenta una segregación predominantemente disómica para tres LGs, herencia intermedia con tendencia disómica para cinco LGs y un tipo de segregación intermedia para un LG. Las estructuras de los gametos diploides interespecíficos resultantes mostraron una alta heterocigosis en *C. medica*/*C. micrantha*, parentales de la lima `Mejicana´.

Las estructuras genéticas observadas en los gametos diploides de la lima `Mejicana´ doble diploide son compatibles con la hipótesis de que las variedades triploides de lima `Tahití´ y `Tanepao´ se obtuvieran a partir de una hibridación interploide en la cual uno de los parentales fuese la lima `Mejicana´ doble diploide. El tipo de segregación disómico conlleva una limitación de la recombinación y la diversidad genética de la población de gametos diploides. Sin embargo la viabilidad del polen de la lima `Mejicana´ DD en comparación con la lima `Mejicana´ diploide permite la utilización de este genotipo como parental para la obtención de nuevas variedades de lima en programas de mejora genética.

Finalmente, se discuten las implicaciones en los programas de mejora genética para la obtención de híbridos triploides el comportamiento meiótico que origina la formación de gametos no reducidos de polen en el tangor `CSO´, la formación de gametos no reducidos de óvulo en los limones y los gametos diploides producidos por la lima `Mejicana´ doble diploide.

Resum

La indústria dels cítrics és una font important d'ingressos tant per als productors individuals com pels països productors. Per tant, la millora de la qualitat, especialment l´obtenció de varietats sense llavors va tindre un paper fonamental per al mercat, ja que els consumidors demanen fruits sense llavors. L'obtenció d'híbrids triploides mitjançant la manipulació de la ploïdia podria ser una metodologia valuosa per a obtindre varietats de cítrics sense llavors.

Els híbrids triploides de cítrics es poden obtindre a partir de creuaments 2x x 2x aprofitant la formació de gàmetes no reduïts (2n). Les hibridacions sexuals 2x x 4x i 2x x 4x són també àmpliament utilitzades. Els mecanismes de producció i les estructures genètiques de les gàmetes diploides son la clau per a l'optimització de les estratègies de millora de poliploides. Quant a les gàmetes no reduïts, s'observen dos mecanismes principals en angiospermes: Restitució en la Primera Divisió (RPD) i Restitució en la Segona Divisió (RSD). D'altra banda, tot i que els portaempelts tetraploids mostren trets agronòmics prometedors, el seu comportament meiòtic i la anàlisi de la seua segregació son encara desconeguts en els cítrics. Els models disómic i tetrasómic es van definir com a models extrems per a la segregació tetraploide, però, el model d'herència intermèdia s'ha descrit per a diversos cultius.

Aquesta tesi ha tingut com a objectius l'estudi de (i) els mecanismes subjacents a la formació de pol·len de gàmetes no reduïts al tangor híbrid diploide `CSO', utilitzat com a progenitor masculí en els programes de millora de triploides 4x x 2x (ii) les freqüències i els mecanismes implicats en la producció gàmetes 2n no reduïts en la femella als genotips de llimona `Eureka Frost´ i Fina' (iii) la recombinació interespecífica i les estructures resultants de gàmetes diploides del doble-diploide de `llima Mèxicana' per avaluar la possibilitat que la hibridació interploid natural potser l'origen de les varietats triploides *C. latifolia* (llima tipus `Tahiti') i *C. aurantifolia* (llima tipus `Tanepao').

La producció de 54 híbrids tetraploides obtinguts d´hibridacions sexuals 4x x 2x va permetre l'anàlisi dels mecanismes de formació de gàmetes no reduïts de pol·len. Els marcadors moleculars SSR i SNP van revelar que la majoria d'aquestes plantes es van obtenir de pol·len 2n no reduït del parental diploide tangor. Llavors, el mètode de màxima probabilitat basat en la restitució de l´heterocigositat parental (RHP) de loci centromèrics va revelar que tant RPD, amb ocurrència predominant, com RSD van ser els mecanismes que condueixen a la formació de gàmetes masculins no reduïts en aquest tangor. Aquestes observacions van ser confirmades per l'anàlisi de patró de RHP al llarg del cromosoma 2. Des del nostre coneixement, aquest és el primer estudi de progènies de cítrics tetraploides derivats de pol·len no reduït i la primera descripció de la coexistència de dos mecanismes de restitució meiòtiques (RPD i RSD) produint pol·len no reduït en els cítrics.

Per tal d'estudiar les freqüències i els mecanismes implicats en la producció de gàmetes 2n sense reduir de la femella, en dos genotips diferents de llimona, vam obtenir 43 híbrids triploides i tetraploides d´hibridacions sexuals 2x x 2x i 4x x 2x utilitzant `Eureka Frost ' i `Fino' com progenitors femenins. Les freqüències de la producció de

gàmetes 2n van ser, respectivament, 4,9% i 8,3%. L'anàlisi de màxima probabilitat i el patró de RHP al llarg del cromosoma 1 van revelar que RSD és el principal mecanisme de gàmetes no reduïts en la llimona utilitzada com parent femení (88%), seguit pels mecanismes RPD o duplicació pre-meiòtica (DPR) (7%) i la duplicació del genoma post-meiòtica (DPM) (5%).

Per primera volta en els cítrics s´ha obtingut un gran nombre de progènie de llimona a partir de gàmetes 2n i s´ha identificat un nou mecanisme, el DPM que poques vegades s'ha descrit en altres espècies herbàcies o llenyoses.

A través dels dos treballs, hem demostrat, a nivell metodològic, l'eficàcia d'utilitzar dos enfocaments complementaris, és a dir, l'anàlisi del patró de RHP en un cromosoma amb el mètode de màxima probabilitat basat en loci centromèrics per distingir entre els diferents mecanismes de la producció de gàmetes no reduïts .

Es van analitzar els mecanismes meiòtics d'un doble-diploide `llima Mèxicana', la recombinació interespecífica i les estructures resultants de gàmetes diploides combinant una anàlisi de segregació de marcadors SSR i SNP, un estudi citogenètic i l'avaluació de la viabilitat del pol·len. Hem arribat a la conclusió que el doble-diploide de `llima Mèxicana' tenia una segregació predominantment disómica en tres cromosomes, herència intermèdia amb tendència disómica en cinc cromosomes i els models intermedis per a un altre. Les estructures resultants de gàmetes diploides interespecífiques mostren alta heterozigositat *C. medica* / *C. micrantha*. Les estructures genètiques revelades dels gàmetes diploides produïts pel doble-diploide de `llima Mèxicana' són compatibles amb la hipòtesi que les varietats triploides `Tahiti´ i `Tanepao´ resulten de la hibridació interploide que impliquen un doble-diploide de tipus `llima Mèxicana'. Aquesta tendència disómica limita la recombinació i la diversitat de la població de gàmetes diploides, però, la restauració de la viabilitat del pol·len observat a nivell tetraploide podria ser avantatjós per a projectes de millora en cultius intensius.

Les implicacions per a projectes de millora triploide, del comportament meiòtic que condueix al pollen no reduït en el tangor `CSO´, els òvuls no reduïts en les llimones i les gàmetes diploides del doble-diploide de llima `Mexicana', es discuteixen en aquesta tesi.

Résumé

L´agrumiculture est une source importante de revenus pour les producteurs à titre individuels ainsi que pour les pays producteurs. L´amélioration génétique de la qualité, et en particulier l´aspermie, est essentielle pour répondre à la demande du marché. En effet, beaucoup de consommateurs préfèrent des fruits sans pépins. La création d´hybrides triploïdes moyennant la manipulation de la ploïdie est une approche très efficace pour sélectionner des variétés sans pépins.

Les hybrides triploïdes d'agrumes peuvent être obtenus par des croisements entre variétés diploïdes en profitant de la formation de gamètes non réduits (2n). Les hybridations inter-ploïdes 2x x 4x et 4x x 2x sont également largement exploitées. La détermination des mécanismes de production de gamètes diploïdes et des structures génétiques en découlant est décisive pour l'optimisation des stratégies d´amélioration des polyploïdes. Deux mécanismes majeurs ont été observés chez les angiospermes pour la production des gamètes non réduits: la restitution de la première division (FDR) et la restitution de la deuxième division (SDR). D'autre part, malgré l´importance agronomique des porte-greffes tétraploïdes d´agrumes, leurs comportements méiotiques et leurs modes de ségrégation sont encore inconnus. Les modèles disomique et tétrasomique ont été définis comme les modèles extrêmes pour la ségrégation des tétraploïdes, mais des modèles de ségrégation intermédiaires ont été décrit pour plusieurs espèces.

Dans ce cadre, cette thèse visait l´étude de trois aspects principaux: (i) mécanismes responsables de la formation des gamètes mâles non réduits pour l'hybride tangor diploïde `CSO´ utilisé comme parent male dans des programmes d´hybridation 4x x 2x, (ii) les fréquences et les mécanismes impliqués dans la production d´ovules non réduits pour deux génotypes de citronnier `Eureka Frost´ et `Fino´, et (iii) la recombinaison interspécifique et les structures génétiques des gamètes diploïdes résultant de la lime `Mexicaine´ diploïde doublée pour vérifier l'hypothèse que des hybridations interploïdes naturels aient pu être à l'origine des variétés triploïdes de *C. latifolia* (lime type `Tahiti´) et *C. Aurantifolia* (lime type `Tanepao´).

La production de 54 hybrides tétraploïdes par croisements 4x x 2x a permis l´analyse des mécanismes responsables de la formation de gamètes non réduits du pollen du tangor `CSO´. Des marqueurs moléculaires SSRs et SNPs ont révélé que la majorité de ces plantes dérivaient de pollen non réduit du parent tangor diploïde. Ensuite, la méthode de maximum de vraisemblance basée sur la restitution de l´hétérozygotie parentale (PHR) des loci centromériques a révélé que le pollen diploïde issu d´une restitution de la première division de la méiose (FDR) était prédominant (64.1%) avec toutefois une proportion non négligeable (18.8%) de 2n pollen issus de SDR. Ces observations ont été confirmées par l'analyse de la distribution de la restitution de l´hétérozygotie parentale (PHR) au niveau du groupe de liaison 2. A notre connaissance, il s'agit du premier rapport sur des hybrides d´agrumes tétraploïdes obtenus à partir de pollen non réduit et une première description de la coexistence de deux mécanismes de restitution méiotique (SDR et FDR) produisant du pollen non réduit chez les agrumes.

Afin d'étudier les fréquences et les mécanismes impliqués dans la production d'ovules non réduits pour deux génotypes de citronnier, nous avons produit 43 hybrides triploïdes et tétraploïdes à partir des croisements 2x x 2x et 2x x 4x en utilisant `Eureka Frost´ et `Fino´ comme parents femelle. Les fréquences de production des gamètes 2n ont été, respectivement, 4,9% et de 8,3%. L'analyse du maximum de vraisemblance basé sur la PHR des loci centromériques et la distribution de la PHR le long du LG1 ont révélé que la majorité des gamètes non réduits résultaient de SDR (88%), suivi par la FDR, le doublement pré-méiotique (PRD) (7%) et le doublement post-méiotique (PMD) (5%).

Il s'agit d´une première description de la production d'un grand nombre d´hybrides polyploïdes de citronnier provenant de gamètes 2n et de la première identification d'un nouveau mécanisme, PMD qui n'avait pas été décrit auparavant chez les agrumes et rarement chez d'autres espèces herbacées ou ligneuses.

Pour les deux études, nous avons confirmé au niveau méthodologique l'efficacité de l'utilisation de deux approches complémentaires (analyse de la distribution de la PHR au niveau d´un LG et méthode de maximum de vraisemblance basée sur des loci centromériques) pour distinguer entre les différents mécanismes pouvant produire des gamètes non réduits.

Nous avons analysé les fonctionnements méiotiques d'un limettier `Mexicain´ diploïde doublée (DD), la recombinaison interspécifique et les structures de gamètes diploïdes en combinant une analyse de ségrégation de marqueurs SSRs et SNPs, une étude cytogénétique et une évaluation de la viabilité du pollen. Nous avons conclu que le limettier `Mexicain´ DD avait une ségrégation essentiellement disomique au niveau de trois groupes de liaison (LGs), intermédiaire avec une tendance disomique pour cinq LGs et intermédiaire pour un LG. Une hétérozygotie *C. medica*/ *C. micrantha* élevée des gamètes diploïdes a été mise en évidence.

Ces structures génétiques des gamètes diploïdes produites par le limettier `Mexicain´ DD sont compatibles avec l'hypothèse proposant que les variétés triploïdes `Tahiti´ et`Tanepao´ résultent d'une hybridation interploïde impliquant un limettier DD de type `Mexicain´. Cette tendance disomique limite la recombinaison et la diversité de la population de gamètes diploïdes. Cependant, la restauration de la viabilité du pollen observée au niveau tétraploïde pourrait être avantageuse pour des programmes d´amélioration du limettier au niveau triploïde.

Les implications pour les programmes d´amélioration des triploïdes des mécanismes méiotique produisant du pollen non réduit pour le tangor `CSO´, des ovules non réduits pour les citronniers et des gamètes diploïdes de la lime `Mexicaine´ DD sont discutés.

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- ABS : Alternaria Brown Spot
- CIRAD: Centre de Coopération Internationale en Recherche Agronomique pour le Développement
- Cl4x: Doubled–Diploid `Clemenules´ Clementine
- CSO: Diploid Hybrid Tangor (clementine x sweet orange)
- CVC: Citrus Variegated Chlorosis
- DC: Distance to the Centromere
- DD: Doubled Diploid
- DR: Double Reduction
- EuFor: Hybridization Between `Eureka Frost´ emon and `Fortune´ mandarin
- EuIch Hybridization between `Eureka Frost´ lemon and *C. ichangensis*
- FCSO: Hybridization between `CSO´ diploid tangor and `Fina´ clementine
- FDR: First Division Restitution
- FinMac: Hybridization between `Fino´ lemon and C. macrophylla
- GMP: Genetic Map Position
- HLB: Huanglongbing Disease
- HTA: Half-tetrad Analysis
- IC: Chromosome Interference Coefficient
- IVIA: Instituto Valenciano de Investigaciones Agrarias
- LG: Linkage Group
- ML4x: Doubled–Diploid `Mexican´ Lime
- MSCO: Hybridization between `CSO´ diploid tangor and `Moncada´ mandarin
- PHR: Parental Heterozygosity Restitution
- PMC: Pollen Mother Cells
- PMD: Post-Meiotic Genome Doubling
- PP: Preferential Pairing
- PRD: Pre-Meiotic Doubling
- SDR: Second Division Restitution
- SNP: Single Nucleotide Polymorphism Markers
- SSR: Simple Sequence Repeat Markers
- UPGMA: Unweighted Pair Group Method with Arithmetic Mean
- τ: Tetrasomic Rate

INTRODUCTION

INTRODUCTION INDEX

I. ECONOMIC IMPORTANCE OF CITRUS

Citrus plants are grown in more than 100 tropical, subtropical and Mediterranean countries and represent the leading fruit crop in the world with more than 137 million tons tons (MT) produced in 2014 with 8.9 million hectares in cultivation. Sweet orange represents a very large part of this production (51.7%), followed by clementines, mandarins and tangerines (21.8%), lemons and limes (11.8%) and grapefruit (6.1%). China is the main producer country followed by Brazil, India, United States of America (USA), Mexico and Spain (FAOSTAT, 2014).

Citrus fruits are used for the fresh fruit market and for processing, mainly for orange juice production. Around 18.6% of citrus fruit production is processed, being Brazil and USA the most important countries covering 75% of the world orange juice markets. For the fresh fruit market, China, Brazil, Mexico, India and Spain are the most important countries, producing around 58% of the total global production (FAOSTAT, 2014).

Spain produced more than 7 million tons in 2014 with a cultivated surface of 300.000 ha, being sweet oranges the 49.5%, 33.8% for mandarins, and 16.7% lemons and grapefruit. Spain exports more than 50% of its citrus fruit production. The Valencia Community is the first Spanish citrus producer with 44.9% of total Spanish citrus production reaching 3.1 million tons and a cultivated surface over 161.000 ha, leaded by sweet oranges (49%), mandarins (43%) and followed by lemons (8%) (GVA, 2015).

II. TAXONOMY, CLASSIFICATION, ORIGIN AND DISTRIBUTION OF CULTIVATED CITRUS

II.1. Taxonomy and classification

The genus *Citrus* was established by Carl Linneaus in 1753 (Swingle and Reece, 1967) within the order *Geraniales*, suborder *Geraniineae*, family *Rutaceae*, subfamily *Aurantioideae*, tribe *Citreae*, and subtribe *Citrinae* which includes *Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus* and *Citrus* genera. The most common citrus cultivars and rootstocks are included in the *Fortunella*, *Poncirus* and *Citrus* genera.
Poncirus includes only the *Poncirus trifoliata* (L.) Raf species and is distinguishable by its trifoliate and deciduous leaves and it could represent the ancestor of the true citrus that spread to the north of China, adapting its morphological and resistance characteristics to extreme conditions of winter cold (Swingle and Reece, 1967). *P. trifoliata* is commonly used as a rootstock in acid soils. Furthermore, due to its resistance to the citrus tristeza virus, it is widely used as a progenitor in rootstock development programs to obtain interspecific hybrids such as citranges (*C. sinensis* x *P. trifoliata*) and citrumelos (*C. paradisi* x *P. trifoliata*).

Fortunella is characterized by small fruits with sweet and edible peel. Its origin is southeast China and is constituted by four species: *F. margarita* (Lour.) Swing., *F. japonica* (Thunb.) Swing., *F. polyandra* (Ridl.) Tan. and *F. hindsii* (Champ.) Swing. They are valued as ornamental plants and used in different breeding programs with the objective to introduce in the progenies cold tolerance and citrus canker and *Phytophtora* resistance (Krueger and Navarro, 2007).

Citrus is the genus with greater economic importance of the subfamily *Aurantioideae*. Two major taxonomic systems for botanical classification are used for this genus. Swingle´s system is relatively simple as it contains 16 species (Swingle and Reece, 1967). It divided *Citrus* into two subgenera, *Eucitrus* and *Papeda*. The former *one* include *C. medica* L., *C. aurantium* L., *C. limon* (L.) Burn. f., *C. aurantifolia* (Christm.) Swing., *C. grandis* (L.) Osb., *C. sinensis* (L.) Osb., *C. reticulata* Blanco., *C. paradisi* Macf., *C. indica* Tan. and *C. tachibana* (Mak.) Tan.

The *Papeda* subgenus include *C. ichangensis* Swing., *C. latipes* (Swing.) Tan., *C. hystrix* D.C., *C. micrantha* Wester., *C. celebica* Koord. and *C. macroptera* Montr. Both subgenera are separated by their morphological characteristics and the chemical components of their flowers, leaves and fruits. *Eucitrus* contains "edible" citrus fruits, while the *Papeda* fruits have high concentrations of acrid oil, rendering them inedible (Spiegel-Roy and Goldschmidt, 1996).

Alternatively, Tanaka´s system divided the *Citrus* genus into two subgenera, *Archicitrus* and *Metacitrus*. They included 162 species and the most important differences in comparison with Swingle´s system affects mandarins, lemons and limes. Indeed, Tanaka subdivided limes to three species, *C. aurantifolia, C. latifolia* Tan. and C. *limettioides* Tan. As for mandarins, Swingle defined only one species *C. reticulata* Blanco, while Tanaka´s system added other species like *C. deliciosa* Ten., *C. unshiu* Marc., *C. clementina* Hort. ex Tan., *C. tangerina* Hort. ex Tan. and *C. nobilis* Lour. (Swingle and Reece, 1967).

From an agronomic point of view, Tanaka´s classification is better adapted to the characteristics of the different agronomic groups, and it is widely used to manage germplasm collections (Krueger and Navarro, 2007).

II.2. Origin and distribution of cultivated citrus plants

Many diversity studies (Tanaka, 1954; Webber *et al.*, 1967; Scora, 1975; 1988) concluded that the center of origin of the majority of citrus species and its related genera was Southeast Asia, especially East India, North Burma, and Southwest China.

The domestication of edible citrus began several thousand years ago, and various biochemical and molecular studies suggested that only citron (*C. medica*), mandarin (*C. reticulata*), pummelo (*C. maxima*) and *C. micrantha* constituted the ancestors of all cultivated *Citrus* species (Nicolosi *et al.*, 2000; Barkley *et al.*, 2006; Ollitrault *et al.*, 2012b; Garcia–Lor *et al.*, 2013b; Curk *et al.*, 2016).

It is well accepted that *C. medica* is native to India and it was the first citrus fruit known by Europeans. However, there are different opinions about the exact period and the steps by which it was first brought from its native land (Nicolosi, 2007). The most probable is following the return of Alexander the Great from India (~300 BC) and then diffused by the Jew civilization in the Mediterranean area (Swingle and Reece, 1967). Its introduction in Spain was around the seventh century (Zaragoza, 2007). As for its genetic origin, Nicolosi *et al.* (2000), combining nuclear and cpDNA data, concluded that *C. medica* is a true species being the male parent in the origin of many hybrid species of *Citrus*, including lemons and limes. Those results have been confirmed by (García-Lor *et al.*, 2013b; Curk *et al.*, 2016).

The earliest record for the mandarin (*C. reticulata*) was in China (2197 - 2205–BC) and in Japan (1278–1346 AD) (Nicolosi, 2007). The first European country to grow the mandarin was England, it was brought from China in 1805 (Nicolosi, 2007). Mandarins were introduced in Italy around the nineteenth century. *C. reticulata* is widely cited as the ancestor of modern cultivated mandarins (Swingle and Reece, 1976). As for its genetic origin, Wu *et al.* (2014) concluded that some mandarin types like `Ponkan´ and `Willowleaf´, result from interspecific introgressions of *C. maxima* (pummelo) into *C. reticulata* (wild mandarin). Later, García-Lor *et al.* (2015) and Curk *et al.* (2015) confirmed that the *C. maxima* genome was the main genome introgressed in the *C. reticulata* background of the mandarin germplasm.

Pummelo is indigenous to the Malayan and East Indian archipelagos (Nicolosi, 2007) and then spread to China, Persia, Palestine (1178 AD) and later in Europe (Spiegel-Roy and Goldschmidt, 1996). Analysis of three Chinese pummelos reported by Wu *et al.* (2014) shows that they derived from domestication of a wild sexual *C. maxima* population.

The four citrus ancestral taxa are fully sexually compatible and all the other cultivated *Citrus* species are considered to originate from hybridization between these ancestral species, (Nicolosi *et al.,* 2000; García-Lor *et al.,* 2013b; Wu *et al.,* 2014; Curk *et al.,* 2016).

The sweet orange (*C. sinensis*) is believed to have originated in Indonesia and southern China (Webber *et al.,* 1967). It was first introduced in Spain first by Italian (1400), then Portuguese, in the middle of the 15th century (Zaragoza, 2007). Genetically, sweet orange is accepted to be an interspecific hybrid (Scora, 1975; Nicolosi *et al.,* 2000; Wu *et al.,* 2014). Barrett and Rhodes, (1976), Torres *et al.* (1978), Scora, (1988), Nicolosi *et al.* (2000) and Moore, (2001) proposed that sweet orange was a direct interspecific hybrid between a pummelo (*C. maxima*) and a mandarin (*C. reticulata*). Whereas recent studies (Roose *et al.,* 2009; Garcia-Lor *et al.,* 2012a, Xu *et al.,* 2013 and Wu *et al.,* 2014) indicated that *C. sinensis* resulted from a backcross 1 (BC1) [(*C. maxima* x *C. reticulata*) x *C. reticulata*]. No more sexual events contributed to the diversification of sweet oranges and moderns orange cultivars share the same genomic organization with little sequences variation (Wu *et al.,* 2014).

The sour orange (*C. aurantium*) is believed to be native to South-east Asia, possibly India (Nicolosi, 2007). The Arab traders introduced the sour orange in Spain towards the fifth and sixth centuries (Zaragoza, 2007). Molecular markers analysis showed that sour orange is a direct hybrid between a pummelo (*C. maxima*) seed parent and a wild mandarin (*C. reticulata*) pollen parent (Wu *et al.,* 2014; Curk *et al.,* 2015).

The exact area of the origin of the lemon (*C. limon*) is still uncertain. Tolkowsky, (1938) suggested that the lemon is native to India while Webber *et al.* (1967) consider southern China and probably Upper Burma to be the native origin of the lemon. The same references affirmed that the lemon had been introduced by the Arabs in North Africa and Spain by 1048-1075 AD (Zaragoza, 2007). Molecular analyses indicate that this species resulted from direct hybridization between *C. aurantium* (sour orange) as the female parent and *C. medica* (citron) as the male parent (Nicolosi *et al.,* 2000; Froelicher *et al.,* 2011; García-Lor *et al.,* 2013b; Curk *et al.,* 2016).

The origin of *C. aurantifolia* is uncertain, but Nicolosi, (2007) proposed that it is native of the Malaysian region of south-western Asia. Molecular data proved that it is a direct hybrid between *C. micrantha* as female parent and *C. medica* as male parent (Scora, 1975; Nicolosi *et al.*, 2000; Ollitrault *et al.*, 2012b; Garcia–Lor *et al.*, 2013b; Curk *et al.*, 2016).

Lime is one of the few citrus species with spontaneous polyploid germplasm. The triploid lime `Tahiti´ (*C. latifolia*) is thought to be a hybrid between a haploid ovule of *C. limon* and a diploid gamete of *C. aurantifolia*, whereas `Tanepao´ (*C. aurantifolia*) triploid genotypes result from a backcross between a diploid ovule of *C. aurantifolia* and *C. medica* (Curk *et al*. 2016).
Tetraploid `Giant Key´ lime, classified as *C. aurantifolia* by Tanaka (1961), is a spontaneous tetraploid selected in a seedling population of the diploid `Key´ lime (`Mexican´ lime type) (Curk *et al*. 2016).

Grapefruit (*C. paradisi*) is a very close species to *C. maxima* and could result from a spontaneous cross between *C. maxima* and *C. sinensis* hybrid produced in the Barbados island (Barrett and Rhodes, 1976; Scora *et al.*, 1982; de Moraes *et al.*, 2007; Ollitrault *et al.*, 2012a). Its initial cultivation in Spain occurred in the first half of the 20th century (Herrero *et al.*, 1996).

III. REPRODUCTIVE BIOLOGY

The citrus flower is perfect containing both female and male sex organs. The structure of the mature flower consists in a pistil in the center of each flower surrounded by, 20-40 stamens, depending on the species (Figure1a).
Citrus fruit is a modified berry (hesperidium) originates as a consequence of single ovary growth (Figure1b,c,d). It consists of 8–16 carpels clustered around the floral axis. The carpels form locules, or segments that contain vesicles (juice sacs) and seeds. The pericarp is divided into exocarp (flavedo) and mesocarp (albedo).

Apomixis is defined as natural replacement of normal sexual reproduction by asexual reproduction, which yields offspring that are genetically identical to the mother plant (Wang *et al.*, 2017). Although three mechanisms of apomixis have been described in plants, the sporophytic adventitious embryony is defined as the mechanism leading to apomixis in citrus (Aleza *et al.*, 2010c). It consists in embryos development initiation directly from the nucellar cells surrounding the embryo sac containing a developing zygotic embryo (Kobayashi *et al.*, 1981; Aleza *et al.*, 2010c). With the exception of citrons, pummelos and clementines cultivars and some mandarin hybrids, the rest of citrus genotypes are apomictic (Aleza *et al.*, 2010c). Recently, Wang *et al*. (2017) identified an insertion of miniature inverted-repeat transposable element in the promoter region of CitRWP gene (one of the 11 candidate genes for apomixis expressed at higher levels in ovules of polyembryonic genotypes) that co-segregates with apomictic seeds. Apomixis allows the conservation of many of the spontaneous mutations and the development of uniform progeny maintaining the characteristics of the mother plant. It is a key character for conform multiplication of rootstocks by seedlings (Cameron and Frost, 1968).
Two types of seeds are distinguishable in citrus depending in the number of embryos, monoembryonic seeds produced by non-apomictic citrus genotypes and polyembryonic

seeds developed by apomictic citrus genotypes. Monoembryonic seeds contain a single embryo of sexual origin, and polyembryonic ones contain an embryo of sexual origin and one or more nucellar embryos. The number of embryos per seed varies greatly depending on the genotype (Frost and Soost, 1968). In seeds of citrus apomictic genotypes, formation of the nucellar embryos can be initiated before fertilization and competition between the zygotic and nucellar embryos often results in the failure of the development of the zygotic one (Frost and Soost, 1968; Wakana and Uemoto, 1988; Koltunow, 1993). This characteristic can be a problem when apomictic genotypes are used as female parents in sexual hybridization. In this context, molecular marker techniques allow the differentiation between zygotic and nucellar seedlings and they have been developed and introduced within citrus breeding programs (Luro *et al., 199*5; Ruiz *et al., 200*0; Aleza *et al.,* 2011). Nevertheless at practical level it is very difficult to recover large population of hybrids using apomictic genotypes as female parents.

Figure 1. Citrus (a) `Pineapple´ flower, (b) `Mexican´ lime pollinated flower and (c;d) fruit set in `Eureka Frost´ lemon and tetraploid *C. clementina* respectively © H. Rouiss-IVIA.

Self-incompatibility is a genetically controlled mechanism by which the genotypes do not produce seeds when they are self-pollinated. Soost (1969) has shown that gametophytic self-incompatibility is the mechanism involved in citrus, which stops the development of the pollen tube in the upper or middle part of the stigma. Nearly all the pummelos, grapefruits and lemons, some mandarins, and several natural or artificial hybrids are self-incompatible (Hearn, 1969). Caruso *et al*. (2012) identified candidate genes involved in self-incompatibility. They suggested that expression of some non-homologous genes located in a restricted genome region could lead to self-compatibility.

IV. MAIN BIOTIC AND ABIOTIC STRESS IN CITRUS

Citrus production is affected by both biotic and abiotic stresses that can severely influence fruit quality and production. In addition, Syvertsen and Levy, (2005) described interactions between both stresses that almost have synergistic effects on citrus.

IV.1. Biotic stress

Although several biotic stress agents are described, six major biotic stresses are considered as threats to the modern citrus industry.

Citrus greening also known as Huanglongbing (HLB) is caused by three uncultured species of α-Proteobacteria, *Candidatus* Liberibacter asiaticus, *Ca*. L. americanus, and *Ca*. L. africanus. Two insect vectors are responsible for the spread of the disease: the African citrus psyllid, *Trioza erytreae* and the Asian citrus psyllid, *Diaphorina citri* (Bové, 2014; Duran-Vila *et al.,* 2014). This disease is present in Asia, South Africa, Brazil and USA and is commonly regarded as the most severe and devastating disease of citrus (Batool, 2007; Gottwald *et al.,* 2007; Tatineni, 2008; Shokrollah, 2010). HLB negatively alters fruit quality and production; it makes fruits unsuitable for juice and fresh market (Bassanezi, 2009; Dagulo, 2010). In USA, the most critical situation is in Florida, where citrus growers are suffering a severe crisis since the identification of the disease in 2005. It caused a loss of 30% from total citrus cultivated superficies in Florida (USDA, 2016) especially in oranges were it destroyed already 20 million orange trees (25%) and caused a 67.4 % decrease of orange production from 2007–08 to 2015–16 seasons (Monzó and Stansly, 2017). The African citrus psyllid, is already present in Spain, in Galicia (Monzó *et al.,* 2015) and the Canary islands (Duran-Vila *et al.,* 2014). However, the citrus greening disease has not been detected yet.

Citrus canker, caused by the bacterium *Xanthomonas citri* pv. citri, is a very important disease in most tropical and subtropical areas where rainfall and warm temperatures are frequent during periods of shoot emergence and early fruit development. Infection can cause defoliation, shoot dieback, and fruit drop (Gottwald et al., 2002). It is present in USA, China, Brasil, Uruguay, Japan, Malaysia and Paraguay (EPPO, 2017). Some areas of the world have eradicated citrus canker like Australia and New Zealand, while the Mediteterranean area remains a free of the disease.

Alternaria Brown Spot (ABS), caused by the fungus *Alternaria alternata* pv. citri, is a major problem for some susceptible mandarin cultivars that induces necrotic brown lesions in fruits, leaves and shoots. The young leaves are very sensitive to the disease, and the appearance of necrotic areas on the leaves is frequent (Akimitsu *et al.,* 2003). The affected fruits present necrotic depressions of variable size and although these lesions only affect the cortex, they commercially depreciate the fruit for fresh consumption (Vicent *et al.,* 2000). This fungus has been detected in all citrus growing areas (Vicent *et al.,* 2000; Timmer *et al.,* 2003; Golmohammadi *et al.,* 2006; Wang *et al.,* 2010) and represents a serious problem in Spain. In fact susceptible cultivars to ABS like `Fortune´, `Nova´ and `Murcott´ mandarins, have been replaced or top-grafted by resistant cultivars. Cuenca *et al.* (2013; 2016) demonstrated that the resistance to ABS is conferred by a recessive locus located within 366 kb region near the centromere of chromosome III.

Citrus Variegated Chlorosis (CVC) symptoms were first observed in, 1984 in Argentina, but not recognized to be CVC until the disease had been characterized in Brazil (He *et al.,* 2000). It is caused by the bacteria *Xylella fastidiosa* subsp. *pauca*, (Almeida *et al.,* 2008). Spain remains a free region however, CVC is producing important damage in Brazil (Ollitrault and Navarro, 2012) being sweet orange varieties more susceptible than limes and grapefruits (Brlansky *et al.,* 2002).

Citrus black spot, caused by *Phyllosticta citricarpa*, (McAlpine) was first described by 1895 in Australia (Carstens *et al.,* 2017). The disease causes external blemishes on the rind which make the fruit unsuitable for the fresh market (Martínez-Minaya *et al.,* 2015). All commercial varieties of sweet orange, mandarin, lemon and grapefruit are susceptible to the disease (Kotzé, 2000; Martínez-Minaya *et al.,* 2015). The pathogen is currently present in the main citrus-growing regions of southern and central Africa, South America and Asia (Kiely, 1948; Kotzé, 2000) however, the Mediterranean Basin is free of the disease (Carstens *et al.,* 2017).

Citrus tristeza virus, is present in most citrus areas, including the Mediterranean countries, usually inducing the quick decline if the used rootstock is sour orange, which is highly susceptible to the decline (Moreno *et al.,* 2008). This decline is associated with a phloem necrosis which blocks translocation of carbohydrates to the root system (Yokomi, 2009). Tristeza virus only infects phloem-associated tissues of species of the genera Citrus and Fortunella within the family Rutaceae (Bar-Joseph *et al.,* 1989). Tristeza has spurred the use of tolerant rootstocks, Poncirus hybrids (Citrange, Citrumelo) to replace sour orange.

IV.2. Abiotic stress

Abiotic stress agents significantly limit citrus production in many areas worldwide. Drought, salinity and soil alkalinity are a major factors reducing citrus production among the world (Álvarez-Fernández *et al.,* 2002; Romero *et al.,* 2006; Ollitrault and Navarro, 2012; Ruiz *et al.,* 2016). The rootstock choice is critical to limit the impact of most abiotic stress.

Drought is considered as a principal factor that limits global citrus production, and its impact depends on the cultivar and rootstock. Citrus can sustain certain levels of water deficit stress (Romero *et al.,* 2006). Drought in citrus trees affects several aspects of plant physiology, such as gas exchanges, hormone relations and mainly water relations (Gomes *et al.,* 2004). Furthermore, it reduces growth and metabolism, leading to a decrease in fruit yield and quality (Gómez-Cadenas *et al.,* 1998; Arbona *et al.,* 2005).

The impact of salinity is critical for citrus, given the preferential accumulation of salts in the aerial part, which may lead to permanent damage of the plantations. The range of salt concentrations tolerated by citrus varies greatly from species to species and citrus are sensitive to specific ions, mainly Cl^- (Maas, 1992; Bañuls *et al.,* 1997). Recently, doubled diploids (DD) selected from different citrus rootstocks, have been studied to evaluate their tolerance to salinity compared to their diploid parent. Ruiz *et al.* (2016) indicated that the lower transpiration rate and thus lower Cl- accumulation in the aerial part are involved in the enhanced salinity tolerance of the tetraploid rootstocks.

Soil alkalinity affects many areas especially in the Mediterranean basin restricting the use of sensitive rootstocks like *P. trifoliata*. Alkaline pH can induce iron deficiency in plants, limiting their absorption and transport (Coulombe *et al.,* 1984; White and Robson, 1990). For this reason, the technique commonly used to avoid iron chlorosis, is to apply to the soil synthetic ferric chelates that are most effective under alkaline conditions (Álvarez-Fernández *et al.,* 2002).

V. MAIN OBJECTIVES OF CITRUS BREEDING PROGRAMS

Systematic oriented breeding programs first began in Florida in 1893 with Swingle and Webber (Davies and Albrigo, 1994). The modern citrus industry is based on grafted plants, with the scion cultivar budded on a rootstock (Khan and Kender, 2007; Ollitrault and Navarro, 2012). So breeders have to keep in mind that scion and rootstock cannot be breaded independently, since many factors can affect the final product such as graft compatibility, fruit quality and productivity (Gmitter *et al.,* 2009; Navarro *et al.,* 2015). Fruit quality, productivity, maturing time and tolerance to diseases are the main objectives of scion breeding programs. For the rootstock, breeders are looking mainly for resistance to soil pathogens and viruses diseases and adaptation to abiotic stress (water deficit, salinity, calcareous soils, etc) (Khan and Kender, 2007; Ollitrault and Navarro, 2012).

V.1. Main objectives of scion breeding programs

For any breeding program, the quality of the final product is the essential criteria. The definition of organoleptic quality can vary according to the consumers. Citrus breeders must therefore endeavor to develop a wide range of varieties likely to meet these diverse demands.

For fresh fruit market, the challenges of new citrus varieties for the next future will be to recover seedless cultivars with high nutritional qualities and health-promoting effects (Ollitrault et al., 2008). Citrus fruits have a multitude of health promoting properties and research has made significant contributions in connecting specific health benefits to a group of secondary metabolites (Butelli *et al.,* 2012; 2017). Otherwise, it´s important to obtain cultivars producing fruits during the whole harvesting season to avoid the need for conservation and in order to fulfill the demand in optimal conditions (Ollitrault *et al.,* 2008; Sdiri *et al.,* 2012; Aleza, 2015).

Resistance to abiotic stress for scion breeding is also a main goal for many breeding programs (Nicotra, 2001). Some diseases cause considerable damages in orchards. Huanglongbing in Asia, South Africa and recently in Brazil and Florida, citrus canker in most tropical and subtropical areas, citrus variegated chlorosis and Sudden death in Brazil. Alternaria also becomes a problem for some mandarin cultivars in several countries, such as `Fortune´ and `Nova´ mandarins and `Murcott´ tangor in Spain. Ranges of varietal susceptibility have been established for most of these diseases and tolerant parents are selected in some breeding programs. (Boscariol *et al.,* 2006; Ollitrault and Navarro, 2012; Cuenca *et al.,* 2013b; 2016).

V.2. Main objectives of rootstocks breeding programs

The need for new rootstocks is of primary concern as they affect all aspects of fruit production and quality (Castle, 2010; Grosser and Gmitter, 2011; Ollitrault and Navarro, 2012). The choice of rootstock breeding objectives is usually based on the specific needs for each region and production area. General considerations as disease tolerance and resistance or soil type are often decisive factors (Gmitter *et al.,* 2009; Ollitrault and Navarro, 2012). Phytophthora and citrus tristeza virus resistance are two essential characters for citrus rootstock.

For instance, for HLB, no ultimate resistance or tolerance has been described in citrus. However, Castle *et al*. (2015) described the `Economical tolerance´ as range of possibilities to breed economic tolerant rootstocks. Grosser and Gmitter, (2014) obtained and are evaluating various tetraploid rootstocks for HLB resistance in Florida. Finally, relative genera, like *Microcitrus* and *Eremocitrus* were reported to be tolerant (Ramadugu *et al.,* 2016). Those genera are compatible with the *Citrus* genera, so they can be used in future breeding programs.

Otherwise, interactions between different types of stress were reported. Salinity stress can inhibit plant defense mechanism against *Phytophthora* (Afek and Sztejnberg, 1993) and decrease root regeneration under pathogen pressure (Syvertsen and Levy, 2005). The development of new rootstocks combining tolerance to biotic (tristeza, *Phytophthora* spp) and abiotic (salinity and alkaline soils) stresses to provide high quality fruits is a major objective for many rootstocks breeding programs.

Other objective for rootstock breeding program is the tree size control for high-density plantings. It was observed that tetraploid rootstocks provided some level of size control (Batra, 1952; Beakbane, 1967). Their potential utility as citrus rootstocks has been suggested in many studies (Cameron and Frost, 1968; Lee, 1988; 1990; Spiegel-Roy and Goldschmidt, 1996; Grosser and Gmitter, 2014) because it allows efficient production and reducing costs (Grosser *et al.,* 2007). Some programs to improve citrus rootstocks at the tetraploid level have been initiated worldwide (Ollitrault *et al.,* 2008; Grosser and Gmitter, 2011; Dambier *et al.,* 2011; Ruiz *et al.,* 2016).

VI. CITRUS BREEDING: STRATEGIES, TOOLS AND MAIN RESULTS

Several serious obstacles exist and complicate the conventional sexual citrus breeding, as the complex reproductive biology (Navarro, 2005; Gmitter *et al.,* 2009; Navarro *et al.,* 2015), high heterozygosity (Ollitrault and Faure, 1992; Gmitter *et al.,* 2009; Navarro *et al.,* 2015), pollen and ovule sterility, incompatibility and long juvenile period (Khan and Kender, 2007). The main strategies used for citrus breeding are identification and clonal selection of spontaneous mutation observed in orchards, induced mutations in elite genotypes, or sexual hybridizations taking advantage of biotechnology tools developed in citrus to solve the problems found in conventional breeding.

VI.1. Spontaneous mutations

It is the oldest and the most efficient breeding method since most of the varieties cultivated worldwide arose from this process (Aleza, 2015). Spontaneous mutation are

identified on adult material, so the obtained genotypes do not display juvenile characteristics (Ollitrault and Navarro, 2012; Aleza, 2015). Additionally, those new cultivars usually have the general characteristics of the parent, with specific change in traits concerning harvesting period, size or fruit color.
The frequency of spontaneous mutations in the field is relatively high and easy to find, especially if the mutation affects fruit morphological characteristics. (Vardi *et al.*, 2008; Ollitrault and Navarro, 2012; Henrique *et al.*, 2016).
In the Mediterranean area, mainly in Spain, many new clementine varieties have been selected, for their harvesting period and fruit size and color. Similar results have been obtained for Satsuma mandarins in Japan and sweet oranges in Australia and South Africa (Aleza, 2015).

VI.2. Induced mutagenesis

Since 1935, various mutagenesis agents have been used to obtain new cultivars, being gamma irradiation the most common method (Aleza, 2015). The main advantage of this method is the preservation of the main genetic background of the initial cultivar and the modification of only one or a small number of traits. Other plus of this technology is its simplicity (it is not necessary previous knowledge on gene control traits), rapidity (resulting trees will not display juvenile phase) and inexpensiveness. The main disadvantages are the large populations needed to find desirable stable mutations and the frequent chimeric status of the mutations. `Star Ruby´ grapefruit was the first commercial cultivar obtained by irradiating seeds of `Hudson´ grapefruit; later, `Rio Red´ grapefruit was obtained by irradiation of `Ruby Red´ grapefruit (Hensz, 1971). This technique is mainly used for obtaining diploid seedless genotypes and there are many examples of recently released seedless cultivars like `Nulessin´ and `Nero´ clementines from `Clemenules´ clementine, `Mor´, `Moria´, Murta´ and `Murina´ from `Murcott´ tangor, `Orri´ from `Orah´ mandarin, `Tango´ from `Nadorcott´ tangor, `Moncalina´ from `Moncada´ mandarin, `DaisySL´ from `Daisy´ mandarin, `Mandanova´ from `Nova´ mandarin and `FairchildSL´ from `Fairchild´ mandarin.

VI.3. Sexual breeding

Diploidy is the general rule in *Citrus* and its related genera, with a basic chromosome number x=9 (Krug, 1943). However, some triploid and tetraploid genotypes have been early detected in citrus germplasm or seedlings (Longley, 1925; Lapin, 1937; Iwasaki, 1943). Conventional breeding in citrus has important limitations due to the complex reproductive biology of these species. Most genotypes are apomictic, and the development of zygotic embryos is hampered by the presence of the nucellar embryos. So in practice apomictic genotypes are avoided as female parents in most breeding programs. Furthermore, male and female sterility, and self and cross-incompatibility are relatively common among many genotypes, which limit the possibilities to select parents for specific crosses.

Once obtained, sexual hybrids display a long juvenile period and they need to undergo a transition from the juvenile to the reproductive phase that often goes on more than six years (Krajewski and Rabe, 1995). This characteristic of the citrus juvenile plants is one of the most important constraints for citrus breeding programs. In addition, there is a lack of knowledge of the genetic mechanisms that control the main organoleptic and

pomological traits. All this aspects complicate the breeding schemes over several generations. (Ollitrault *et al.,* 2008; Navarro *et al*, 2005; 2015; Aleza, 2015).

The absence of seeds is appreciated in citrus fruits because many consumers do not accept seedy fruits. Seedlessness can contribute to the increase of some fruits quality when when seeds are hard or have a bad taste. In the case of citrus, seeds are associated with unfavorable aromatic compounds and bitterness (Ollitrault *et al.,* 2007b). Thus, the breeding for seedless cultivars has become a major goal in many citrus breeding programs around the world either at diploid or triploid levels (Recupero *et al.,* 2005; Ollitrault *et al.,* 2007b; Roose and Williams, 2007; Aleza *et al.,* 2010a,b; Cuenca *et al.,* 2010; Navarro *et al.,* 2015).

VI.3.1. Sexual breeding for seedlessness at diploid Level

Sexual breeding is mainly used for diversification of the main important genotypes. Most of the obtained diploid hybrids are fertile and thus seedy, which limit the use of this methodology. Moreover, sterility was managed at diploid level to obtain seedless hybrids. Indeed, most of the commonly cultivated diploid citrus species have some degree of ovule or pollen sterility (Frost, 1948). Male and female sterility may be due to different genetic factors such as, sterility genes, and chromosomal abnormalities like reciprocal translocations and inversions (Iwamasa, 1966; Iwamasa and Iwasaki, 1963; Del Bosco *et al.,* 1999; Ollitrault *et al.,* 2012). The strongest female sterility reported in citrus concern the `Mukaku Kishu´ mandarin (*C. kinokuni* hort. Ex. Tanaka) caused by an arrested seed development at early stage (Yamasaki *et al.,* 2009).

Male sterility in `Eureka´ lemon and `Mexican´ lime is induced by chromosome aberration (Nakamura, 1943; Iwamasa and Iwasaki, 1963; Iwamasa, 1966) and reciprocal translocation is the main cause of male sterility in `Valencia´ sweet orange (Iwamasa, 1966). In satsuma and `Encore´ mandarins different genetic analysis have been performed with the objective to study the process of anther abortion. Different works have been published (Iwamasa, 1966; Nakano *et al.,* 2001; Yamamoto *et al.,* 2001) indicating that this male sterility is produced by a nucleo-cytoplasmic interaction and is probably controlled by more than two major nuclear genes. `Kiyomi´ tangor and `Queen´ mandarins are two hybrids with satsuma as female parent and both genotypes produce androsterile flowers.

The gametophytic self-incompatibility described in some citrus genotypes (Soost, 1969) avoids the development of pollen tube in the style of the pistil. These genotypes produce seedless fruits if growing in isolation, but if compatible cultivars are planted in the proximity seedy fruits can be produced as a consequence of their female fertility and cross-pollination. This situation occurs, for example, with clementines and other mandarin genotypes like `Nadorcott´ tangor. Despite those limitations, seedless or low seed diploid hybrids have been obtained in breeding programs with importance at commercial level in different countries, such as `Gold Nugget´ and `Kiyomi´ mandarins (Aleza, 2015).

VI.3.2. Sexual breeding for seedlessness at triploid Level

Triploid plants are generally considered an evolutionary dead-end, since they, commonly, give rise to aneuploid gametes with very low fertility (Otto and Whitton,

2000) due to the trivalents and univalent associations that are formed during meiosis of citrus triploid hybrids (Cameron and Frost, 1968). Moreover, abortion of the megasporogenesis between the embryo-sac first divisions and the fertilized egg cell is common (Fatta del Bosco *et al.,* 1992). For these reasons, citrus triploid hybrids are generally sterile, although they can occasionally produce fruits with very few seeds. An important exception concern the triploid *C. aurantifolia* lime (`Tanepao´ type) producing seedy fruits.

Different triploid breeding program were started during the eighties and nineties of the last century with the objective to produce new high quality seedless triploid cultivars. Citrus triploid plants can be obtained by 2x x 2x (Esen and Soost, 1971; 1973a; Ollitrault *et al.,* 2008; Aleza *et al.,* 2010b; Cuenca *et al.,* 2015; Navarro *et al.,* 2015) or 2x x 4x and 4x x 2x sexual hybridizations (Esen and Soost, 1973b; Cameron and Burnett, 1978; Starrantino and Recupero, 1981; Ollitrault *et al.,* 2008; Grosser and Gmitter, 2011; Aleza *et al.,* 2012a, b; Navarro *et al.,* 2015). Sexual hybridization between diploid parents exploits natural events of polyploidization such as unreduced gametes, using embryo rescue and flow cytometry to select triploid hybrids (Ollitrault *et al.,* 1996, 2007b; Aleza *et al.,* 2010a,b). Another strategy is to cross diploid non-apomictic female parents with tetraploid male parents (Starrantino and Recupero, 1981). Such tetraploid plants can be obtained in apomictic seedlings as a consequence of the spontaneous duplication of chromosomes in nucellar cells (doubled-diploids (Aleza *et al.,* 2011), by somatic hybridization (Grosser *et al.,* 2000; 2010a) or by artificial chromosome doubling by colchicine or oryzalin treatments. The application of the last technique to monoembryonic parents opens the avenue to 4x x 2x crosses with tetraploid female parents. This strategy displays the highest efficiency for triploid hybrids production in comparison with the other two (Aleza *et al.,* 2012b).

Several triploid hybrids recovered in breeding programs have been released worldwide. The first two selections of the IVIA (Instituto Valenciano de Investigaciones Agrarias) triploid breeding program (Navarro *et al.,* 2015) were released in, 2008: `Garbi´ and `Safor´ mandarins (Aleza *et al.,* 2010a; Cuenca *et al.,* 2010). More than 600.000 plants of both mandarins have been sold in the last years (Aleza, 2015). Otherwise, `Shasta Gold®´, `Tahoe Gold®´ and `Yosemite Gold®´ triploid mandarins were released by the Riverside breeding program in California (Roose *et al., 200*2; Williams and Roose, 2004); and other triploid mandarins like `Tacle´, `Clara´, `Mandared´ and `Mandalate´ mandarins and `Lemox´ lemon were released by Istituto Sperimentale per l´Agrumicoltura di Acireale, Italy (Starrantino and Recupero, 1981; Russo *et al.,* 2004; Recupero *et al.,* 2005).

More details on unreduced gametes and tetraploid meiosis and resulting genetic structure of unreduced and diploid gametes are given in sections VII, Sexual and somatic citrus polyploidy.

VI.3.3. Tetraploid citrus genetic pool diversification

Scarcity of tetraploid parents represents a major limitation to create triploid hybrids from interploid crosses. Thus, several research groups are working to diversify the tetraploid gene pool by identification and selection of spontaneous tetraploids (doubled-diploids; Aleza *et al.,* 2009b, 2011)) and somatic hybridization by protoplast fusion (Grosser *et al.,* 2000; Ollitrault *et al.,* 2007a; Navarro *et al.,* 2015).

Doubled-diploids

Doubled-diploids are obtained through two ways, either selection of spontaneous tetraploids and induction of tetraploid plants with antimitotic chemicals. Spontaneous tetraploidization seems to occur frequently in apomictic citrus genotypes. Frost and Soost, (1968) and Kobayashi *et al.* (1981) proposed that chromosome doubling in nucellar tissue might be the general mechanism underlying this process. It was confirmed by Aleza *et al.* (2011). This characteristic could be under genetic control (Barrett and Hutchison, 1978) and/or affected by environmental conditions (Hutchison and Barrett, 1981; Aleza *et al.*, 2011).

At IVIA (Spain) and in the CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement-France) breeding programs, many doubled-diploids plants have been identified analyzing a high number of seedlings by flow cytometry. The handicap of this methodology is the juvenile period displayed for the obtained plants which limit their direct use for hybridization (Navarro *et al.*, 2015).

In non-apomictic citrus genotypes spontaneous tetraploidization does not occur. Aleza *et al.* (2009) established a new technique to produce autotetraploid genotypes based on in vitro shoot-tips grafting combined with colchicine or oryzalin treatment and selection of non-chimeric plants by flow cytometry. This methodology gave rise to stable non apomictic tetraploid plants of different mandarins (Aleza *et al.*, 2009b; Navarro *et al.*, 2015). This methodology, allows to recover plants without juvenile characters (Navarro *et al.*, 2015) permitting consequently its use for triploid hybrids production through 4x x 2x and 2x x 4x sexual hybridizations.

Sexual hybrids

Sexual tetraploid hybrids were reported in 2x x 4x sexual hybridizations (Tachikawa *et al.*, 1961; Cameron and Soost, 1969; Esen and Soost, 1972; Ollitrault *et al.*, 2008). Esen and Soost (1972) suggested that they were originated from unreduced female gametes fertilized by diploid pollen. It was confirmed later by Aleza *et al.* (2012a) with molecular markers. In 4x x 2x hybridizations, Aleza *et al.* (2012b) displayed the production of tetraploid hybrids originated by self-pollination. However, until now, no tetraploid hybrids have been recovered in 4x x 2x sexual hybridizations by fertilization of diploid female gametes with unreduced male gametes.

These tetraploid hybrids are of great value for triploid cultivars and tetraploid rootstock breeding programs.

Somatic hybrids

Somatic hybridization is an important tool in citrus breeding programs (Ollitrault *et al.*, 2008; Grosser *et al.*, 2010; Grosser and Gmitter, 2011; Navarro *et al.*, 2015; Aleza, 2015). Somatic hybrids can be obtained through the bind of two non-sexual cells, one from an embryonic callus and the other from a mesophyll-cell. The fusion is carried out by the use of polyethylene glycol (PEG) or by electro-fusion. In case of symmetric fusion, somatic hybrids are tetraploid plants possessing the entire genetic configuration of their parents with no recombination (Louzada *et al.*, 1993; Ollitrault *et al.*, 2007a;

Grosser *et al.,* 2010; Grosser and Gmitter, 2011; Dambier *et al.,* 2011; Aleza *et al.,* 2016b). One advantage of this methodology is the possibility to produce allotetraploid somatic hybrids between sexual incompatible genotypes and to produce new genetic combinations overcoming the complex citrus reproductive biology (Grosser and Gmitter, 1990; Grosser *et al.,* 2000; Ollitrault *et al.,* 2007a; Dambier *et al.,* 2011; Grosser and Gmitter, 2011; Aleza *et al.,* 2016b).

In citrus, the most important application of somatic hybridization is the production of autotetraploid and allotetraploid hybids that can be used either as rootstocks or as tetraploid parents in interploid sexual hybridizations for the production of seedless triploid cultivars (Grosser and Gmitter, 2005; Ollitrault *et al.,* 2007a; Grosser *et al.,* 2010).

VI.4. Biotechnological tools for citrus breeding

VI.4.1. Genetic transformation

Applications of the biotechnological techniques such as genetic engineering are useful for the genetic improvement of many of the citrus cultivars avoiding the barriers of the traditional sexual hybridization (Gmitter *et al.,* 1992; 2009; Peña *et al.,* 2001; 2008; Pons et *al.,* 2011; Navarro *et al.,* 2015). The most used methods are through *Agrobacterium tumefaciens* or PEG (polyethylene glycol) treatment of protoplasts (Peña *et al.,* 2008). This methodology can open the way to the introduction of specific traits associated with a known characters into elite genotypes without altering their genetic background. Genetic engineering has been experimentally applied to an increasing number of traits to try inducing resistance to the CTV virus (Soneji *et al.,* 2007; Soler *et al.,* 2012), tolerance to HLB disease (Dutt *et al.,* 2016) and repellency to its psyllid vector (Alquézar *et al.,* 2017), and to enhance tolerance to salinity (Cervera *et al.,* 2000) or for reproductive biology investigation purposes (Pons *et al.,* 2011). In addition, genetic transformation has been used in attempts for the introgression of seedlessness to some elite genotypes as `Mexican´ lime (Koltunow *et al.,* 2000) and `Ponkan´ and `Valencia´ sweet orange (Li *et al.,* 2002; 2003).

VI.4.2. Genome Editing

Biotechnological tools are developed for genome engineering through edition and they are expected to take place in all fields of future plant breeding. It is a set of molecular tools for cells, tissues and whole organism editing (Barrangou and Doudna, 2016). Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9/single guide RNA (sgRNA) have been already successfully used to obtain genetically modified rice, wheat, Arabidopsis, tobacco and sorghum (Nekrasov *et al.,* 2013; Shan *et al.,* 2013). In citrus, genetic modification has been used either for general objectives like gene function detection (Jia and Wang, 2014) or for specific objectives as citrus canker resistance (Peng *et al.,* 2017).

VI.4.3. Viral Vectors

Plant virus vectors have been used for both expression of foreign genes (Gleba *et al.,* 2007) and suppression of endogenous target genes by virus-induced gene silencing (VIGS) in the infected plants (Senthil-Kumar and Mysore, 2011). Velázquez *et al.*

(2016) developed a viral vector to promote the transition from vegetative to the reproductive phase in juvenile citrus plants by expression of *Arabidopsis thaliana* or citrus FLOWERING LOCUS T (FT) gene based on *Citrus leaf blotch* virus vector. Triploid citrus hybrids were inoculated with this viral vector. All of them flowered in one year from the inoculation date (Aleza *et al.,* 2016c) providing helpful tool to speed up genetic studies and breeding programs.

VI.4.4. Genetic and genomic resources

Molecular markers are specific DNA sequence transmitted by the standard laws of inheritance from one generation to the next. Numerous forms of molecular markers have been developed for *Citrus*. Isozymes markers (Torres *et al.,* 1978; 1982; Roose, 1988), Random Amplified Polymorphic DNA [RAPDs; (Luro *et al.,* 1994)], Sequence Characterized Amplified Regions [SCARs; (Nicolosi *et al.,* 2000)], Restriction Fragment Length Polymorphism [RFLPs; (Federici *et al.,* 1998)], Intersimple Sequence Repeat [ISSRs, (Fang *et al.,* 1997)], Amplified Fragment Length Polymorphism (AFLPs; (Liang *et al.,* 2007; Pang *et al.,* 2007) and Cleaved Amplified Polymorphic Sequences (CAPs) from ESTs (Lotfy *et al.,* 2003) were used mainly for diversity studies.

Since Kijas *et al*. (1995), SSRs or microsatellites markers have been introduced in Citrus genetic studies. They are often helpful for phylogenetic studies (Luro *et al.,* 2001; Garcia-Lor *et al.,* 2012; Garcia-Lor *et al.,* 2013b), somatic hybrids characterization (Aleza *et al.,* 2016b), discrimination between zygotic and nucellar seedlings (Ruiz *et al.,* 2000; Ruiz and Asins, 2003), control of the origin of plants obtained by induced gynogenesis (Froelicher *et al.,* 2007), molecular characterization of triploid cultivars (Cuenca *et al.,* 2010), the analysis of the origin of unreduced gametes (Luro *et al.,* 2004; Chen *et al.,* 2008; Cuenca *et al.,* 2011; Aleza *et al.,* 2016a), mapping (Ollitrault *et al.,* 2012a), and marker assisted selection in breeding (Cuenca *et al.,* 2016). The homoplasic phenomena (identical allelic size arising from independent genetic events) observed by Barkley *et al.* (2009) can limit the use of those markers for phylogenetic studies.

The availability of large set of sequencing data has opened the way for SNP (Single Nucleotide Polymorphism) marker development (García-Lor *et al.,* 2012a; Ollitrault *et al.,* 2012a; 2012b; García-Lor *et al.,* 2013b). SNP markers have been used for genetic diversity studies (Chen and Gmitter, 2013; Cuenca *et al.,* 2013a; Garcia-Lor *et al.,* 2013a and b, Curck *et al.,* 2016), marker assisted selection (MAS) for resistance against *Alternaria alternata* (Cuenca *et al.,* 2013b, 2016), discrimination of zygotic and nucellar plants in seedlings (Zhu *et al.,* 2013) and mapping (Ollitrault *et al.,* 2012a).

At polyploid level, SSR markers have been used in citrus to infer the genetic origin and allelic configurations of triploid and teraploid hybrids using markers with total differentiation between the parents. Indeed, conclusive results can be obtained using only one marker. Otherwise, in case of shared alleles between parents for a given marker, the allele dosage of the obtained triploid and tetraploid hybrids could be estimated by the MacPr method (REF) validated in citrus by Cuenca *et al.* (2011). SNPs are also very useful for the identification of allele doses in heterozygous triploid and tetraploid hybrids as described by Cuenca *et al*. (2013a). SNP genotyping could be performed using the KASPar technique. This methodology allows the identification of

allele doses in heterozygous triploid and tetraploid hybrids via the relative allele signals (Cuenca *et al.*, 2013a).

Ollitrault *et al.* (2012a) published the clementine reference genetic map and later, Aleza *et al.* (2015) located the centromere positions for all LGs. This genetic map has been used to enable the chromosome assembly of the reference whole genome citrus sequence (Wu *et al.*, 2014), that used a haploid clementine for sequencing (Aleza et al., 2009a).

Xu *et al.* (2013) sequenced and assembled the dihaploid genome of sweet orange. Recently Wang *et al.* (2017) published the draft genomes of a citrus relative species, *Atalantia buxifolia*, *C. ichangensis, C. medica* and *C. maxima* (Wang *et al.*, 2017). The availability of these whole genome sequences provides a valuable genomic resource for citrus genetics and breeding improvement.

VII. SEXUAL AND SOMATIC CITRUS POLYPLOIDY

Polyploidy is a very common phenomenon in plants, particularly in angiosperms, where 60-70% of the species have a polyploid ancestor (Grant, 1981; Van de Peer *et al.*, 2009). Even the first polyploid was discovered over a century ago (Strasburger, 1910), the genetic and evolutionary implications of polyploidy are still being studied and discussed (De Storme and Geelen, 2013b). Many cultivated species are polyploid; potato varieties include triploids, tetraploids and pentaploids. Oats are hexaploid, wheat species are tetraploid and hexaploid, banana is triploid and strawberry species and hybrids can be diploid, tetraploid, pentaploid, hexaploid, heptaploid, octoploid, or decaploid. Polyploidy also exists in wild species such as oak and bluegrass (Wendel, 2000). Several anatomic and physiologic characters that advantage polyploidy species against stress and adaptation to environmental conditions were attributed to polyploidy (Warner and Edwards, 1989; 1993; Li *et al.*, 1996; Ramsey, 2011; Manzaneda *et al.*, 2012).

For breeding, there are many opportunities for exploitation of polyploidy as a valuable tool (Ortiz, 1997; Ollitrault *et al.*, 2008; Cuenca *et al.*, 2015). The two main mechanisms of polyploid formation are somatic doubling of chromosome set (somatic polyploidization) and meiotic nuclear restitution leading to unreduced gamete production (sexual polyploidization).

VII.1. Unreduced gametes

Unreduced gametes formation is widespread across numerous eukaryotic taxa, including yeasts, plants, insects, amphibians, reptiles, and fish (Dowling and secor, 1997; Brownfield and Köhler, 2011; Mable *et al*., 2011; Albertin and Marullo, 2012). Indeed, several studies suggested that the majority of polyploidization events in both plants and animals have been produced from unreduced gametes (Ramsey and Schemske, 1998; Husband, 2004; Ramsey, 2007). Environmental stress often promote the formation of unreduced gametes, suggesting that these may facilitate polyploid speciation in response to changing environments, thus, it can be considered as a mechanism for evolutionary speciation (Mason and Pires, 2015).

The normal meiosis involves DNA replication followed by two rounds of chromosome division to produce cells with half the chromosome number of the mother cell. In the first meiotic division, the homologous chromosomes are separated, so it is called a reductional division. For the second meiotic division, it involves the separation of sister chromatids and referred as an equational division. Unreduced gametes arise through meiotic defects so called meiotic nuclear restitution. It was described for the first time by Rosenberg, (1927) and up to seven major mechanisms of unreduced gamete formation have been cytogenetically characterized: pre-meiotic doubling (PRD); post-meiotic doubling (PMD); first-division restitution (FDR); chromosome replication during the meiotic interphase; second-division restitution (SDR); indeterminate meiotic restitution and apospory (Peloquin *et al.,* 1989; Lim *et al.,* 2001; Dewitte *et al.,* 2012). Although 2n gamete formation through pre-meiotic genome doubling is rare in plants, it has been observed in *Solanum lycopersicum* by De Storme and Geelen, (2013b). The post-meiotic restitution is characterized by the formation of fully homozygous 2n gametes after an extra round of genome duplication. This mechanism was observed in *Solanum tuberosum* (Bastiaanssen *et al.,* 1998) in some *Rubus* species (Dowrick, 1966) and in Alstroemeria (Ramanna and Jacobsen, 2003).

FDR and SDR mechanisms are considered the principal mechanisms of 2n gamete formation (Bretagnolle and Thompson, 1995; Tavoletti *et al.,* 1996; Cai and Xu, 2007). These mechanisms arise through meiotic defects. If an equational mitosis of all chromosomes occurs in the first division instead of a reductional mitosis, an FDR 2n gamete will be produced. As a result, the non-sister chromatids are included in the same gamete (Gallais, 2003; Park *et al.,* 2007; Cuenca *et al.,* 2011). Alternatively, if the first mitosis occurs normally, but an omission of the second meiotic division occurs, an SDR 2n gamete will be produced with sister chromatids included in the same gamete (Gallais, 2003; Park *et al.,* 2007; Cuenca *et al.,* 2011). The cytological processes leading to meiotic restitution can be divided in three classes: alterations in spindle biogenesis and polarity, cytokinetic defects and complete omission of a meiotic cell division (De Storme and Geelen, 2013a).

The identification of the mechanisms underlying 2n gametes formation is complex. Cytological techniques have been used initially to determine the mechanism of 2n gamete formation by genomic in situ hybridization-GISH or/and fluorescent in situ hybridization-FISH (Lim *et al.,* 2001; Crespel and Gudin, 2003; Dewitte *et al.,* 2012). However, the small and indistinguishable citrus chromosomes and the small frequency of unreduced gametes represented a major constrain for these techniques in citrus (Barba Gonzalez *et al.,* 2005; Jaskani *et al.,* 2007). In contrast, molecular marker analysis have been proved as a very helpful tool to estimate the heterozygosity restitution in the unreduced gametes in polyploid progenies and paves the way for the identification of mechanism underlying 2n gametes formation (Barone *et al.,* 1995; Vorsa and Rowland, 1997; Bastiaanssen *et al.,* 1998; Barcaccia *et al.,* 2003; Luro *et al.,* 2004; Chen *et al.,* 2008; Hayashi *et al.,* 2009).

The unreduced gametes frequency is under genotype and environmental control. The genetic control of 2n gamete formation has been observed in peach, *Medicago sativa*, *Trifolium pratense and S. tuberosum* (Dermen, 1938; Mok and Peloquin, 1975; Parrott and Smith, 1986; Tavoletti *et al.,* 1996). In addition, it was observed that interspecific and intergeneric hybrids produce unreduced gametes more frequently than their parents (Ramsey and Schemske, 1998) supporting the idea that the underlying cytological

anomalies mentioned above are regulated by a monogenic allele (Bretagnolle and Thompson, 1995; Ortiz, 1997).

Bretagnolle and Thompson, (1995) added that the unreduced gamete formation is sex specific leading to probably a different mechanism for each sex. d´Erfurth *et al.* (2008) identified the protein AtPS1 from *A. thaliana* (PARALLEL SPINDLES 1) that induces a restitution of male meiosis (up to 65%), and not in the female. Recent studies have revealed a genetic background for the pre-meiotic genome doubling. Two proteins have been found to be involved in meiotic ploidy control: the 40S ribosomal protein (rp) S6 kinases S6K1 and S6K2 (De Storme and Geelen, 2013a).

The different mechanisms of 2n gamete formation have different genetic consequences and particulary affects the transmission of the parental heterozygosity in relation to centromere distance. FDR 2n gametes contain non-sister chromatids, which in the absence of crossover maintain the parental heterozygosity. When crossing over occurs, the parental heterozygosity restitution (PHR) rates vary from 100% for loci close to the centromere to 60–70% for loci far from the centromere, depending on the level of chromosome interference (Cuenca *et al.*, 2011). For SDR, the 2n gametes contain two sister chromatids, which reduces the parental heterozygosity level (Bastiaanssen *et al.*, 1998; Cuenca *et al.*, 2011; De Storme and Geelen, 2013b). When crossing over occurs, the PHR rate varies from 0% for loci close to the centromere to 60–75% for loci far from the centromere, depending on the level of chromosome interference (Cuenca *et al.*, 2011).

Pre-meiotic genome doubling produces 2n gametes equivalent to the meiosis of doubled diploid genotypes. Therefore, PHR depends mainly on the chromosomal preferential pairing rate (Stift *et al.*, 2008), which should vary between 66% for fully tetrasomic meiosis to 100% for fully disomic meiosis. Little variation can occur along the chromosome due to double reduction events. In the case of post-meiotic doubling, haploid gametes undergo an extra round of genome duplication, leading to the formation of fully homozygous 2n gametes (Bastiaanssen *et al.*, 1998; Ramanna and Jacobsen, 2003; De Storme and Geelen, 2013b; Cuenca *et al.*, 2015). Thus, 100% homozygosity for all loci is expected among the 2n gametes (Ramanna and Jacobsen, 2003). SDR can also produce 100% homozygosity for centromeric markers, but not for telomeric ones (Cuenca *et al.*, 2011). Therefore, in order to distinguish between both mechanisms, Cuenca *et al.* (2015) genotyped telomeric loci to determine whether diploid gametes fully homozygous for centromeric markers resulted from post-meiotic doubling or SDR. Alternatively, Bastiaanssen *et al.* (1998) obtained hybrids originated from fully homozygous 2n female gametes, they used RFLP markers to prove the existence of recombination of homozygous alleles originated from its ancestor´s parents for the same linkage group (LG), and thus they concluded that it originated from post-meiotic genome doubling.

Molecular marker analysis is used for the estimation of parental PHR through unreduced gametes in polyploid progenies (Barone *et al.*, 1995; Vorsa and Rowland, 1997; Luro *et al.*, 2004; Bastiaanssen *et al.*, 1998; Cuenca *et al.*, 2011; 2015). Most of the previously developed methodologies are based on the genetic analysis of a high number of random molecular markers. Markers with PHR lower than 50% indicate that the progeny was originated by SDR (Park *et al.*, 2007) whereas with PHR over 50%, for

all analyzed markers, no definitive conclusion between SDR or FDR can be obtained without previous knowledge of their genetic distance to the centromere. Tavoletti *et al.,* (1996) developed a multilocus maximum-likelihood method of half-tetrad analysis (HTA) to estimate the relative frequencies of FDR and SDR that was also useful for mapping centromere position. In citrus, taking advantage of the centromeres location (Aleza *et al.,* 2015) in the reference genetic map (Ollitrault *et al.,* 2012a), Cuenca *et al.* (2015) developed a maximum-likelihood methodology to identify the unreduced gamete formation mechanism both at the population and individual levels using independent centromeric markers.

Comparing to other species, citrus produce a high percentage of unreduced gametes. Aleza *et al.* (2010a) and Cuenca *et al.* (2011) reported that several citrus species produce unreduced gamete in frequencies ranging from 1% to over, 20%. As a consequence, triploid plants (2n=3x=27) can be obtained through unreduced gametes and it has been used in several plant-breeding programs for the development of seedless commercial citrus varieties (Ollitrault *et al.,* 1996; 2008; Navarro *et al.,* 2005;2015; Aleza *et al.,* 2010a,b; 2011; 2012a,b).

Untill recently, only two mechanisms have been detected in citrus, FDR and SDR, and many published papers affirmed that SDR mechanism is the main mechanism of 2n megagametophytes in citrus (Esen *et al.,* 1979; Luro *et al.,* 2000; Cuenca *et al.,* 2011, 2015; Aleza *et al.,* 2016a). Chen *et al.* (2008) proposed FDR mechanism as the principal mechanism for the 2n eggs formation in sweet orange and Ferrante *et al.* (2010) in lemon. However, their results may be questionable because they were based on the analysis of a few numbers of individuals, with few markers and without previous knowledge of centromere location. Later, Cuenca *et al.* (2015) revealed that FDR-2n gametes were implicated in three over 543 triploid hybrids analyzed (0.6 %), one in `Ellendale´ tangor and two in `Fortune´ mandarin.

A few cases of unreduced pollen gametes have been reported in citrus although that the the most important naturally cultivated triploid variety (`Tahiti´ lime) was probably originated through unreduced pollen from a diploid `Mexican´ lime (Curk *et al.,* 2015). Luro *et al.* (2004) identified a few triploid hybrids produced by 2n pollen from three different mandarins, `King´, `Hansen´ and `Ananas´, `Star Ruby´ grapefruit and `Tarroco Rosso´ and `Sanguinelli´ sweet oranges. Later, Chen *et al.* (2008) recognized triploid hybrids resulting from 2n-pollen of *P. trifoliata* in hybridizations with sweet orange. Recently, Honsho *et al.* (2016), identified giant pollen grains in `Nishiuchi Konatsu´ mandarin (*C. tamurana* Hort. ex Tanaka) and based in single-pollen genotyping, revealed that FDR was the mechanism for the 2n pollen gamete formation, although no plants were recovered.

VII.2. Diploid gametes produced by tetraploid plants

The main function of meiosis, in a generative reproduction concept, consists in creation of genetic variability during pairing, recombination, and segregation (Sybenga, 2012). In diploids, each pair of homologous chromosomes consists of one chromosome inherited from the female parent and the other one from the male parent. Chromosome association tends to align with strict bivalents formation (Otto, 2007). The increase of chromosome number by tetraploidization events results in duplicated sets of chromosomes. There is a basic classification of tetraploid plants based on the

origin and type of chromosomes, autopolyploids and allopolyploids. Autopolyploids are produced from a variation of ploidy within a single species, and chromosomes display the same type and have the same origin. In contrast, allopolyploids contain two differentiated genomes, through the process of interspecific hybridization and subsequent chromosome doubling, being different the type and the origin of the chromosomes (Gallais, 2003).

In allotetraploids, there are two sets of homologous chromosomes and during meiosis, each chromosome pairs only with its homologous (Sybenga, 2012), and only bivalents are formed (Stebbins, 1947). It results in a disomic inheritance with 100% of the interspecific heterozygosity transmitted by each gamete (Stift *et al.*, 2008). In autotetraploids, the presence of four homologous chromosomes instead of two, results in equal opportunities to pair at meiosis leading to multivalent formation and tetrasomic inheritance (Jackson and Jackson, 1996; Sybenga, 1996). For autotetraploid (doubled diploids), tetrasomic inheritance leads, hypothetically, to 66% of restitution of the heterozygosity of the diploid that originated the tetraploid (Sanford *et al.*, 1983; Aleza *et al.*, 2016a). Allo and autotetraploids (with disomic and tetrasomic inheritance, respectively) are the extremes of a range. In cases where parents are divergent but have retained enough homology to prevent exclusive preferential pairing, inheritance patterns intermediate `segmental patter´ between di– and tetrasomic can be expected (Stebbins, 1947; Sybenga, 1996; Stift, *et al.*, 2008; Jeridi *et al.*, 2012). Many different studies (Danzmann and Bogart, 1983; Hickok, 1978; Stift *et al.*, 2008; Kamiri *et al.*, 2011; Jeridi *et al.*, 2012) have displayed that polyploid taxa presented inheritance patterns intermediate among disomic and tetrasomic.

Newly formed auto- and allopolyploids exhibit considerable meiotic complexity, including, double reduction (DR), asynapsis, inversions, reciprocal translocation and the production of unbalanced gametes (Sybenga, 1975).

In *Rutaceae*, Froelicher *et al*. (2000) were the first to study the meiotic behavior of the tetraploid *Clausena excavata*, belonging to the subfamily Aurantioideae, using molecular markers and displayed a strict disomic inheritance. Later, interspecific somatic hybrids has been studied (Fatta Del Bosco *et al.*, 1999; Chen *et al.*, 2004; Kamiri *et al.*, 2011; Xie *et al.*, 2015). For example, Del Bosco *et al*. (1999), Chen *et al*. (2004) and Kamiri *et al*. (2011) analyzed various allotetraploid somatic hybrids. The results obtained using cytogenetic techniques and molecular markers segregation was compatible with tetrasomic and intermediate between disomic and tetrasomic inheritance. Aleza *et al*. (2016a) produced an artificial doubled-diploid clementine by colchicine treatment. Molecular marker analysis revealed tetrasomic segregation although three LGs displayed intermediate segregation and one LG had a tendency for disomy.

OBJECTIVES

Ploidy manipulation is an attractive strategy in the modern citrus breeding programs aiming to obtain triploid and tetraploid plants. The creation of triploid hybrids is an important breeding strategy to develop new seedless citrus commercial varieties and tetraploid plants can be used as parents for triploids recovery through interploid sexual hybridizations. Rootstock breeding at tetraploid level is also considered as promising for increased adaptation to biotic and abiotic stresses.

In the last years, new methodologies have been developed enhancing the knowledge about genetics of citrus polyploid plants, especially in some mandarin elite cultivars. However it is necessary to continue enhancing the research and knowledge about genetic of citrus polyploid plants with economic relevancy like lemons and limes.

Sexual polyploidization by 2n female gametes is a relative frequent event in citrus and it has also been used worldwide for triploid breeding by 2x x 2x sexual hybridizations. SDR mechanism has been identified as the main mechanism of 2n female gametes formation in mandarins although very few FDR 2n gametes have been described. Nevertheless a small number of unreduced pollen gametes have been reported in citrus. In the framework of our triploid breeding program several 4x x 2x hybridizations have been performed. Among these hybridizations, numerous tetraploid progenies have been recovered in two 4x x 2x sexual hybridizations suggesting the occurrence of 2n pollen gametes. These progenies are of great value to study 2n pollen gametes, the mechanism underlying 2n gametes formation and their implications in citrus triploid breeding programs based on sexual polyploidization.

Other mechanisms leading to unreduced gamete formation have been described, such as pre-meiotic and post-meiotic genome doubling and both mechanisms have rarely been documented in plants. These mechanisms produce 2n gametes with different genetic structure. Pre-meiotic genome doubling originate 2n gametes equivalent to the meiosis of doubled diploid genotypes and PHR depends mainly on the chromosomal preferential pairing rate. In the case of post-meiotic genome doubling, haploid gametes undergo an extra round of genome duplication, leading to the formation of fully homozygous 2n gametes. Lemon is a direct hybrid between two genetically distant genotypes, sour orange and citron, and the specific origins of the homozygous alleles can easily be distinguished and recombination analysed. For this reason, 2n gametes produced by different genotypes of lemons can be used as a good model to test if other mechanism of 2n gametes can occur in citrus plants.

Somatic polyploidization is a relative frequent event in citrus and adventitious embryony from nucellar cells is the apomictic mechanism involved in citrus, so tetraploid plants can be produced by spontaneous duplication of chromosomes in nucellar cells. Some of these tetraploid are used for interploid breeding for the production of seedless varieties and “tetrazyg” hybridization for rootstock breeding. Understanding the meiotic behavior of these DD parents is fundamental to optimize these breeding strategies. Indeed, there are two extreme models for diploid gametes produced by tetraploid plants, disomic and tetrasomic, although some intermediate model have also been described. Lime is the only *Citrus* horticultural group with natural triploid germplasm. Spontaneous DD occurs and it was proposed that *C. aurantifolia* and *C. latifolia* triploid varieties resulted from the combination of a diploid gamete of a DD *C. aurantifolia* (`Mexican´ lime type) with haploid gametes of *C. medica* and *C. limon* respectively. Limes market is currently very important and it has increased

dramatically since eighties of the last century. However, lime production is based on a very narrow genetic basis including a few diploid and triploid cultivars and varietal diversification is needed. Knowledge about meiosis of tetraploid `Mexican´ lime is a key step for the development of new lime triploid varieties and it would greatly improve the efficiency of triploid lime breeding programs. It is moreover an interesting model for DD meiotic study considering the important genomic differentiation between its two ancestral progenitors: *C. micrantha* and *C. medica*.

The specific objectives of this PhD thesis are the following:

Objective 1: To define the mechanisms underlying unreduced pollen gamete formation in 4x x 2x sexual hybridizations.

In citrus there are no evidences about progenies obtained by 2n pollen gametes. In this PhD thesis we have recovered two different progenies of tetraploid hybrids by 4x x 2x sexual hybridizations. We have analysed:

1. The origin of tetraploid progenies in tetraploid clementine by diploid tangor hybridization
2. The mechanisms underlying 2n pollen gamete formation in the diploid tangor
3. The implications of the identified mechanisms in citrus triploid breeding programs.

Objective 2: To define the frequencies and the mechanisms involved in the unreduced gametes production in two different genotypes of lemon

For lemon, the frequencies and the mechanisms of unreduced gametes production have been poorly studied. In this work, we have analyzed:

1. The frequencies of 2n gamete formation in two different genotypes of lemon, `Eureka Frost´ and `Fino´.
2. The mechanisms leading to 2n gamete formation in these two genotypes.
3. The implications in lemon breeding programs based on sexual polyploidization by 2n gametes.

Objective 3: To gain knowledge about meiosis of the doubled diploid `Mexican´ lime and the implications for lime triploid breeding programs.

Doubled-diploid `Mexican´ lime is a direct hybrid of two genetically distant species, *C. medica* and *C. micrantha*. Cytogenetic and molecular marker analysis of the DD `Mexican´ lime meiosis would greatly improve the efficiency of lime triploid breeding programs. In this context, we have analysed:

1. The inheritance model, disomic, tetrasomic or intermediate segregation, of the DD `Mexican´ lime
2. The interspecific recombination pattern and the genetic structure of the resulting diploid gametes
3. The possibility that the `Tahiti ´ and `Tanepao´ limes types derived from interploid hybridization based on their phylogenomic structure and the ones of the diploid gametes produced by the DD `Mexican´ lime.

The manuscript is structured in three chapters, corresponding to published or submited scientific articles as follows:

CHAPTER 1. **Tetraploid citrus progenies arising from FDRand SDR unreduced pollen in 4x x 2x hybridizations.** *Tree Genetics & Genomes* (2017) 13:10

CHAPTER 2. **Unreduced Megagametophyte Production in Lemon Occurs via Three Meiotic Mechanisms, Predominantly Second-Division Restitution.** *Frontiers in Plant Science***.** *Frontiers in Plant Science* (2017) doi: 10.3389/fpls.2017.01211.

CHAPTER 3. **Doubled diploid `Mexican´ lime display preferential disomic segregation compatible with interploid crosses origin of *C. Latifolia* and *C. aurantifolia* triploid limes.** *Annals of Botany***,** Submitted

CHAPTER I

Tetraploid citrus progenies arising from FDR and SDR unreduced pollen in 4x x 2x hybridizations

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Abstract

Polyploid citrus arise by somatic and sexual polyploidization, and both have been used in triploid breeding programs. Sexual polyploidization is mainly achieved by First Division Restitution (FDR) or Second Division Restitution (SDR) meiotic restitution mechanisms. In citrus, mostly SDR producing 2n ovule has been described. However, we obtained 72 tetraploid hybrids from 4x x 2x sexual hybridizations using two doubled-diploid mandarins as female parents (`Moncada´ mandarin and `Fina´ clementine) and a diploid hybrid tangor as male parent (clementine x sweet orange - `CSO´) suggesting 2n pollen formation. This material was used to confirm the existence of 2n pollen in Citrus and to analyze its origin. SSR and SNP molecular marker analyses revealed that 64 out of the72 recovered tetraploid plants resulted from the fertilization of a reduced diploid female gamete by unreduced (diploid) pollen from `CSO´, whereas eight tetraploid plants arose from self-pollination of the tetraploid parent. The maximum-likelihood method based on parental heterozygosity restitution (PHR) of centromeric loci identified both FDR and SDR as the mechanisms leading to unreduced male gamete formation. From the 64 unreduced gametes produced by diploid `CSO´ tangor, 41 (64.1%) were originated by FDR, whereas 12 (18.8%) were significant for SDR. Non-conclusive results were obtained for 11gametes (17.2%). The pattern of PHR variation of markers located along the linkage group 2 confirmed our results at population level. To our knowledge, this is the first report of tetraploid citrus progenies arising from unreduced pollen and the first description of the coexistence of two meiotic restitution mechanisms (SDR and FDR) producing unreduced pollen in citrus.

Keywords
Citrus, First-division restitution, Second-division restitution, SSR and SNP markers, Parental Heterozygosity Restitution

Introduction

Polyploidy is an important pathway for plant evolution and speciation (Gallais, 2003). Although the first polyploid was discovered over a century ago (Strasburger, 1910), the genetic and evolutionary implications of polyploidy are still being studied and discussed (De Storme and Geelen, 2013). On a practical level, there are many opportunities for exploitation of polyploidy as a valuable tool in plant breeding programs (Ortiz, 1997; Ollitrault *et al*., 2008; Cuenca *et al*., 2015). The two main mechanisms of polyploid formation are somatic doubling of chromosomes (somatic polyploidization) and meiotic nuclear restitution leading to unreduced gamete production (sexual polyploidization). In somatic polyploidization, chromosome restitution occurs during mitosis and all the chromosomes of a somatic cell are included in one daughter nucleus, giving rise to a cell with a doubled chromosome number (Carputo *et al*., 2003). Sexual polyploidization is originated through gametic non reduction, including three different mechanisms to produce unreduced gametes (De Storme and Geelen, 2013); pre and post-meiotic genome doubling, and meiotic restitution. Pre and post-meiotic doubling mechanisms are not as frequent an event in plants, whereas meiotic restitution have been identified in several, and is the main mechanism of 2n gamete formation (De Storme and Geelen, 2013). Occurrence of unreduced gametes have been observed in potato (Mok *et al*., 1975; Mendiburu and Peloquin, 1977 a, b), *Achillea borealis* (Ramsey, 2007), *Ipomoea trifida* (Iwanaga *et al*., 1991), *Brassica spp*. (Mason *et al*., 2011), *Anthoxanthum alpinum* (Bretagnolle, 2001), *Musa spp.* (Ortiz, 1997) *Dactylis* (Maciera *et al*., 1992), *Rosa spp* (Zlesak, 2009), maize (Rhoades *et al*., 1966), *Populus* (Liesebach *et al*., 2015) and *Citrus* (Frost and Soost, 1968; Esen and Soost, 1971; Geraci *et al*., 1975, Cuenca *et al*., 2015). If an equational mitosis of all chromosomes occurs in the first division instead of a reductional mitosis, a First-Division Restitution (FDR) will be produced. As a result, the non-sister chromatids are included in the same gamete (Gallais, 2003; Park *et al*., 2007; Cuenca *et al*., 2011). Furthermore, if the first mitosis occurs normally, but an omission of the second meiotic division occurs, a Second Division Restitution (SDR) will be produced with sister chromatids included in the same gamete (Gallais, 2003; Park *et al*., 2007; Cuenca *et al*., 2011). These two mechanisms gave rise to highly diverse genetic structures of gamete populations and therefore of breeding material. Understanding the origin and mechanisms underlying unreduced gamete formation open an exciting way to convert this knowledge into practical benefits for plant breeding programs (Brownfield and Köhler, 2011).

Citrus and related genera of *Aurantioideae* are generally diploid, usually x = 9 (Krug, 1943), but some higher euploid genotypes are extant in the citrus germplasm. The most common euploid variations are triploids and tetraploids (Lee, 1988). Citrus polyploidy occurs through somatic or sexual polyploidization. Adventitious embryony from nucellar cells is the apomictic mechanism involved in citrus, so tetraploid plants can be produced by spontaneous duplication of chromosomes in nucellar cells (Aleza *et al*., 2011). Also, in citrus, artificial tetraploid plants have been obtained with antimitotic chemicals like colchicine and oryzalin (Aleza *et al*., 2009b). Tetraploid plants have been used as parents in 2x x 4x and 4x x 2x interploid hybridizations (Cameron and Burnett, 1978; Starrantino and Recupero, 1981; Ollitrault *et al*., 2008; Grosser and Gmitter, 2011; Aleza *et al*., 2012a, b) with the objective to produce triploid seedless cultivars that are desirable for the fresh-fruit market. Sexual polyploidization by 2n female gametes is a relative frequent event in citrus (Esen and Soost, 1971; Luro *et al*., 2004; Aleza *et al*., 2015; Cuenca *et al*., 2015) and it has also been used for triploid breeding

by 2x x 2x sexual hybridizations (Esen and Soost 1971; 1973; Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Cuenca *et al*., 2015). SDR mechanism has been identified by Cuenca *et al*. (2015) as the main mechanism of 2n female gametes formation in mandarins. From 543 2n gametes analyzed, only three triploid plants were obtained by FDR-2n female gametes and no 2n pollen gametes were identified. A few cases of unreduced pollen gametes have been reported in citrus. Luro *et al*. (2004) reported that 2n pollen gametes production is a rare event in citrus (less than 2%). Recently, Honsho *et al*. (2012; 2016), identified giant pollen grains in `Nishiuchi Konatsu´ mandarin (*Citrus tamurana* Hort. ex Tanaka) and based in Single-pollen genotyping, revealed that FDR is the mechanism for the 2n pollen gamete formation, although no plants were recovered. Within the framework of the triploid breeding program carried out at IVIA since 1995, (Navarro *et al*., 2015) several 4x x 2x hybridizations have been performed (Aleza *et al*., 2012b). Among these, numerous tetraploid progenies have been recovered in two sexual hybridizations between tetraploid female parents and a diploid hybrid between clementine and sweet orange [*C. sinensis* L. Osb.; hereafter referred to as `CSO´ tangor] used as pollinator, suggesting the frequent occurrence of 2n pollen in `CSO´ tangor.

Different methodologies have been used for the identification of the mechanism underlying unreduced gamete formation. Cytological techniques were the first to be used (Karlov *et al*., 1999), but the small size of the chromosomes, like in citrus, is the major handicap for the implementation of these techniques (Barba-Gonzalez *et al*., 2005; Jaskani *et al*., 2007). Molecular marker analysis is used for the estimation of parental heterozygosity restitution (PHR) through unreduced gametes in polyploid progenies (Barone *et al*., 1995; Vorsa and Rowland, 1997; Luro *et al*., 2004; Bastiaanssen *et al*., 1998; Cuenca *et al*., 2011, 2015). Most of the previously developed methodologies are based on the genetic analysis of a high number of random molecular markers. Markers with PHR lower than 50% indicate that the progeny was originated by SDR (Park *et al*., 2007) whereas with PHR over 50%, for all analyzed markers, no definitive conclusion between SDR or FDR can be obtained without previous knowledge of their genetic distance to the centromere. Tavoletti *et al*. (1996) developed a multilocus maximum-likelihood method of Half Tetrad Analysis (HTA) to estimate the relative frequencies of FDR and SDR that was also useful for mapping centromere position. In citrus, taking advantage of the centromeres location (Aleza *et al*., 2015) in the reference genetic map (Ollitrault *et al*., 2012a), Cuenca *et al*. (2015) developed a maximum-likelihood methodology to identify the unreduced gamete formation mechanism both at the population and individual levels using independent centromeric markers.

The objective of this work was to confirm the hypothesis of 2n pollen formation in `CSO´ tangor and to analyze the mechanisms underlying 2n pollen formation. We applied the maximum likelihood methodology based on centromeric molecular markers to identify the unreduced gamete formation mechanisms at individual level and validated our results by the analysis of PHR along one linkage group. Finally we discuss the applications and implications of such mechanisms in citrus triploid breeding programs.

Materials and Methods

Plant material

Tetraploids of 'Moncada' mandarin(*C. clementina* x (*C. unshiu* x *C. nobilis*)) and `Fina´ clementine (*C. clementina*) were obtained at IVIA by shoot-tip grafting *in vitro* combined with colchicine treatment as described by Aleza *et al*. (2009). They are therefore doubled diploid genotypes. They were pollinated with `CSO´ diploid tangor. Hereafter hybridization between `Moncada´ mandarin and `Fina´ clementine by diploid `CSO´ tangor we referred to as MCSO and FCSO respectively. Ploidy level analysis of the obtained plants were performed by flow cytometry as described in Aleza *et al*. (2012b). Respectively, ten and 62 tetraploid plants were recovered from normal seeds in the MCSO and FCSO hybridization. These 72 tetraploid plants and their parents were used in this work.

SSR and SNP genotyping

The female and male parents together with the progenies recovered from both 4x x 2x sexual hybridizations were genotyped using SSR and SNP markers. The markers are distributed across the nine LGs of the clementine genetic map (Ollitrault *et al*., 2012a). Markers that indicated heterozygosity for the `CSO´ tangor and polymorphism with `Moncada´ mandarin and `Fina´ clementine were selected and used for the progenies genotyping (Table 1.1). As `CSO´ is a hybrid between clementine and sweet orange and, as clementine is a hybrid itself between `Common´ mandarin and sweet orange (Ollitrault *et al*., 2012a; Garcia-Lor *et al*., 2012; Wu *et al*., 2014), it was difficult to find heterozygous markers for `CSO´ tangor with polymorphism with clementine. Ninety-eight SSR and six SNP markers were tested and 100 new centromeric SSR markers were designed. From the 100 new centromeric SSR markers designed, only 5AT21 and 9TAA22 SSR markers, located in LGs 5 and 9, respectively, displayed the appropriate configuration to be used in this study (Table 1.1). We found a total of twelve SSR and four SNP markers with adequate allelic configuration between parents from the 198 SSR and six SNP markers as tested.

DNA from leaves of the recovered plants and their parents was isolated using the Plant DNAeasy kit from Qiagen Inc. (Valencia, CA, USA), following the manufacturer´s protocol. PCR amplifications, using SSR markers, were performed using a Thermocycler rep gradient S (Eppendorf®) in 10 µL final volume containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng/mL of citrus DNA, 0.2 mM of wellRED (Sigma®) dye-labelled forward primer, 0.2 mM of non dye-labelled reverse primer, 0.2 mM of each dNTP, 10X PCR buffer and 1.5 mM MgCl2. The PCR protocol was as follows: denaturation at 94°C for 5 min followed by 40 repeats of 30 s at 94°C, 1 min at 50°C or 55°C, 45 s at 72°C; and a final elongation step of 4 min at 72°C. Capillary electrophoresis was carried out using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc.). PCR products were initially denatured at 90°C for 2 min, injected at 2 kV for 30 s and subsequently separated at 6 kV for 35 min. Alleles were sized, based on a DNA size standard (400 bp). The GenomeLab™ GeXP v.10.0 genetic analysis software was used for data collection. Allele dosage was calculated using the MAC-PR (microsatellite DNA allele counting-peak ratio) method (Esselink *et al*., 2004), validated in citrus by Cuenca *et al*. (2011).

Table 1.1. Information on used molecular markers with their Gene Bank or Phytozome accesion, position in the Clementine reference genetic map (Ollitrault *et al*., 2012b), parental genotypes and bibliographic references

Locus	Gene Bank/ Phytozome Accesion	Linkage Group	Genetic map *locus* position (cM)	Distance to centromere (cM)	Noted alleles [1]			Bibliographic reference
					Clementine	Moncada	CSO	
CIBE5720	ET082224	1	58,45	2,2	325-337	329-337	325-340	Ollitrault *et al*. (2010)
MEST539	DY294904	1	61,82	1,2	104-108	98-104	104-108	In preparation
mCrCIR03C08	FR677576	2	82,19	25,3	208-226	221-225	208-212	Cuenca *et al*. (2011)
CIBE6006	ET084205	2	124,01	67,1	176-200	176-200	197-200	Ollitrault *et al*. (2010)
2p21022555	Ciclev10018135 m.g	2	57,00	0,1	A-A	T-T	A-T	Curk *et al*. (2015)
CX6F23	CF417259	2	49,53	7,3	149-161	149-161	155-161	Chen *et al*. (2006)
mCrCIR04H06	FR677579	2	23,65	33,2	190-196	190-196	184-196	Cuenca *et al*. (2011)
3p35931624	Ciclev10023979 m.g	3	95,10	4,5	G-G	G-G	G-A	This manuscript [2]
TC01	CK934237	3	96,00	5,4	333-348	329-333	333-351	In preparation
CF-ACA01	CN181701.1	4	24,41	8,3	335-338	335-335	335-338	In preparation
5AT21	none	5	17,53	5,6	254-254	254-262	240-254	This manuscript [3]
CiC4356-06	ET111465	6	6,21	0,2	C-T	C-C	C-T	Ollitrault *et al*. (2012b)
mCrCIR01C06	FR692356	6	88,92	82,5	133-165	131-165	159-165	Cuenca *et al*. (2011)
Ci07C07	AJ567409	7	98,02	1,6	228-240	228-234	228-240	Froelicher *et al*. (2008)
LCY2-M-376	FJ516403	8	58,10	3,9	A-G	G-G	A-G	Ollitrault *et al*. (2012b)
9TAA22	none	9	62,57	10,4	150-203	151-157	164-203	This manuscript [4]

1. Noted alleles. The numbers indicate the size of alleles in nucleotides for SSR markers and letters correspond to SNP markers
2. SNP flanking sequence:
GAAGAGTTTCTTCTTAACAGTGGCCAAATTTTTCGAGTGGCCTGTGACAA[G/A]TACGGAAACTATGTGATTCAAAC AGCATTGATCGAGACAATGCGACCGAA
3. Primer´s sequence of 5AT21 SSR marker: Forward: TGGTAGAAAATGTTGAATTGACG, Reverse: AATCAAATTGGCTTTTTGGAA.
4. Primer´s sequence of 9TAA22 SSR marker: Forward: ATGACGACCCACCAAAGAAA, Reverse:

Parents and progenies were also genotyped with SNP markers using KASPar technology by LGC Genomics (http://www.lgcgenomics.com). The KASPar™ Genotyping System is a competitive, allele-specific dual Förster Resonance Energy Transfer (FRET)-based assay for SNP genotyping. Primers were designed by LGC Genomics Company based on the SNP locus flanking sequence (approximately 50 nt on each side of the SNP). SNP genotyping was performed using the KASPar technique. Detailed explanation on specific conditions and reactives can be found in Cuppen, (2007). Identification of allele doses in heterozygous tetraploid hybrids has been carried out from the relative allele signals as described by Cuenca *et al*. (2013a).

Data analysis

Identification of the origin of tetraploid plants and inference of the unreduced gamete genotype

Determination of the origin of tetraploid plants was performed by molecular marker analysis. The two hypotheses tested were (i) self-fertilization of the tetraploid female parent and (ii) fertilization by a diploid pollen of the diploid `CSO´ tangor. Markers with total differentiation between the parents (A1A1A1A1 x A2A2; A1A1A1A1 x A2A3, A1A1A2A2 x A3A4) were more useful for this purpose as only one marker was sufficient to conclude. For MCSO hybridization, we have used the mCrCIR03C08 SSR marker. However, for FCSO hybridization, as `CSO´ is a hybrid itself of clementine, the two parents share at least one allele and five kinds of allelic configurations encountered from the two parents (A1A1A1A1 x A1A1; A1A1A2A2 x A1A2; A1A1A2A2 x A1A1; A1A1A1A1 x A1A2: A1A1A2A2 x A1A3). Only the last two configurations provide the opportunity to demonstrate fertilization by diploid `CSO´ tangor and were used for the FCSO hybridization. The probability that the specific allele of the `CSO´ parent does not pass to the tetraploid progeny in case it arise from an unreduced pollen of `CSO´ is 0.5. With independent marker, the probability that such an event being unidentified, decreases to 0.5^n.
When it was demonstrated that a tetraploid plant resulted from the fertilization by unreduced pollen of CSO, we performed the inference of the unreduced gamete genotype for the markers in heterozygosity for CSO. For a locus bearing completely different parental allelic configurations (case of MCSO hybridization), A1A1A2A2 x A3A4 and A1A1A1A1 x A3A4, the genotype of the unreduced gamete was deduced directly from the observation of the A3 and A4 alleles in the tetraploid hybrids. When the male and female genitor shared one allele (A1A1A1A1 x A1A2 and A1A1A2A2 x A1A3), for the tetraploid hybrids that have inherited the common allele from the female genitor, the inference of the unreduced male gamete structure was carried out from the estimated allele dosage in the tetraploid hybrid.

Mechanism of unreduced gamete formation

Once 2n pollen of CSO were identified, the maximum-likelihood method developed by Cuenca *et al*. (2015) was used to identify the mechanism of unreduced gamete formation at population and individual levels using PHR values of centromeric markers.
Taking advantage of the reference genetic map of clementine (Ollitrault *et al*., 2012a) and the centromeres location (Aleza *et al*., 2015), three SSR markers (CIBE5720, 5AT21 and 9TAA22) and two SNP markers (2p21022555 and 3p35931624), located in five different LGs (1, 2, 3, 5and 9 respectively), were used to identify the mechanism underlying 2n gametes formation in tetraploid plants recovered from FCSO hybridization while seven SSR markers (MEST539, CX6F23, JC-TC01, CF-ACA01, 5AT21, Ci07C07 and 9TAA22) and two SNP markers (CiC4356-06 and LCY2-M-376) located in the nine LGs, were used for MCSO tetraploid plants (Table 1.1). All markers used are located less than 10 cM from the centromere. At individual level, the probabilities of a heterozygous or a homozygous diploid gamete occurring at a locus, under the two models (SDR/FDR), were calculated. Next, LOD values were estimated from the probabilities of a marker being inherited as heterozygous or homozygous under the SDR or FDR mechanisms (LOD=log (pSDR/pFDR). LOD

scores greater than 2 or below −2 were considered as thresholds indicating that SDR and FDR, respectively, were the mechanism involved in the single unreduced gamete formation. For LOD scores between 2 and −2, no significant conclusions were considered (Cuenca *et al*., 2015).

Pattern of PHR along the LG 2 for the unreduced pollen population

Genetic analysis with markers distributed along a LG can also be used to identify the mechanism underlying 2n gamete formation (Park *et al*., 2007), as we have previously corroborated in `Fortune´ mandarin (Cuenca *et al*., 2011) and clementine (Aleza *et al*., 2015). Thus, as a complementary study of the centromeric markers analysis, we analyzed the PHR pattern along the LG2 in the 2n pollen gamete progenies with four SSR markers (mCrCIR04H06, CX6F23, mCrCIR03C08 and CIBE6006) and one SNP marker (2p21022555). mCrCIR04H06 and CIBE6006 are telomeric markers, 2p21022555 is located very close to the centromere and the last two markers are located between telomere and centromere at each side of the LG.

Population diversity organization

Population diversity organization was examined by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis. DARwin5 software (Perrier and Jacquemoud-Collet, 2006) was used to compute the simple matching dissimilarity index (di-j) between pairs of *loci* (units):

$$d_{i-j} = 1 - \frac{1}{L}\sum_{l=1}^{L}\frac{m_l}{\pi}$$

where d_{i-j} is the dissimilarity between units *i* and *j*, *L* is the number of *loci*, and m_l is the number of matching alleles for *locus l*. Then the UPGMA tree was computed with MEGA 6 software from the dissimilarity matrix.

Results and Discussion

Genetic origin of tetraploid hybrids recovered from 4x x 2x sexual hybridizations

Tetraploid plants from MCSO hybridization were first analyzed with CIBE5720 and mCrCIR03C08 SSR markers. Results revealed that they arise from the fertilization of diploid female gametes by unreduced pollen gametes of the `CSO´ diploid tangor (Figure 1.1).

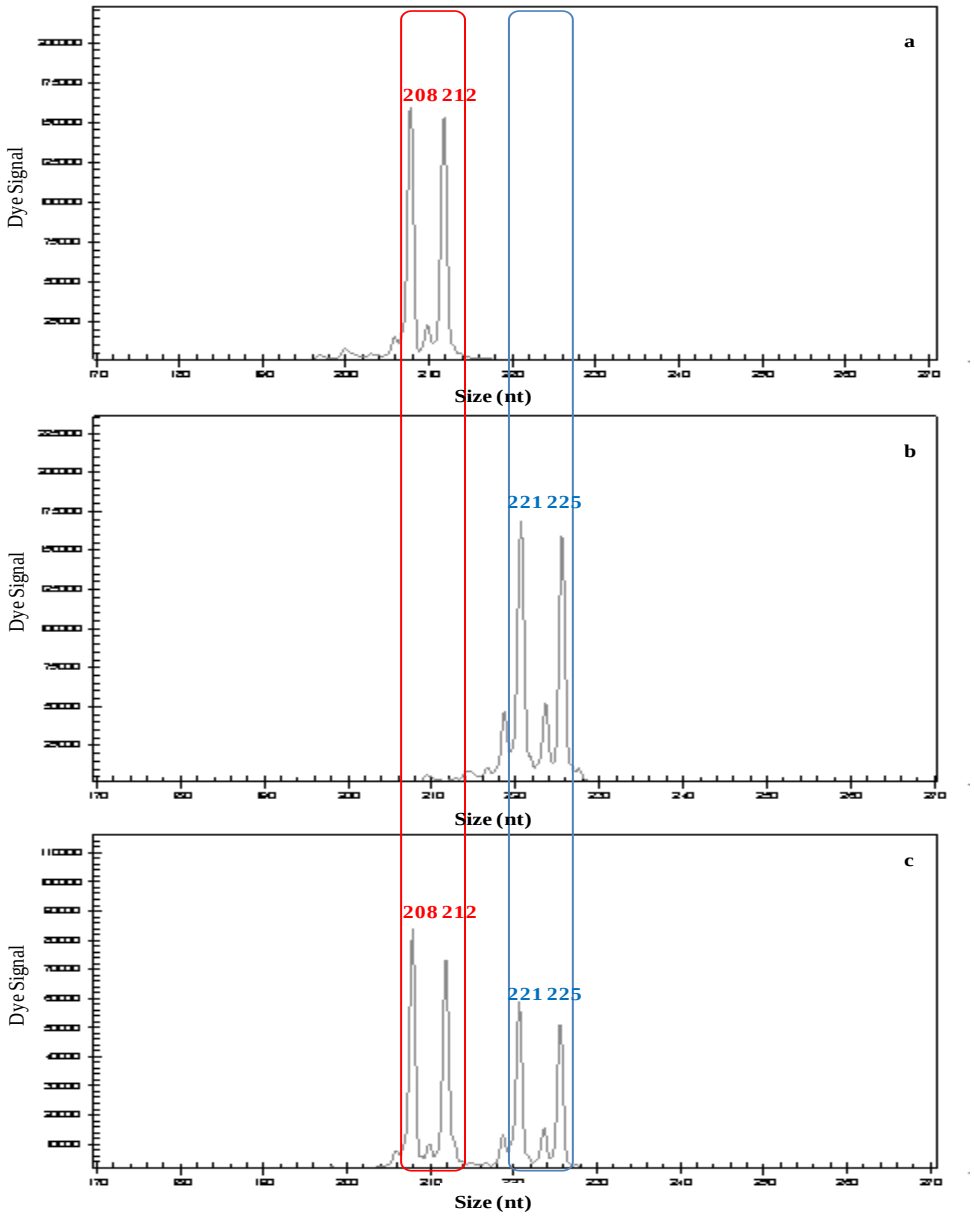

Figure 1.1. Electropherograms obtained using mCrCIR03C08 SSR marker in a: diploid tangor `CSO´, b: tetraploid `Moncada´ mandarin, c: tetraploid hybrid displaying the alleles of both parents recovered from MCSO hybridization. The numbers indicate the size of alleles in nucleotides (nt) for each genotype.

In the FCSO hybridization, from 62 tetraploid plants, eight displayed only specific alleles of the DD parent and never exhibited the specific alleles of the `CSO´ parent

(supplementary file 1). Under the hypothesis of cross pollination with CSO 2n gametes arising from FDR or SDR, the probabilities to observe, at individual level, such configurations without the CSO specific alleles are respectively P=8.03E-12 and P=5.18E-4. Therefore the hypothesis of cross-pollination with CSO 2n gametes can be rejected for these plants. Moreover none of these plants are identical to the DD Clementine (Additional table 1.1) as they display different allele doses (0/4; 3/1; 1/3 or 4/0) for the loci heterozygous in Clementine (2/2 dose in the DD). Therefore they are not tetraploid nucellar plants and the presence of allelic recombination proved that these plants were originated by self-pollination of the DD female parent. The remaining 54 tetraploid hybrids resulted from the fertilization of reduced diploid female gamete by unreduced pollen gametes of the `CSO´ diploid tangor (Figure 1.2). This is the first report of citrus tetraploid progenies recovered from unreduced pollen gametes in 4x x 2x sexual hybridizations. This phenomenon has been observed and studied in other species like *Lilium* (Lim *et al*., 2004), potato (Mendiburu and Peloquin, 1977b; Hutten *et al*., 1994; Carputo *et al*., 2003; Park *et al*., 2007) *Boecheraspp*. (Mau *et al*., 2013) and *Arabidopsis* (d'Erfurth *et al*., 2008).

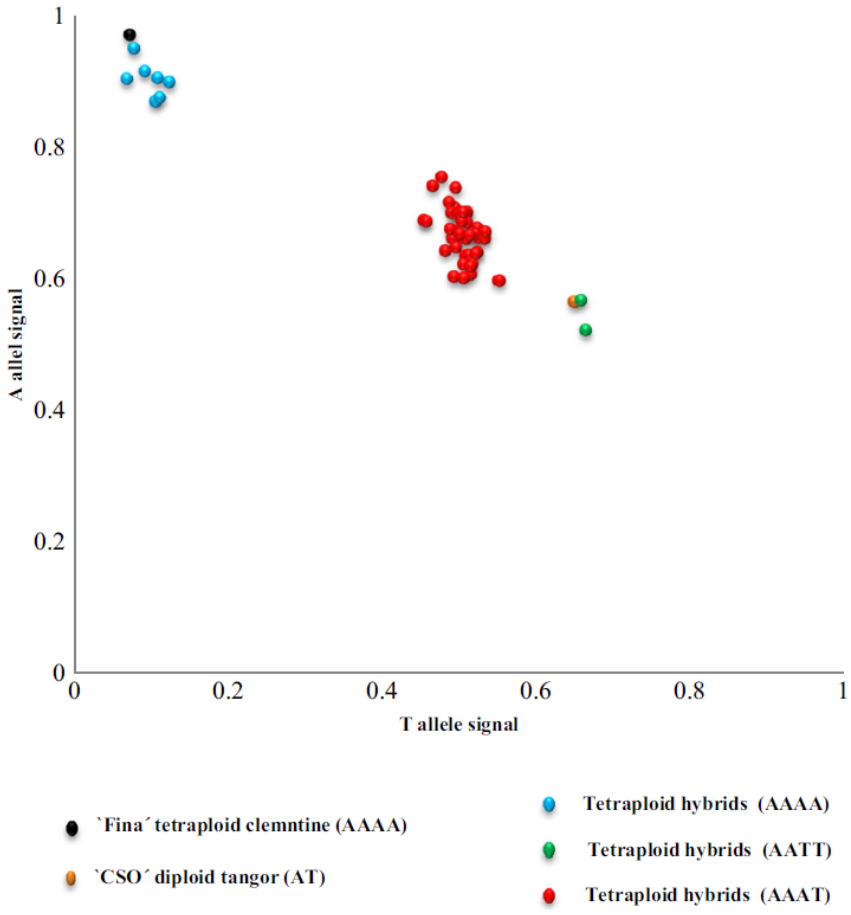

Figure 1.2. Plot of normalized A, T allele signals from cluster analysis over 54 tetraploid hybrids from the hybridization between tetraploid `Fina´ clementine and diploid tangor `CSO´ with the 2p21022555 SNP marker. Tetraploid hybrids (AAAA) and (AATT) originated from homozygous unreduced pollen grain (AA and TT) and tetraploid hybrids (AAAT) originated from heterozygous unreduced pollen grain (AT).

Tetraploid plants in tentative 4x x 2x sexual hybridizations have been previously reported in citrus and were originated by selfing of the tetraploid female parent (Aleza *et al*., 2012b). We have observed the same result in eight over the 72 tetraploid recovered plants. Xie *et al*. (2014) also recovered tetraploid plants from the reciprocal hybridization, 2x x 4x, and tetraploid plants were originated as a consequence of 2n female gametes. Here, we have obtained a high number of tetraploid hybrids from 2n pollen gametes (64 over 72 tetraploid recovered plants from only 50 and 32 collected fruits of MCSO and FCSO respectively). Ramsey and Schemske; (1998) suggested that the frequency of polyploid plants is related to the effect of environmental conditions and their genotypes. Great differences in frequencies of 2n gametes between genotypes have been observed in citrus (Aleza *et al*., 2010b), peach (Dermen, 1938) and potato (Mok and Peloquin, 1975). Watanable and Peloquin, (1993) indicated that 2n-pollen gamete frequencies can range between 1.9 to 36% in diploid, tetraploid and hexaploid *Solanum* species. As well as the high variability reported in *Dactylis*, *Trifolium*, *Manihot* and *Medicago* (Bregtanolle and Thompson, 1995).

Most studies on 2n citrus gametes were performed from 2x x 2x hybridizations where triploid embryos are included in small seeds with a 3:5 embryo-endosperm ploidy level ratio. Esen and Soost, (1971; 1973) indicated that the ratio between the ploidy level of embryos and endosperm was responsible for seed size reduction, since pentaploid endosperms grow slower and stop seed development prematurely. In this context there are very few reports about polyploid plants produced by unreduced pollen gametes. Luro *et al*. (2004) described a few triploid plants recovered from unreduced male gametes of `Ananas´ (*C. reticulata* Blanco), `Hansen´ (*C. reticulata* Blanco) and `King´ mandarins (*C. nobilis* Lour.), `Star Ruby´ grapefruit (*C. paradisi* (Macf.), `Tarocco Rosso´ and `Sanguinelli´ sweet oranges. The very low rates of triploids arising from unreduced pollen in 2x x 2x hybridization was confirmed later by Cuenca *et al*. (2015). In a diploid plant, when pollinated by diploid pollen, the embryo/endosperm ratio (3/4) is less favorable than the 2/3 occurring in normal embryos in diploid hybridization, while the pollination of a tetraploid plant by a diploid pollen provides a correct embryo/endosperm ploidy ratio (4/6=2/3) leading to normal seed development. Tetraploid x diploid hybridization is therefore the better situation to reveal the existence of unreduced pollen by the production of tetraploid embryos in normal seeds.

Mechanism of unreduced pollen formation

For heterozygous markers at parental level, meiotic cells without crossing over between the centromere and the considered marker will produce heterozygous FDR-2n gametes and homozygous SDR-2n gametes. While when one crossing over occurs between the centromere and the considered locus, 50% of FDR-2n gametes will be heterozygous (and 50% homozygous), but all SDR gametes will be heterozygous at this locus (Park *et al*., 2007). Therefore, the probabilities of a 2n gamete being heterozygous or homozygous for a marker, as a consequence of FDR or SDR mechanisms are direct functions of the marker-centromere distance (Park *et al*., 2007). Moreover for one meiotic cell, the occurrence of crossing over is totally independent between the different chromosomes; therefore the restitution of heterozygosity in 2n gametes for markers in different chromosome is also independent.
Maximum-likelihood method based on PHR of the centromeric markers developed by Cuenca *et al*. (2015) was used to identify the mechanism of unreduced pollen gamete

formation in tetraploid plants recovered from 4x x 2x sexual hybridizations. For each 4x hybrid, the LOD score for SDR/FDR probabilities was done from individual´s multilocus allelic configuration (Additional tables 1.2 and 1.3).

LODs values for the ten 4x hybrids of MCSO hybridization (Table 1.2), indicated that six plants displayed LOD values between -8.28 and -4.39, being conclusive for FDR, two plants showed LOD values higher than 2, significant for SDR, and two plants had LOD values of -0.96 and 0.30, not allowing to conclude between the FDR and SDR mechanisms.

Table 1.2. Analysis at individual and population level of the origin of `CSO´ tangor 2n gametes recovered from DD `Moncada´ mandarin by `CSO´ diploid tangor sexual hybridization using markers close to the centromeres of all LGs and the LOD score test probability ratio for SDR/FDR

Closest marker to the centromere	**MEST 539**	**CX6F2 3**	**TC01**	**CF-ACA01**	**5AT21**	**CiC4356 -06**	**Ci07C0 7**	**LCY2-M-376**	**9AAT2 2**	
LG	1	2	3	4	5	6	7	8	9	
Centromere Position (cM)	60.7	56.9	90.6	16.1	23.1	6.4	96.4	54.2	52.2	
Marker Position (cM)	61.8	49.5	96.0	24.4	17.5	6.2	98.0	58.1	62.6	
Marker distance to centromere (cM)	1.2	7.3	5.4	8.3	5.6	0.2	1.6	3.9	10.4	
Individuals analyzed	**Individual multilocus allelic configuration**									**LOD (SDR/ FDR)**
MCSO 03	HE	HE	HE	HE	HE	HE	HE	HE	HE	-8.28
MCSO 09	HE	HE	HE	HE	HE	HE	HE	HE	HE	-8.28
MCSO 05	HE	HE	HE	HE	HE	HE	HE	HE	HO	-6.68
MCSO 08	HE	HE	HE	HE	HE	HE	HE	HO	HE	-6.40
MCSO 10	HO	HO	HE	HE	HE	HE	HE	HE	HE	-5.16
MCSO 02	HE	HE	HE	HE	HO	HE	HO	HE	HE	-4.39
MCSO 06	HE	HE	HE	HE	HE	HO	HE	HO	HO	-0.96
MCSO 07	HE	HO	HE	HE	HO	HO	HE	HO	HE	0.30
MCSO 04	HE	HE	HO	HO	HO	HE	HO	HO	HE	2.06
MCSO 01	HO	HO	HO	HE	HO	HE	HO	HE	HO	2.62
Population LOD										**-35.17**

LODs > 2 are significant for SDR, LODs> -2 are significant for FDR and, LODs between 2 and -2 do not allow to conclude between SDR and FDR hypotheses. cM. Centimorgans. HO Homozygous and HE heterozygous

Regarding the 54 tetraploid plants recovered from FCSO hybridization (Table 1.3), LOD valuesrangedfrom-6.36 to 7.75. Thirty-five plants displayed a LOD value between -6.36 to -2.28 and were considered significant for the FDR 2n gamete formation mechanism. Ten plants showed LOD values between 2.04 and 7.75, being significant for the SDR mechanism.

Table 1.3. Analysis at individual level of the origin of `CSO´ tangor 2n gametes recovered from DD 'Fina' clementine by `CSO´ diploid tangor sexual hybridization using markers close to the centromeres of five different LGs and the LOD score test probability ratio for SDR/FDR.

Closest marker to the centromere	**CIBE5720**	**2p21022555**	**3p35931624**	**5AT21**	**9TAA22**	
LG	1	2	3	5	9	
Centromere Position (cM)	60.7	56.9	90.6	23.1	52.2	**LOD (SDR/FDR)**
Marker Position (cM)	58.4	57.0	95.1	17.5	62.6	
Marker distance to centromere (cM)	2.2	0.1	4.5	5.6	10.4	
Individuals analyzed	**Individual multilocus allelic configuration**					
FCSO 05	HE	HE	HE	HE	HE	-6.36
FCSO 12	HE	HE	HE	HE	HE	-6.36
FCSO 13	HE	HE	HE	HE	HE	-6.36
FCSO 27	HE	HE	HE	HE	HE	-6.36
FCSO 33	HE	HE	HE	HE	HE	-6.36
FCSO 36	HE	HE	HE	HE	HE	-6.36
FCSO 58	HE	HE	HE	HE	HE	-6.36
FCSO 59	HE	HE	HE	HE	HE	-6.36
FCSO 02	HE	HE	HE	HE	HO	-4.77
FCSO 09	HE	HE	HE	HE	HO	-4.77
FCSO 20	HE	HE	HE	HE	HO	-4.77
FCSO 21	HE	HE	HE	HE	HO	-4.77
FCSO 01	HO	HE	HE	HE	HE	-4.30
FCSO 11	HO	HE	HE	HE	HE	-4.30
FCSO 19	HO	HE	HE	HE	HE	-4.30
FCSO 23	HO	HE	HE	HE	HE	-4.30
FCSO 29	HO	HE	HE	HE	HE	-4.30
FCSO 37	HO	HE	HE	HE	HE	-4.30
FCSO 38	HO	HE	HE	HE	HE	-4.30
FCSO 45	HO	HE	HE	HE	HE	-4.30
FCSO 07	HE	HE	HE	HO	HE	-4.10
FCSO 08	HE	HE	HE	HO	HE	-4.10
FCSO 25	HE	HE	HO	HE	HE	-3.88
FCSO 43	HE	HE	HO	HE	HE	-3.88
FCSO 44	HE	HE	HO	HE	HE	-3.88
FCSO 06	HO	HE	HE	HE	HO	-2.70
FCSO 18	HO	HE	HE	HE	HO	-2.70
FCSO 39	HO	HE	HE	HE	HO	-2.70
FCSO 46	HO	HE	HE	HE	HO	-2.70
FCSO 52	HO	HE	HE	HE	HO	-2.70
FCSO 54	HO	HE	HE	HE	HO	-2.70
FCSO 16	HE	HE	HE	HO	HO	-2.51
FCSO 40	HE	HE	HO	HE	HO	-2.28
FCSO 41	HE	HE	HO	HE	HO	-2.28
FCSO 47	HE	HE	HO	HE	HO	-2.28
FCSO 56	HO	HE	HO	HE	HE	-1.82
FCSO 42	HE	HE	HO	HO	HE	-1.62
FCSO 17	HO	HE	HE	HO	HO	-0.44
FCSO 50	HO	HE	HE	HO	HO	-0.44
FCSO 48	HO	HE	HO	HE	HO	-0.22
FCSO 22	HE	HE	HO	HO	HO	-0.02
FCSO 35	HE	HO	HE	HE	HO	0.94
FCSO 53	HE	HO	HE	HE	HO	0.94
FCSO 32	HO	HO	HE	HE	HE	1.41
FCSO 60	HO	HE	HO	HO	HO	2.04
FCSO 24	HE	HO	HO	HE	HO	3.43
FCSO 51	HE	HO	HO	HO	HE	4.09
FCSO 15	HO	HO	HE	HO	HO	5.27
FCSO 34	HO	HO	HE	HO	HO	5.27
FCSO 04	HO	HO	HO	HE	HO	5.49
FCSO 26	HO	HO	HO	HE	HO	5.49
FCSO 31	HO	HO	HO	HO	HE	6.15
FCSO 28	HO	HO	HO	HO	HO	7.75
FCSO 57	HO	HO	HO	HO	HO	7.75
	Population LOD					**-98.29**

LODs > 2 are significant for SDR, LODs> -2 are significant for FDR and, LODs between 2 and -2 do not allow to conclude between SDR and FDR hypoteses . cM Centimorgans, HO Homozygous and HE heterozygous

For nine plants displaying LOD values between -2 and 2, it was not possible to conclude between SDR and FDR hypotheses. At population level, LOD values were –35.17 and -98.29 for MCSO and FCSO hybridizations, respectively. Taking together both hybridizations, out of the 64 unreduced pollen gametes produced by diploid `CSO´ tangor, 41 (64.1%) were obtained by FDR, 12 (18.8%) were significant for SDR, and eleven (17.2%) yielded non-conclusive results.

For the analysis of the evolution of PHR percentages along the LG2, unreduced pollen gamete populations were classified as significant for SDR or FDR according to their LOD values. Then, they were analyzed with four SSR markers (mCrCIR04H06, Cx6F23, mCrCIR03C08 and CIBE6006) and one SNP marker (2p21022555) (Figure 1.3.). FDR progeny consisted in 41 2n pollen gametes; 06 and 35 from MCSO and FCSO hybridizations, respectively, and SDR progeny included 12 unreduced pollen gametes, 02 from the MCSO hybridization and 10 from the FCSO hybridization. For heterozygous loci for the parent producing the 2n gamete, the probabilities of a 2n gamete being heterozygous or homozygous as a consequence of FDR or SDR mechanisms are direct functions of the marker-centromere distance (Park *et al*., 2007). For heterozygous markers close to the centromere, FDR-2n gametes will be heterozygous and SDR-2n gametes will be homozygous but when crossing over takes place between centromere and the considered locus, FDR-2n gametes will be 50% heterozygous and 50% homozygous, but all these loci will be heterozygous in SDR gametes (Park *et al*., 2007). Forty of 41 FDR-2n pollen gametes analyzed displayed heterozygous allelic configuration for markers close to the centromere (CX6F23 SSR makers and 2p21022555 SNP marker located at 7.3 and 0.1 cM from left and right side of the centromere, respectively) whereas with the telomeric markers, 17 of the FDR-2n pollen gametes displayed homozygous allelic configurations. For SDR-2n pollen gametes, ten over 12 SDR-pollen gametes displayed homozygous allelic configurations for 2p21022555 SNP marker located at 0.1 cM from the centromere whereas with markers located between 7.3 and 67.1 cM from the centromere, 09 of 12 SDR-2n pollen gametes displayed heterozygosity.

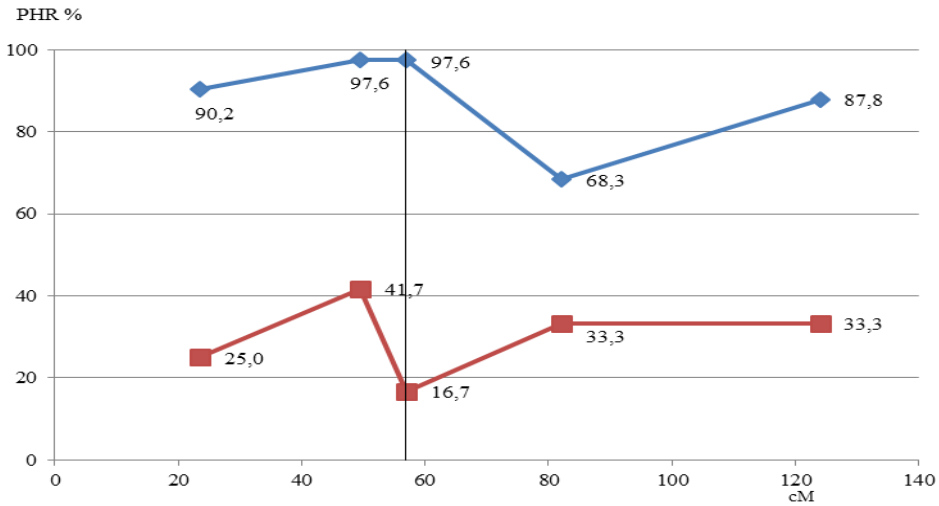

Figure 1.3. Observed parental heterozygosity restitution values for 2n pollen gametes classified by mechanism of unreduced gamete formation for markers in LG 2. Positions, according to clementine genetic map (Ollitrault *et al*., 2012). Blue line corresponds to FDR and red line corresponds to SDR mechanism and vertical line corresponds with the centromere location (Aleza *et al*., 2015).

Regarding the FDR population, PHR value increases from 90.2% for the mCrCIR04H06 marker (located in one telomere) to 97.6% for the CX6F23 and 2p21022555 centromeric markers. Subsequently, PHR value decreased to68.3% for the mCrCIR03C08 marker (at 25,3cM from the centromere) and increased again to 87.8% for the CIBE6006 telomeric marker (Figure 1.3). The average of PHR for FDR-2n gamete population was 88.29%. Considering all the 2n gametes with LOD significant for SDR, the PHR increases from 25% for the mCrCIR04H06 telomeric marker to 41.7% for the CX6F23 marker and decreases to 16.7% for the 2p21022555 centromeric marker. Also, the PHR value increases again to33% for the remaining two markers. The average of PHR for SDR-2n gamete population was 30%. Assuming that a random distribution of heterozygous loci along the chromosomes occurred, global restitution of heterozygosity is expected to be near 80 % for FDR and 40 % for SDR (Peloquin, 1983; Hutten *et al*. 1994; Carputo *et al*., 2003). In a previous work (Aleza *et al*., 2015), the maternal heterozygosity restitution average produced by clementine SDR-2n gametes was 42.5%. The present results obtained for FDR-2n pollen gametes are in agreement with those obtained in potato (Peloquin, 1983; Hutten *et al*., 1994; Carputo *et al*., 2003) whereas for the SDR-2n pollen gametes, the average restitution is lower than those expected. Considering the low number of markers and SDR individuals analyzed, this average could be considered as normal for a SDR gamete population.

This is the first report in *Citrus* of coexistence of two different meiotic restitution mechanisms (SDR and FDR) producing unreduced pollen in the same genotype. FDR appeared predominant in the studied 2n pollen, while SDR have been identified as the main mechanism of 2n megagametophytes in mandarins. Cuenca *et al*. (2015) analyzed 543 triploid hybrids obtained from nineteen different genotypes of mandarins and only three triploid plants were obtained by FDR-2n female gametes and no 2n pollen gametes contributed to the production of triploid hybrids. In a `Nishiuchi Konatsu´, a Japanese mandarin (*C. tamurana*), Honsho *et al*. (2016) reported the production of pollen grains with different sizes. They demonstrated that the biggest pollen grains were diploid resulting from FDR, although no progenies were recovered. In potato, genotypes that produce FDR-2npollen gametes and SDR-2n female gametes and genotypes that produce a mixture of SDR and FDR-2n female gametes have been described (Hutten *et al*., 1994). Such variability of mechanism of 2n gamete production has also been observed in other woody specie like *Populus* L. Liesebach *et al*. (2015) observed that 2n male gametes were originated by both mechanisms in similar frequencies. However, frequencies from 2n female gametes were uncoupled with a strong deviation to SDR. Most alleles conferring sexual polyploidization appear to be highly sex specific, indicating that unreduced gamete formation in male and female sporogenesis are largely uncoupled (Bretagnolle and Thompson, 1995; De Storme and Geelen, 2013).

Implications for polyploid breeding programs

The *Citrus* genus can be used as a model for meiosis, unreduced gamete mechanism studies, and polyploid research (Ollitrault *et al*., 2008; Cuenca *et al*., 2015; Aleza *et al*., 2009b; 2016a). The associated development of molecular and cytological techniques will lead to rapid advancements in the field in coming years (De Storme and Geelen, 2013). The breeding value of sexual polyploidy has been convincingly demonstrated in the case of potato (Peloquin, 1982), alfalfa (Bingham and McCoy, 1979) and red clover (Parrot *et al*., 1985) among other crops.

Determination of mechanisms underlying 2n pollen formation is a key result for breeding programs based onploidy manipulation. Indeed the different mechanisms imply major differences in the genetic structure of triploid progenies and hence, in the efficiency of breeding schemes. Several previous publications discussed the relative advantages of SDR and FDR gametes in polyploid breeding (Mendiburu and Peloquin, 1977a, b; Hutten *et al*., 1994). Genetic and economic importance of any obtained population goes through its genetic structure in relation with the objectives of the breeding program. For example, if the objective is to create progenies more similar to the parent producing the unreduced gamete, FDR-2n gametes will be a better strategy because the resulting 2n gametes will be heterozygous as their parent from the centromere to the first crossing over. Therefore 2n gametes retain most parental heterozygosity and epistatic interactions as it has been demonstrated in potato by Mendiburu and Peloquin (1977a, b). On the contrary, SDR-2n gametes provide the opportunity to create a larger number of new multilocus genotypic combinations and a higher number of polymorphic progenies, providing new products that meet commercial market segmentation strategies (Cuenca *et al*., 2011; Aleza *et al*., 2016a).

The main strategies used to create triploid hybrids in citrus are sexual polyploidization by 2n gametes, as has been discussed above, and interploid sexual hybridizations using tetraploid parents (doubled diploids, DD) (Aleza *et al*., 2010b; 2012a, b). Aleza *et al*. (2016a) demonstrated the complementarity of diploid gamete population produced by a DD clementine displaying a predominant tetrasomic segregation (PHR average around 65%) and SDR-2n gametes of clementine with less PHR value. The distribution of PHR along each chromosome was also different. In SDR-2n pollen gametes, loci between the centromere and the first crossover are homozygous, but parental heterozygosity restitution is favored for the telomeric loci. By contrast, PHR is relatively constant across a chromosome for DD gametes with genotypic combinations that are closer to clementine. In the present study, the UPGMA analysis of the 41 FDR 2n pollen and 12 SDR 2n pollen based on the five markers of LG2 clearly displayed differentiated clusters between FDR and SDR-2n pollen (Figure 1.4) and an even higher differentiation than the one revealed by Aleza *et al*. (2016a) between DD Clementine gametes and SDR 2n gametes. Indeed, for FDR-2n gametes the PHR is higher than the one obtained with a doubled-diploid parent and pattern of PHR along a chromosome are opposed between FDR and SDR. For FDR, loci between the centromere and the first crossover are heterozygous, and half of the parental heterozygous loci beyond the first crossing over are also heterozygous. Thus, with the objective to develop new citrus cultivars that are phenotypically close to one parent, the exploitation of 2n FDR pollen in diploid crosses should be the best strategy; followed by interploid hybridization with a DD of the considered parent. The exploitation of SDR 2n pollen or ovules will produce more polymorphic progenies and should be more adapted to new product selection and market segmentation strategies. The tetraploid plants obtained in 4x x 2x hybridization may be used as parents for further triploid breeding. The ones arising from FDR should be more interesting to provide increased gametic diversity and heterosis due to their higher level of heterozygosity, particularly in centromeric regions.

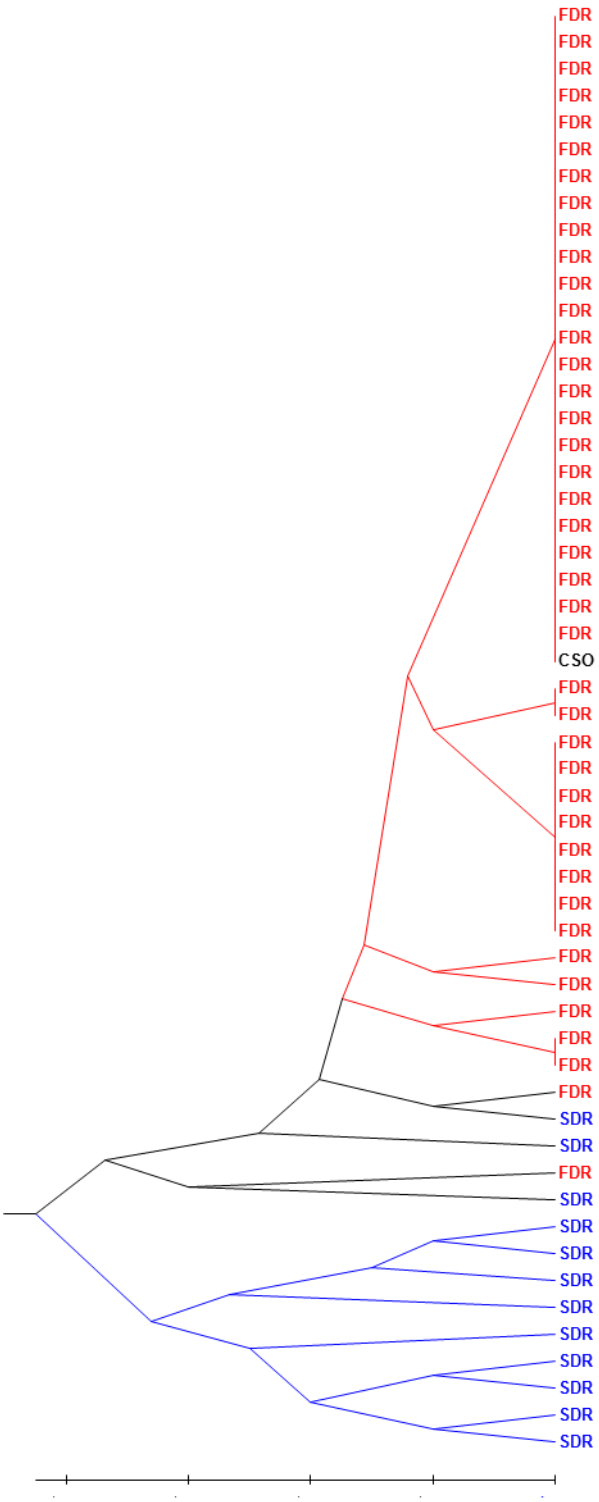

Figure 1.4. UPGMA tree obtained from allelic data of 2n pollen gametes produced by the diploid `CSO´ tangor (black) originated by FDR (red) and SDR (blue) mechanisms.

Conclusion

SSR and SNP analysis revealed that 89% of 72 tetraploid hybrids obtained in 4x x 2x hybridization using CSO tangor as male parents resulted from 2n pollen of CSO. Maximum likelihood method based on PHR of centromeric loci was applied to determine the FDR or SDR origin at individual level. Among the 53 plants with conclusive results FDR was the predominant mechanism (77%), but 23% of SDR deriving plants were also identified. To our knowledge, this is the first report for citrus of tetraploid progenies arising from unreduced pollen and the first description of the coexistence of two meiotic restitution mechanisms (SDR and FDR) producing unreduced pollen. Progenies arising from FDR and SDR pollen displayed complementary genetic diversity. FDR pollen should be more efficient to develop new cultivars closer to the pollen parent while SDR should allow developing more polymorphic progeny with the perspective of selection of new products.

Supplementary information

Additional table 1.1. Evidence of the origin by selfertilisation of tetraploid clementine for eight progenies.

Table 1.1.a. Allelic configuration of the eight tetraploid plants recovered from self-pollination of tetraploid `Fina´ clementine.

Individuals	CIBE5720				mCrCIR04H06				CX6F23				mCrCIR03C08			
4x `Fina´ clementine	**325**	**325**	**337**	**337**	**190**	**190**	**196**	**196**	**149**	**149**	**161**	**161**	**208**	**208**	**226**	**226**
2x `CSO´	**325**	**340**			**184**	**196**			**155**	**161**			**208**	**212**		
FCSO 61	325	325	337	337	190	196	196	196	149	161	161	161	208	208	208	226
FCSO 62	325	325	337	337	190	196	196	196	149	161	161	161	208	208	226	226
FCSO 63	325	325	337	337	190	190	196	196	149	149	161	161	208	208	208	226
FCSO 64	325	325	337	337	190	190	196	196	161	161	161	161	208	208	208	226
FCSO 65	325	325	337	337	190	190	196	196	149	161	161	161	208	208	208	208
FCSO 66	325	325	337	337	190	196	196	196	149	161	161	161	208	208	226	226
FCSO 67	325	325	337	337	190	196	196	196	149	161	161	161	208	208	208	226
FCSO 68	325	325	337	337	190	196	196	196	149	149	161	161	208	208	226	226

Individuals	2p21022555				CIBE6006				3p35931624				5AT21			
4x `Fina´ clementine	**A**	**A**	**A**	**A**	**176**	**176**	**200**	**200**	**G**	**G**	**G**	**G**	**254**	**254**	**254**	**254**
2x `CSO´	**T**	**A**			**197**	**200**			**G**	**A**			**240**	**254**		
FCSO 61	A	A	A	A	176	176	200	200	G	G	G	G	254	254	254	254
FCSO 62	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254
FCSO 63	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254
FCSO 64	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254
FCSO 65	A	A	A	A	176	176	200	200	G	G	G	G	254	254	254	254
FCSO 66	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254
FCSO 67	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254
FCSO 68	A	A	A	A	176	200	200	200	G	G	G	G	254	254	254	254

Individuals	9TAA22				mCrCIR01C06				TC01			
4x `Fina´ clementine	**150**	**150**	**203**	**203**	**133**	**133**	**165**	**165**	**333**	**333**	**348**	**348**
2x `CSO´	**164**	**203**			**159**	**165**			**333**	**351**		
FCSO 61	150	150	203	203	133	133	165	165	333	333	348	348
FCSO 62	150	150	203	203	133	133	165	165	333	333	333	348
FCSO 63	150	150	203	203	133	165	165	165	333	333	348	348
FCSO 64	150	150	203	203	133	133	165	165	333	333	333	348
FCSO 65	150	150	203	203	133	133	165	165	333	333	348	348
FCSO 66	203	203	203	203	133	133	165	165	333	333	348	348
FCSO 67	203	203	203	203	133	133	165	165	333	333	348	348
FCSO 68	150	150	203	203	133	133	165	165	333	333	348	348

Numbers indicate the size of alleles in nucleotides (nt). Squares painted in green indicate the presence of sexual recombination and testify that the tetraploid plants do not arise from nucellar embryony (apomixis).

The specific alleles of the `CSO´ hybrid (in red) were never observed in these eight tetraploid plants (Table 1.1.b).

For loci close to the centromere, according to the distance to the centromere (d) of each marker and the function of heterozzygosity restitution under total interference model (Cuenca *et al*., 2015) we estimated the probability that the common allele with Clementine (CA) pass in homozygosity trough FDR or SDR 2n gametes as following:
PFDR (CA)= d/2
PSDR (CA)= 0.5-d

For telomeric loci we considered the thresold of 2/3 for heterozygosity restitution obtained for no interference and partial interference models under SDR and FDR mechanisms (Cuenca *et al*., 2011)
Therefore for telomeric markers:
PFDR (CA)= 1/6
PSDR (CA)=1/6

We considered 5 independant centromeric loci (in different chromosomes) and 2 telomeric independant loci (Table 1.1.b).

Table 1.1.b. Probability of transmission of the `CSO´ specific allele through unreduced gametes.

Markers	CiBE6006	TC01	5AT21	CiBE5720	9TAA22	2p21022555	mCrCIR01C06	Total Probability
LG	2	3	5	1	9	2	6	
Centromere Position	0.569	0.906	0.231	0.607	0.522	0.569	0.064	
Marker Position	1.240	0.960	0.175	0.584	0.626	0.570	0.889	
Distance	0.671	0.054	0.056	0.023	0.104	0.001	0.825	
Prob He SDR	0.667	0.108	0.112	0.045	0.208	0.003	0.667	
Prob Ho SDR	0.333	0.892	0.888	0.955	0.792	0.997	0.333	
Prob Ho SDR specific allele	0.167	0.446	0.444	0.477	0.396	0.499	0.167	5.18E-04
Prob He FDR	0.667	0.946	0.944	0.977	0.896	0.999	0.667	
Prob Ho FDR	0.333	0.054	0.056	0.023	0.104	0.001	0.333	
Prob Ho FDR specific allele	0.167	0.027	0.028	0.011	0.052	0.001	0.167	8.03E-12

Under the hypothesis of cross pollination with `CSO´ 2n gametes arising from FDR or SDR, the probabilities to observe, at individual level, such configurations without the `CSO´ specific alleles are respectively P=8.03E-12 and P=5.18E-04. Therefore the hypothesis of cross-pollination with `CSO´ 2n gametes can be rejected for these plants.

Additional Table 1.2. Analysis at individual and population level of the origin of `CSO´ tangor 2n gametes recovered from DD `Moncada´ mandarin by `CSO´ diploid tangor sexual hybridization using markers close to the centromeres of all LGs and the LOD score test probability ratio for SDR/FDR.

Closest marker to the centromere	**MEST539**	**CX6F23**	**TC01**	**CF-ACA01**	**5AT21**	**CiC4356-06**	**Ci07C07**	**LCY2-M-376**	**9AAT22**	**LOD (SDR/FDR)**	**Conclusion**
LG	1	2	3	4	5	6	7	8	9		
Centromere Position (cM)	60,7	56,9	90,6	16,1	23,1	6,4	96,4	54,2	52,2		
Marker Position (cM)	61,8	49,5	96,0	24,4	17,5	6,2	98,0	58,1	62,6		
Marker distance to centromere (cM)	1,2	7,3	5,4	8,3	5,6	0,2	1,6	3,9	10,4		
CSO genotype	104 108	155 161	333 352	336 340	240 254	C T	227 240	A G	163 202		
Individuals analyzed	**Multilocus allelic configuration of the CSO 2n gametes**										
MCSO 03	104 108	155 161	333 352	336 340	240 254	C T	227 240	A G	163 202	-8,28	FDR
MCSO 09	104 108	155 161	333 352	336 340	240 254	C T	227 240	A G	163 202	-8,28	FDR
MCSO 05	104 108	155 161	333 352	336 340	240 254	C T	227 240	A G	163 163	-6,68	FDR
MCSO 08	104 108	155 161	333 352	336 340	240 254	C T	227 240	A A	163 202	-6,40	FDR
MCSO 10	104 104	155 155	333 352	336 340	240 254	C T	227 240	A G	163 202	-5,16	FDR
MCSO 02	104 108	155 161	333 352	336 340	254 254	C T	227 227	A G	163 202	-4,39	FDR
MCSO 06	104 108	155 161	333 352	336 340	240 254	C C	227 240	A A	163 163	-0,96	UN
MCSO 07	104 108	155 155	333 352	336 340	254 254	C C	227 240	A A	163 202	0,30	UN
MCSO 04	104 108	155 161	333 333	340 340	240 240	C T	240 240	A A	163 202	2,06	SDR
MCSO 01	104 104	155 155	333 333	336 340	240 240	C T	240 240	A G	163 163	2,62	SDR
Population LOD										**-35,17**	

LODs > 2 are significant for SDR, LODs> -2 are significant for FDR and, LODs between 2 and -2 do not allow to conclude between SDR and FDR hypotheses . cM Centimorgans, Numbers indicate the size of alleles in nucleotides (nt) and letters correspond to SNP markers alleles.

Additional table 1.3. Analysis at individual level of the origin of `CSO´ tangor 2n gametes recovered from DD 'Fina' clementine by `CSO´ diploid tangor sexual hybridization using markers close to the centromeres of five different LGs and the LOD score test probability ratio for SDR/FDR.

Closest marker to centromere	**CIBE5720**	**2p21022555**	**3p35931624**	**5AT21**	**9TAA22**	**LOD (SDR/FDR)**	**Conclusion**
LG	1	2	3	5	9		
Centromere Position (cM)	60,7	56,9	90,6	23,1	52,2		
Marker Position (cM)	58,5	57,0	95,1	17,5	62,6		
Marker distance to centromere (cM)	2,2	0,1	4,5	5,6	10,4		
`CSO´ genotype	325 340	AT	GA	240 254	164 203		
Individuals analyzed	**Multilocus allelic configuration of the CSO 2n gametes.**						
FCSO 05	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 12	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 13	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 27	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 33	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 36	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 58	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 59	325 340	AT	GA	240 254	164 203	-6,36	FDR
FCSO 02	325 340	AT	GA	240 254	203 203	-4,77	FDR
FCSO 09	325 340	AT	GA	240 254	203 203	-4,77	FDR
FCSO 20	325 340	AT	GA	240 254	164 164	-4,77	FDR
FCSO 21	325 340	AT	GA	240 254	203 203	-4,77	FDR
FCSO 01	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 11	340 340	AT	GA	240 254	164 203	-4,30	FDR
FCSO 19	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 23	340 340	AT	GA	240 254	164 203	-4,30	FDR
FCSO 29	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 37	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 38	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 45	325 325	AT	GA	240 254	164 203	-4,30	FDR
FCSO 07	325 340	AT	GA	254 254	164 203	-4,10	FDR
FCSO 08	325 340	AT	GA	240 240	164 203	-4,10	FDR
FCSO 25	325 340	AT	AA	240 254	164 203	-3,88	FDR
FCSO 43	325 340	AT	AA	240 254	164 203	-3,88	FDR
FCSO 44	325 340	AT	AA	240 254	164 203	-3,88	FDR
FCSO 06	340 340	AT	GA	240 254	164 164	-2,70	FDR
FCSO 18	325 325	AT	GA	240 254	203 203	-2,70	FDR
FCSO 39	340 340	AT	GA	240 254	203 203	-2,70	FDR
FCSO 46	340 340	AT	GA	240 254	164 164	-2,70	FDR
FCSO 52	325 325	AT	GA	240 254	203 203	-2,70	FDR
FCSO 54	340 340	AT	GA	240 254	203 203	-2,70	FDR
FCSO 16	325 340	AT	GA	254 254	164 164	-2,51	FDR
FCSO 40	325 340	AT	AA	240 254	164 164	-2,28	FDR
FCSO 41	325 340	AT	AA	240 254	164 164	-2,28	FDR
FCSO 47	325 340	AT	AA	240 254	164 164	-2,28	FDR
FCSO 56	325 325	AT	AA	240 254	164 203	-1,82	UN
FCSO 42	325 340	AT	AA	254 254	164 203	-1,62	UN
FCSO 17	325 325	AT	GA	254 254	203 203	-0,44	UN
FCSO 50	340 340	AT	GA	254 254	203 203	-0,44	UN
FCSO 48	340 340	AT	AA	240 254	203 203	-0,22	UN

Additional table 1.3. –cont. Analysis at individual level of the origin of `CSO´ tangor 2n gametes recovered from DD 'Fina' clementine by `CSO´ diploid tangor sexual hybridization using markers close to the centromeres of five different LGs and the LOD score test probability ratio for SDR/FDR.

Closest marker to centromere	**CIBE5720**	**2p21022555**	**3p35931624**	**5AT21**	**9TAA22**	**LOD (SDR/FDR)**	**Conclusion**
LG	1	2	3	5	9		
Centromere Position (cM)	60,7	56,9	90,6	23,1	52,2		
Marker Position (cM)	58,5	57,0	95,1	17,5	62,6		
Marker distance to centromere (cM)	2,2	0,1	4,5	5,6	10,4		
`CSO´ genotype	325 340	AT	GA	240 254	164 203		
Individuals analyzed	**Multilocus allelic configuration of the CSO 2n gametes.**						
FCSO 22	325 340	AT	GG	254 254	164 164	-0,02	UN
FCSO 35	325 340	AA	GA	240 254	203 203	0,94	UN
FCSO 53	325 340	TT	GA	240 254	164 164	0,94	UN
FCSO 32	340 340	AA	GA	240 254	164 203	1,41	UN
FCSO 60	325 325	AT	GG	254 254	203 203	2,04	SDR
FCSO 24	325 340	TT	AA	240 254	164 164	3,43	SDR
FCSO 51	325 340	AA	GG	254 254	164 203	4,09	SDR
FCSO 15	340 340	AA	GA	240 240	164 164	5,27	SDR
FCSO 34	325 325	AA	GA	254 254	203 203	5,27	SDR
FCSO 04	325 325	AA	GG	240 254	203 203	5,49	SDR
FCSO 26	325 325	AA	AA	240 254	164 164	5,49	SDR
FCSO 31	340 340	AA	GG	254 254	164 203	6,15	SDR
FCSO 28	325 325	AA	GG	254 254	203 203	7,75	SDR
FCSO 57	325 325	AA	AA	254 254	203 203	7,75	SDR
Population LOD						**-98,29**	

LODs > 2 are significant for SDR, LODs> -2 are significant for FDR and, LODs between 2 and -2 do not allow to conclude between SDR and FDR hypotheses (UN) . cM Centimorgans, Numbers indicate the size of alleles in nucleotides (nt) and letters correspond to SNP markers alleles.

CHAPTER II

Unreduced Megagametophyte Production in Lemon Occurs via Three Meiotic Mechanisms, Predominantly Second-Division Restitution

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Abstract

Unreduced (2n) gametes have played a pivotal role in polyploid plant evolution and are useful for sexual polyploid breeding in various species, particularly for developing new seedless citrus varieties. The underlying mechanisms of 2n gamete formation were recently revealed for *Citrus reticulata* but remain poorly understood for other citrus species, including lemon (*C. limon* [L.] Burm. f.). We investigated the frequency and causal meiotic mechanisms of 2n megagametophyte production in lemon. We genotyped 48 progeny plants of two lemon genotypes, `Eureka Frost´ and `Fino´, using 16 Simple Sequence Repeats (SSR) and 18 Single Nucleotide Polymorphism (SNP) markers to determine the genetic origin of the progenies and the underlying mechanisms for 2n gamete formation. We utilized a maximum-likelihood method based on parental heterozygosity restitution (PHR) of centromeric markers and analysis of PHR patterns along the chromosome. The frequency of 2n gamete production was 4.9% for `Eureka Frost´ and 8.3% for `Fino´, with three meiotic mechanisms leading to 2n gamete formation. We performed the maximum-likelihood method at the individual level via telomeric marker analysis, finding that 88% of the hybrids arose from second-division restitution (SDR), 7% from first-division restitution (FDR) or pre-meiotic doubling (PRD), and 5% from post-meiotic genome doubling (PMD). The pattern of PHR along LG 1 confirmed that SDR is the main mechanism for 2n gamete production. Recombination analysis between markers in this LG revealed partial chiasma interference on both arms. We discuss the implications of these restitution mechanisms for citrus breeding and lemon genetics.

Keywords

Citrus, Unreduced gametes, Meiotic restitution, Second-division restitution (SDR), First-division restitution (FDR), Post-meiotic genome doubling (PMD) mechanisms, Seedlessness

Introduction

The exact area of origin of lemon (*Citrus limon*) is uncertain, but, likely, it originated in Northern India and South East China or in northern Myanmar (Curk *et al*., 2016). Molecular analyses indicate that this species resulted from direct hybridization between *C. aurantium* (sour orange) as the female parent and *C. medica* (citron) as the male parent (Nicolosi *et al*., 2000; Froelicher *et al*., 2011; García-Lor *et al*., 2013a; Curk *et al*., 2016).
The Mediterranean Basin is a major area of lemon production, accounting for 48% of production worldwide (Duportal *et al*., 2013). Turkey is the most important lemon-producing country in this area (annual production greater than 1,000,000 tons), followed by Spain (900,000 tons) and Italy (500,000 tons) (Martín and González, 2014). Seedless lemons with high organoleptical qualities and resistance to important diseases, such as Mal secco caused by *Phoma tracheiphila*, are in high demand by consumers and growers (Uzun *et al*., 2008; Perez-Tornero *et al*., 2012; Licciardello *et al*., 2006; Migheli *et al*., 2009). Several lemon-breeding programs worldwide are focused on meeting this demand (Calabrese *et al*., 2000; Recupero *et al*., 2005; Spiegel-Roy *et al*., 2007; Uzun *et al*., 2008; Pérez-Tornero *et al*., 2012), despite the difficulties imposed by the high heterozygosity and low genetic variation of this species (Krueger and Navarro, 2007).

In *Citrus*, diploidy is the general rule, with a basic chromosome number x = 9 (Krug, 1943), although triploid and tetraploid genotypes are present in the citrus germplasm (Lee, 1988). Triploid citrus plants are currently being produced in various breeding programs for the development of new seedless commercial citrus varieties (Starrantino and Recupero, 1981; Ollitrault *et al*., 2008; Grosser *et al*., 2010; Navarro *et al*., 2015). Triploid citrus plants can be recovered from interploid hybridizations, 2x x 4x and 4x x 2x (Esen and Soost, 1973b; Cameron and Burnett, 1978; Starrantino and Recupero, 1981; Ollitrault *et al*., 2008; Grosser and Gmitter, 2011; Aleza *et al*., 2012a, b; Navarro *et al*., 2015), or by 2x x 2x sexual hybridizations as a consequence of unreduced (2n) gamete formation (Esen and Soost, 1971, 1973a; Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Cuenca *et al*., 2015; Navarro *et al*., 2015). The sexual 2x x 2x hybridization strategy was used by Geraci *et al*. (1975) and Esen and Soost (1975) to obtain triploid progenies using `Lisbon´ and `Eureka´ lemons as female parents. Viloria and Grosser (2005) and Recupero *et al*. (2005) recovered progenies of triploid lemon-like hybrids via 2x x 4x sexual hybridizations. Pérez-Tornero *et al*. (2012) started a lemon-breeding program in 2008 aimed at obtaining triploid hybrids of higher quality than `Fino´ and `Verna´ lemons, the most important lemon varieties in Spain.

The frequency of 2n female gametes, an intrinsic characteristic of citrus genotypes, can vary from less than 1% to over 20% (Esen and Soost, 1971; Ollitrault *et al*., 2008). For *C. limon*, 1% and 5% of triploid progenies were recovered from 2x x 2x sexual hybridizations using `Lisbon´ and `Eureka´ lemons as the female parents, respectively (Esen and Soost, 1975; Geraci *et al*., 1975). Moreover, Pérez-Tornero *et al*. (2012) obtained 5.8% to 8.6% of triploid hybrids from a 2x x 2x cross between `Verna´ and `Fino´ genotypes. Various meiotic aberrations can result in unreduced gamete formation. First-division restitution (FDR) and second-division restitution (SDR) are the predominant mechanisms of 2n gamete formation in plants (De Storme and Geelen, 2013). These gametes are produced as a consequence of the failure of the first or second meiotic division, respectively, leading to the formation of restitution nuclei with a

somatic chromosome number (Mendiburu and Peloquin, 1976; Park *et al*., 2007). As a result, FDR and SDR have different genetic implications. FDR 2n gametes contain non-sister chromatids, which in the absence of crossover maintain the parental heterozygosity. When crossing over occurs, the parental heterozygosity restitution (PHR) rates vary from 100% for loci close to the centromere to 60–70% for loci far from the centromere, depending on the level of chromosome interference (Cuenca *et al*., 2011). For SDR, the 2n gametes contain two sister chromatids, which reduces the parental heterozygosity level (Bastiaanssen *et al*., 1998; Cuenca *et al*., 2011; De Storme and Geelen, 2013). When crossing over occurs, the PHR rate varies from 0% for loci close to the centromere to 60–75% for loci far from the centromere, depending on the level of chromosome interference (Cuenca *et al*., 2011). SDR is the dominant mechanism involved in the origin of unreduced female gametes in clementines and mandarins (Luro *et al*., 2004; Cuenca *et al*., 2011, 2015; Aleza *et al*., 2016a). Ferrante *et al*. (2010) reported that FDR is the main mechanism for unreduced female gamete formation in lemon. However, their results were based on the analysis of only a few individuals with few markers and without previous knowledge of centromere location. Other mechanisms leading to unreduced gamete formation have been described, such as pre-meiotic (PRD) and post-meiotic genome doubling (PMD). Although PMD was identified in potato (Bastiaanssen *et al*., 1998), both mechanisms have only rarely been documented in plants (De Storme and Geelen, 2013). PRD produces 2n gametes equivalent to the meiosis of doubled diploid genotypes. Therefore, PHR depends mainly on the chromosomal preferential pairing rate (Stift *et al*., 2008), which should vary between 66% for fully tetrasomic meiosis to 100% for fully disomic meiosis. Little variation can occur along the chromosome due to double reduction events. In the case of PMD, haploid gametes undergo an extra round of genome duplication, leading to the formation of fully homozygous 2n gametes (Bastiaanssen *et al*., 1998; Ramanna and Jacobsen, 2003; De Storme and Geelen, 2013; Cuenca *et al*., 2015). Thus, 100% homozygosity for all loci is expected among the 2n gametes (Ramanna and Jacobsen, 2003). SDR can also produce 100% homozygosity for centromeric markers, but not for telomeric ones (Cuenca *et al*., 2011). Therefore, in order to distinguish between both mechanisms, Cuenca *et al*. (2015) genotyped telomeric loci to determine whether diploid gametes fully homozygous for centromeric markers resulted from PMD or SDR. Moreover, Bastiaanssen *et al*. (1998) identified 2n female gametes of potatoes fully homozygous for RFLP markers. The evidence for recombination between alleles originating from the two ancestors of the parent producing 2n gametes indicated that these gametes originated from PMD. Molecular marker analyses can be used to estimate the PHR rates for diploid gametes in polyploid progenies and, therefore, to identify the mechanisms underlying unreduced gamete formation (Cuenca *et al*., 2011). Cuenca *et al*. (2015) took advantage of known citrus centromere locations (Aleza *et al*., 2015) to develop a maximum-likelihood method that distinguishes between SDR and FDR mechanisms at both the population and individual levels based on the PHR patterns of unlinked markers located close to the centromeres of different chromosomes.

In this study, we analyzed the frequencies of 2n gamete formation and the meiotic mechanisms leading to 2n gamete formation in two varieties of lemon, `Eureka Frost´ and `Fino´, through genetic analysis of triploid and tetraploid hybrids recovered from 2x x 2x and 2x x 4x sexual hybridizations. We used the maximum-likelihood method based on centromeric molecular markers in conjunction with a telomeric loci study and analysis of the pattern of PHR variation along LG 1 to identify the mechanisms underlying unreduced gamete formation at the individual and population level. Cross

over interference was also analysed. We discuss the implications for breeding programs based on sexual polyploidization.

Materials and Methods

Plant material

Triploid and tetraploid citrus hybrids were obtained via 2x x 2x and 2x x 4x sexual hybridizations using diploid `Eureka Frost´ and `Fino´ lemon genotypes as female parents pollinated with diploid `Fortune´ mandarin (*C. clementina* x *C. tangerina*) and *C. ichangensis* and tetraploid *C. macrophylla*. Flowers in pre-anthesis were emasculated, pollinated, and enclosed with a cloth bag. A total of 115 `Eureka Frost´ lemon flowers were pollinated, including 55 with `Fortune´ mandarin (named EuFor) and 60 with *C. ichangensis* (named EuIch), while 15 `Fino´ lemon flowers were pollinated with tetraploid *C. macrophylla* (named FinMac). The detailed methods used for plant recovery via *in vitro* embryo rescue and ploidy level analysis via flow cytometry can be found in Aleza *et al*. (2010a, b; 2012a, b).

Genotyping of progenies using Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphism (SNP) markers

The male and female parents and 48 hybrids were genotyped using 34 molecular markers (16 Simple Sequence Repeats [SSRs] and 18 Single Nucleotide Polymorphisms [SNPs]) showing heterozygosity for the lemon genotypes and polymorphism with the male parents. These markers are distributed across all LGs of the clementine genetic map (Ollitrault *et al*., 2012a) (Table 2.1).

Genomic DNA was isolated using a Plant DNeasy kit from Qiagen Inc. (Valencia, CA, USA) following the manufacturer´s protocol. PCR amplifications using 16 SSR markers were performed using a Thermocycler rep gradient S (Eppendorf®) in a 10 µL final volume containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng/mL citrus DNA, 0.2 mM welled (Sigma®) dye-labeled forward primer, 0.2 mM non-dye-labeled reverse primer, 0.2 mM of each dNTP, 10× PCR buffer, and 1.5 mM $MgCl_2$. The PCR protocol was as follows: denaturation at 94°C for 5 min followed by 40 cycles of 30 s at 94°C, 1 min at 50°C or 55°C, and 45 s at 72°C; and a final elongation step of 4 min at 72°C. Capillary electrophoresis was carried out using a CEN™ 8000 Genetic Analysis System (Beckman Coulter Inc.). The PCR products were initially denatured at 90°C for 2 min, injected at 2 kV for 30 s, and separated at 6 kV for 35 min. Alleles were sized based on a DNA size standard (400 bp). Genome Lab™ Gap v.10.0 genetic analysis software was used for data collection. Allele dosage was calculated using the MAC-PR (microsatellite DNA allele counting-peak ratio) method (Esselink *et al*., 2004), validated in citrus by Cuenca *et al*. (2011).

Table 2.1. Information about the molecular markers used in this study, including GenBank accession numbers, genetic distances, noted alleles, and references.

Locus	Phytozome / Gene Bank Accesion	LG	GMP (cM)	DC	Noted alleles [1]					Used to identify the				Bibliographic reference
					Eureka Frost'	Fino'	C. *macrophylla*	C. *ichangensis*	Fortune	2n gamete origin	LOD	HR pattern LG1	PMD	
CIBE6126	ET084980	1	2.69	57.97	218-220	218-220	218-218	223-230	230-244			1		Ollitrault *et al.* (2010)
CiC2110-02	ET099643	1	29.61	31.05	A-C	A-C	A-A	A-A	C-C			1		Ollitrault *et al.* (2012b)
mCrCIR06B05	AM489744	1	50.27	10.39	187-199	187-199	187-187	185-185	187-187			1		Froelicher *et al.* (2008)
MEST001	DY262452	1	70.61	9.95	176-192	176-192	187-199	190-190	172-172	1	1	1		Luro *et al.* (2008)
CiC5950-02	ET083949	1	91.37	30.71	A-G	A-G	A-A	A-A	G-G			1		Ollitrault *et al.* (2012b)
MEST431	DY291553	1	119.00	58.34	331-348	331-348	345-348	340-342	331-331			1		Garcia-Lor *et al.* (2012a)
JK-CAC15	none	2	43.51	13.36	160-163	160-163	152-160	160-160	151-163		1	1		Kijas *et al.* (1997)
mCrCIR03C08	FR677576	2	82.19	25.32	210-214	210-214	198-214	210-214	226-226				1	Cuenca *et al.* (2011)
CiC3712-01	ET079481	2	93.92	37.05	AC	AC	AA	AA	AA				1	Ollitrault *et al.* (2012b)
JK-TAA41	none	2	131.86	74.99	145-150	145-150	132-154	147-162	137-147	1			1	Kijas *et al.* (1997)
3P165889	Ciclev10023360m.g	3	1.00	89.59	AG	AG	AA	AA	AG				1	Curk *et al.* (2015)
3P11355960	Ciclev10023509m.g	3	88.50	2.09	AG	AG	AG	AA	AA		1			Curk *et al.* (2015)
CiC1459-02	ET073328	3	118.06	27.47	AC	AC	CC	CC	AA				1	Ollitrault *et al.* (2012b)
MEST131	DY276912	3	179.33	88.74	135-147	135-147	147-147	135-141	141-141	1				Garcia-Lor *et al.* (2012a)
CiC4240-04	ET106812	4	7.09	9.05	AG	AG	GG	GG	AG		1			Ollitrault *et al.* (2012b)
mCrCIR07D06	FR677581	4	16.33	0.19	164-168	164-168	168-168	166-178	166-168		1			Cuenca *et al.* (2011)
mCrCIR03G05	FR677578	4	75.06	58.92	226-229	226-229	218-218	218-218	199-228				1	Cuenca *et al.* (2011)
5p22687304	Ciclev10001185m.g	5	21.00	2.12	AC	AC	AC	AA	AA		1			Curk *et al.* (2015)
CiC5842-02	ET083106	5	77.34	54.22	AC	AC	CC	CC	AC				1	Ollitrault *et al.* (2012b)
CiC4356-06	ET107540	6	6.21	0.19	CT	CT	CT	CC	CT		1			Ollitrault *et al.* (2012b)
6p7496245	Ciclev10013603m.g	6	6.50	0.10	GC	GC	GC	CC	CC		1			Curk *et al.* (2015)
LapXcF238	EU719653	6	11.00	4.60	GC	GC	GG	GG	GC		1			Ollitrault *et al.* (2012b)
MEST488	DY297637	6	68.48	62.08	119-133	119-133	119-127	143-153	147-155				1	Garcia-Lor *et al.* (2012a)
JK-TAA1	none	6	93.49	87.09	170-180	170-180	146-162	146-162	160-164	1				Kijas *et al.* (1997)
mCrCIR03B07	FR677573	7	83.39	13.04	269-273	269-273	273-277	267-282	267-282		1			Cuenca *et al.* (2011)
CiC3674-02	ET079224	7	23.56	72.87	AG	AG	AA	AA	AG				1	Ollitrault *et al.* (2012b)
8P18684429	Ciclev10028449m.g	8	56.00	1.79	CT	CT	CT	CC	CC		1			Curk *et al.* (2015)
8P16570424	Ciclev10029557m.g	8	50.00	4.21	AG	AG	GG	GG	AA		1			Curk *et al.* (2015)
8P2427684	Ciclev10029965m.g	8	20.69	33.52	AT	AT	TT	TT	AA				1	Curk *et al.* (2015)
Ci02B07	AJ567403	9	0.00	52.16	164-170	164-170	170-172	162-172	178-182	1				Froelicher *et al.* (2008)
CiC4876-07	ET080580	9	2.69	49.47	AT	AT	TT	TT	AT				1	Ollitrault *et al.* (2012b)
9p4699283	Ciclev10005777m.g	9	50.00	2.16	AG	AG	AG	AA	AA		1			Curk *et al.* (2015)
CIBE3966	ET105040	9	52.27	0.11	106-118	106-118	118-N	106-118	106-118		1			Ollitrault *et al.* (2010)
Ci07C09	AJ567410	9	53.00	0.84	242-250	242-250	242-252	240-242	242-242		1			Froelicher *et al.* (2008)

1. Noted alleles. The numbers indicate the size of alleles in nucleotides for SSR markers and letters correspond to SNP markers alleles. N. Indicate null alleles.

LG: linkage group; GMP: genetic map position; DC: distance to the centromere, PMD: post-meiotic doubling mechanism

Triploid and tetraploid hybrids were also genotyped with 18 SNP markers using KASPar™ technology by LGC Genomics (http//www.lgcgenomics.com). 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 Company based on the SNP locus-flanking sequence (approximately 50 nt on each side of the SNP). SNP genotyping was performed using the KASPar technique. A detailed description of specific conditions and reagents can be found in Cuppen (2007). Identification of allele doses in heterozygous triploid and tetraploid hybrids was carried out based on the relative allele signals, as described by Cuenca *et al*. (2013a) and Aleza *et al*. (2015).

Identification of the parent producing the unreduced gamete and inference of the unreduced gamete genotype

For triploid and tetraploid hybrids, the 2n gamete origin was determined by identifying the parent that passed double genetic information onto the hybrid. Markers with total differentiation between the parents (A_1A_1 x $A_2A_2A_2A_2$, A_1A_2 x $A_3A_3A_3A_3$, and A_1A_2 x $A_3A_3A_4A_4$ in 2x x 4x crosses) for tetraploids and (A_1A_1 x A_2A_2, A_1A_2 x A_3A_3, and A_1A_2 x A_3A_4 in 2x x 2x crosses) for triploids were the best allelic configurations, as described by Aleza *et al*. (2015) and Cuenca *et al*. (2015). Indeed, conclusive results can be obtained using only one marker, as was the case for FinMac hybridization using the JK-TAA41 SSR marker. However, for EuFor and EuIch hybridizations, more than one marker had to be analyzed to observe both alleles from the female parent at least once for each hybrid. The SSRs JK-TAA1, JK-TAA41, and MEST131 were used for EuFor hybridization, and JK-TAA1, JK-TAA41, MEST001, and Ci02B07 were used for EuIch.

Once the female origin of the diploid gamete was demonstrated, inference of the allelic configurations of the 2n gametes from hybrid genotyping was performed as described by Cuenca *et al*. (2011). In the case of FinMac tetraploid hybridization, for the A_1A_2 x $A_3A_3A_3A_3$ and A_1A_2 x $A_3A_3A_4A_4$ allelic configurations, the genotype of the unreduced gamete was deduced directly from observation of both A_1 and A_2 alleles in the tetraploid hybrids. However, when the male and female parents shared one allele (A_1A_2 x $A_1A_1A_1A_1$ and A_1A_2 x $A_1A_1A_3A_3$), for the tetraploid hybrids that inherited the common allele (A_1), inference of the unreduced female gamete structure was carried out based on the estimated allele dosage in the tetraploid hybrid.

In the case of triploid hybrids obtained from EuFor and EuIch hybridizations, for A_1A_2 x A_3A_3 and A_1A_2 x A_3A_4, the genotype of the 2n gamete was deducted directly from the triploid hybrid structure. When the male and female genitors shared one allele (A_1A_2 x A_2A_2 and A_1A_2 x A_2A_3), the 2n female gamete structure for the triploid hybrids with a common allele from the male genitor was inferred from the estimated allele dosage in the triploid hybrid.

Identification of the mechanism underlying unreduced gamete formation

For the EuFor and EuIch progenies, nine SSR and SNP molecular markers within 20 cM of the centromere (Aleza *et al*., 2015) located in all nine LGs of the clementine genetic map (Ollitrault *et al*., 2012a) were genotyped to determine the mechanism of 2n

gamete formation for each population. The molecular markers used included MEST001, JK-CAC15, 3p11355960, mCrCIR07D06, 5p22687304, 6p7496245, mCrCIR03B07, 8p18684429, and Ci07C09 for EuFor hybridization and MEST001, JK-CAC15, 3p11355960, mCrCIR07D06, 5p22687304, CiC4356-06, mCrCIR03B07, 8p18684429, and 9p4699283 for EuIch hybridization. For FinMac hybridization, seven molecular markers distributed in seven LGs were used, including MEST001, JK-CAC15, CiC4240-04, LapXcF238, mCrCIR03B07, 8P16570424, and CIBE3966.

To distinguish between the SDR and FDR hypotheses, the maximum-likelihood method based on the LOD score test described by Cuenca *et al*. (2015) was employed. LODs >2 were considered to be significant for SDR, those <-2 were considered to be significant for FDR, and those between 2 and -2 were considered not to be significant. To compare the SDR hypothesis with the PRD hypothesis using LOD scores, we considered the minimum value of 66% of PHR as the theoretical value for the PRD hypothesis.

Additionally, a set of six SSR and SNP molecular markers distributed along LG 1 were used to analyze PHR evolution, including SSR markers MEST001, mCrCIR06B05, CIBE6126, and MEST431 and SNP markers CiC5950-02 and CiC2110-02. Moreover, a complementary experiment was performed to differentiate between PMD and SDR mechanisms using 11 telomeric molecular markers in LG 2 to LG 9. These included SSR markers mCrCIR03C08, JK-TAA41, MEST488, and mCrCIR03G05 and SNP markers CiC4876-07, CiC3674-02, CiC5842-02, CiC1459-02, CiC3712-01, 3p165889, and 8p2427684 (Table 2.1).

Interference analysis

Taking into account the centromere position, three-point linkage mapping was performed to estimate chiasma interference for each chromosome arm of chromosome I. The centromere was used as the first point, and two markers were selected on each arm (MEST001 and MEST431 on one arm and mCrCIR06B05 and CIBE6126 on the other arm). The chromosome interference coefficient (IC) is defined as follows (Griffiths *et al*., 1996):

$$\mathrm{IC} = 1 - \left[\frac{rd}{r_{C\,M_1} \cdot r_{M_1 M_2}} \right]$$

Where r_{CM1} indicates the observed recombination rate (heterozygous to homozygous and vice versa) between the centromere and locus 1; r_{M1M2}, the observed recombination between locus 1 and 2; and rd, the observed rate of double recombination between the centromere and locus 2.

Results and Discussion

Parental origin of recovered plants and frequencies of unreduced gametes

For sexual hybridizations between `Eureka Frost´ lemon as the female parent and `Fortune´ mandarin and *C. ichangensis* as the male parents, the average fruit set was 45.5% and 36.7%, respectively (Table 2.2), yielding 250 and 464 seeds, respectively, from both hybridizations. We classified the seeds by size, since, according to Aleza *et al*. (2010a), seed size is highly correlated to ploidy level. While small seeds are expected to contain triploid embryos, tetraploids are generally observed in normal size seeds. Thus, we selected 45 and 40 small seeds from the EuFor and EuIch hybridizations, respectively, for plant regeneration by embryo rescue.

Table 2.2. Plant regeneration and ploidy level of plants recovered from `Eureka Frost´ x `Fortune´ mandarin (EuFor), `Eureka Frost´ x *C. ichangensis* (EuIch) and `Fino´ x *C. macrophylla* (FinMac)

Hybridization	Pollinated flowers	Fruits set	Total number of seeds	Normal seeds	Undeveloped seeds	Small seeds	Cultured embryos	Recovered plants	Diploid plants	Triploid plants	Tetraploid plants
EuFor	55	25	464	419	0	45	54	53	32	21	0
EuIch	60	22	250	210	0	40	40	35	21	14	0
FinMac	15	8	156	36	154	36	36	36	0	23	13

From the 45 small seeds obtained in the EuFor hybridization, 54 embryos were cultured *in vitro*, with an average of 1.2 embryos per seed, indicating a low rate of polyembryony in `Eureka Frost´ lemon. Of the 53 plantlets recovered, 32 were diploid and 21 triploid. All 40 small seeds recovered from the EuIch hybridization contained only a single embryo. Of the 35 plants regenerated, 21 were diploid and 14 were triploid. For the FinMac 2x x 4x sexual hybridization, the average fruit set was 53.3%, and 36 normal seeds were obtained according to the size classification of Aleza *et al*. (2012b). Of the 36 plants recovered, 23 were triploid and 13 were tetraploid (Table 2.2).

To determine which parent passed double genetic information onto the hybrids, we genotyped triploid hybrids recovered from the 2x x 2x hybridizations using markers that displayed total allelic differentiation between `Eureka Frost´ lemon and the male parents, `Fortune´ mandarin and *C. ichangensis* (Figure 2.1): SSR markers JK-TAA1, JK-TAA41, and MEST131 for the EuFor hybridization and SSR markers JK-TAA1, JK-TAA41, MEST001, and Ci02B07 for the EuIch hybridization. Genetic analysis enabled us to unequivocally identify the hybrid origins of all triploid plants, except for one plant from the EuFor sexual hybridization and four from the EuIch sexual hybridization, which were rejected since they could have originated from autopollination of the female parents. Genetic analysis showed that `Eureka Frost´ lemon produced the 2n gametes for all triploid hybrids, as shown in Figure 2.1.

For the tetraploid hybrids, the JK-TAA41 SSR marker displayed total allelic differentiation between `Fino´ lemon and tetraploid *C. macrophylla*, allowing us to conclude that all plants were hybrids and that `Fino´ lemon produced the 2n gametes (Figure 2.1). Analysis of the genetic origins of the 23 triploid plants recovered from this

2x x 4x hybridization showed that, as expected, they were obtained from the union of a normal reduced haploid female gamete and a normal reduced diploid pollen gamete, as previously observed in other citrus species (Aleza *et al*., 2012a).

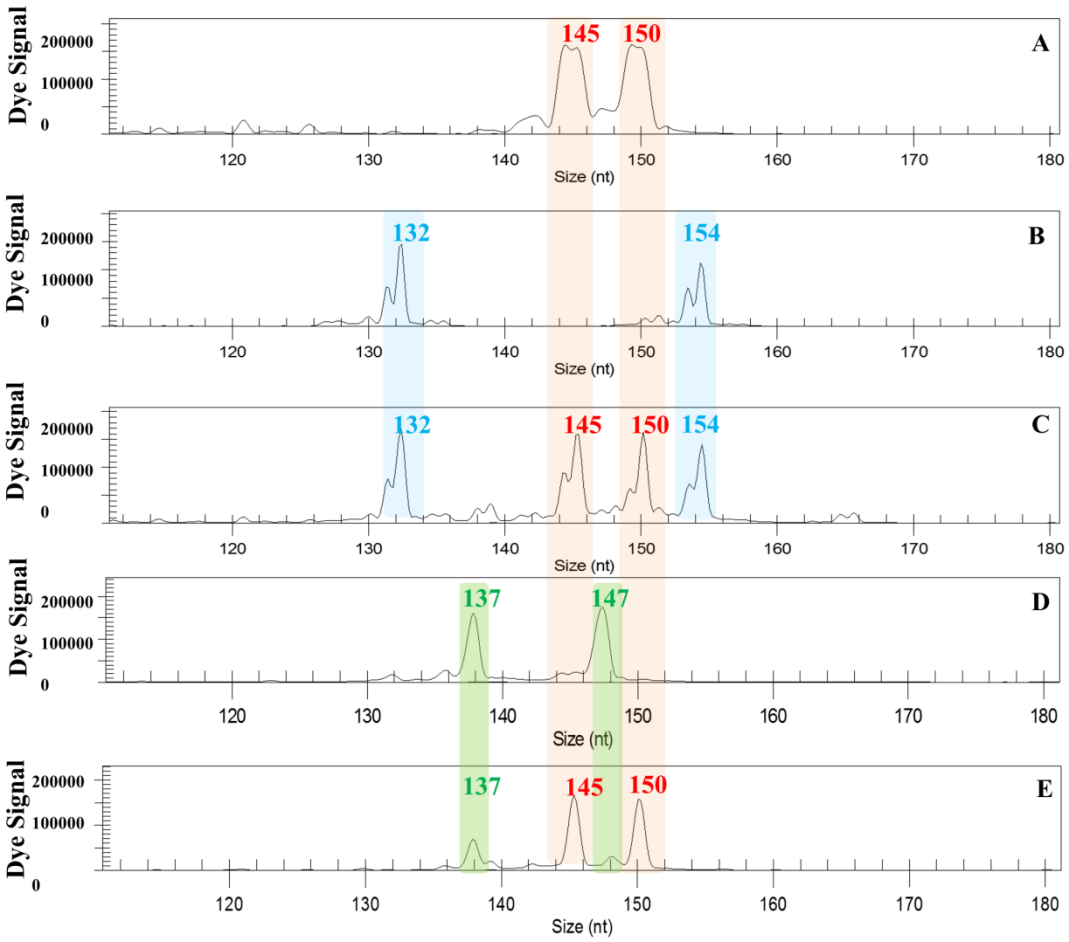

Figure 2.1. Electropherograms of a triploid and a tetraploid hybrid recovered from EuIch and FinMac hybridizations using SSR marker JK-TAA 41. **A.** `Fino´ and `Eureka Frost´ lemons displayed the same allelic configuration for this marker; **B.** *C. macrophylla*; **C.** tetraploid hybrid with four different alleles from `Fino´ x 4x *C. macrophylla* hybridization. **D.** *C. ichangensis*; **E.** Triploid hybrids with two alleles from the female parent `Eureka Frost´ lemon and one from the male parent *C. ichangensis*. nt: nucleotides.

Lemon hybrids were obtained from 2n gametes at a frequency of 4.9% for `Eureka Frost´ and 8.3% for `Fino´. Geraci *et al*. (1975) reported frequencies of 1% and 5% for triploid hybrids assumed to be obtained through unreduced gametes of `Lisbon´ and `Eureka´ lemons, respectively. Pérez-Tornero *et al*. (2012) obtained triploid hybrids at a frequency of 5.8% to 8.6% in hybridizations between diploid plants of `Verna´ as the female parent and `Fino´ as the male parent. In mandarins, greater differences between genotypes have been observed, ranging from less than 1% for clementines to over 22% for `Sukega´ and `Ortanique´ tangor (Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Wakana *et al*., 1982; Esen and Soost, 1971; Xie *et al*., 2014).

The frequency of 2n gametes was shown to be genotype-dependent in citrus and in other herbaceous and woody plants such as *Brassica*, potato, and peach (Dermen, 1938; Mok and Peloquin, 1975; Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Mason *et al*., 2011; Younis *et al*., 2014). This hypothesis is supported by the genetic improvement of unreduced gamete rates for Trifolium (frequencies increased from 0.04% to 47%) and *Medicago sativa* (from 9% to 78%) in only three generations of recurrent selection (Gallais, 2003).

In the current study, we observed a rate of 4.9% 2n gametes in the 2x x 2x hybridizations (EuFor and EuIch), whereas, in the 2x x 4x hybridization (FinMac), the percentage was higher (8.3%). These differences might be due to a genotypic effect of the parents, but are more likely due to the modification of the embryo/endosperm ploidy level ratio in interploid hybridizations. Esen and Soost (1971) reported that, in diploid plants, when an unreduced gamete is pollinated with normal reduced pollen, the embryo/endosperm ploidy level ratio (3/5) is less favorable for embryo development than that for normal diploid embryos (2/3), whereas the pollination of a 2n female gamete with diploid pollen in 2x x 4x sexual hybridizations provides the correct embryo/endosperm ploidy level ratio (4/6 = 2/3), leading to normal seed development. Therefore, 2x x 4x hybridization appears to be a more favorable situation for revealing unreduced gametes via the development of tetraploid embryos in normal seeds.

Mechanism of unreduced gamete formation

To determine the mechanism leading to unreduced gamete formation, we used nine unlinked molecular markers localized in the nine LGs for EuFor and EuIch and seven such markers in seven different LGs for FinMac to perform a LOD score test for SDR/FDR and SDR/PRD probability ratios for all genotypes analyzed (Tables 2.3, 2.4, 2.5). The analysis of six markers covering LG 1 and additional telomeric loci allowed us to distinguish between SDR and PMD when the inferred gametes were totally homozygous for the centromeric loci.

LOD score analysis

For the EuFor hybridization, 20 triploid hybrids were genotyped using nine centromeric loci found in all LGs. Ten of the inferred 2n gametes were totally homozygous for these markers. However, all displayed at least one heterozygous marker when six markers covering LG 1 were analyzed, allowing the PMD hypothesis to be rejected for all inferred 2n gametes. For the SDR/FDR hypothesis test at the individual level, 19 inferred 2n gametes displayed LOD values >2 (ranging from 12.05 to 15.22; Table 2.3). For the same 19 gametes, the LOD values for SDR/PRD were also >2. Therefore, these 19 plants were considered to have originated from SDR. One plant obtained negative LODs of -4.52 and -6.86 for the SDR/FDR and SDR/PRD hypotheses, respectively, suggesting that this plant is of FDR or PRD origin. At the population level, the LOD values were 267.82 and 57.03 for the SDR/FDR and SDR/PRD hypotheses, respectively, revealing a high rate of SDR.

Table 2.3. Heterozygous and homozygous profiles for 2n gametes from EuFor hybridization analyzed using SSR and SNP markers close to the centromere of each LG and the LOD score test for SDR/FDR and SDR/PRD probability ratio.

MARKER	**MEST001**	**JK-CAC15**	**3p 11355960**	**mCrCIR07D06**	**5p 22687304**	**6p 7496245**	**mCrCIR03B07**	**8p 18684429**	**Ci07C09**		
LG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**LOD (SDR/FDR)**	**LOD (SDR/PRD)**
Centromere Position (cM)	**0.607**	**0.569**	**0.906**	**0.161**	**0.231**	**0.064**	**0.964**	**0.542**	**0.522**		
Marker Position (cM)	**0.706**	**0.435**	**0.885**	**0.163**	**0.210**	**0.065**	**0.834**	**0.560**	**0.530**		
Distance to the centromere (cM)	**0.099**	**0.134**	**0.021**	**0.002**	**0.021**	**0.001**	**0.130**	**0.018**	**0.008**		
Genotypes analyzed	**2 n gamete genetic configuration**										
EuFor 1	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 2	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 3	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 4	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 5	HO	HO	HO	HO	HO	HO	HO	HE	HO	12.05	2.14
EuFor 6	HE	HO	HO	HO	HO	HO	HO	HO	HO	13.66	2.97
EuFor 7	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 8	HE	HO	HO	HO	HO	HO	HO	HO	HO	13.66	2.97
EuFor 9	HO	HO	HE	HO	HO	HO	HO	HO	HO	12.19	2.21
EuFor 10	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 11	HO	HE	HO	HO	HO	HO	HO	HO	HO	13.97	3.13
EuFor 12	HO	HE	HO	HO	HO	HO	HO	HO	HO	13.97	3.13
EuFor 13	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 14	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 15	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 16	HO	HE	HO	HO	HO	HO	HO	HO	HO	13.97	3.13
EuFor 17	HO	HE	HO	HO	HO	HO	HE	HO	HO	12.70	2.38
EuFor 18	HO	HE	HO	HO	HO	HO	HO	HO	HO	13.97	3.13
EuFor 19	HO	HO	HO	HO	HO	HO	HO	HO	HO	15.22	3.87
EuFor 20	HE	HO	HE	HE	HE	HO	HO	HE	HE	-4.52	-6.86
Population LODs	**267.82**	**57.03**									

LODs > 2 are significant for SDR. LOD < -2 are significant for FDR or PRD. LODs between 2 and -2 are not significant.

HE: Heterozygous; HO: homozygous

For EuIch hybridization, 10 triploid hybrids were genotyped with nine centromeric markers located on all LGs. Two inferred 2n gametes were totally homozygous for these markers, but at least one heterozygous locus was observed for each 2n gamete in LG 1, discarding the PMD hypothesis. At the individual level, eight plants displayed LOD values >2 for SDR/FDR (from 8.69 to 14.53), rejecting the FDR hypothesis (Table 2.4). Among these, seven displayed a LOD >2 for SDR/PRD (ranging from 2.13 to 3.86) and were considered to have arisen from SDR. The LOD value for the remaining 2n gamete was 0.55, suggesting that this 2n gamete had arisen from SDR rather than PRD, but, since this value is below our threshold, this result is not conclusive. Two plants produced negative LOD values (<-2) in both the SDR/FDR and SDR/PRD tests, suggesting that they originated by FDR or PRD. The population LODs were 80.21 and 2.77, respectively, for SDR/FDR and SDR/PRD, confirming the predominance of the SDR mechanism.

For FinMac, 13 tetraploid hybrids were genotyped with seven centromeric markers (LGs 1, 2, 4, 6, 7, 8, and 9). Six inferred 2n gametes were totally homozygous for these markers (Table 2.5). Among these, two unreduced gametes (from FinMac 12 and FinMac 13) remained totally homozygous after analyzing six markers covering LG 1 and were subjected to additional analysis to distinguish between the SDR and PMD hypothesis. The 11 2n gametes with at least one heterozygous locus produced LOD values >2 for SDR/FDR, rejecting the FDR hypothesis. Among these, four displayed LOD values of 2.81 for the SDR/PRD test and were therefore considered to have arisen from SDR. The seven remaining 2n gametes displayed positive values ranging from 0.52 to 1.91. These gametes had a higher probability of arising from SDR than from PRD, but this result is not conclusive because the values are below our threshold. The population LOD values were 78.84 and 19.81 for SDR/FDR and SDR/PRD, respectively, again confirming the prevalence of SDR. The seven 2n gametes with inconclusive individual LODs display a population LOD of 43.12 and 8.56 for SDR/FDR and SDR/PRD, respectively. It is therefore highly probable that they also arose from SDR.

Table 2.4. Heterozygous and homozygous profiles for 2n gametes from EuIch hybridization analyzed using SSR and SNP markers close to the centromere of each LG and the LOD score test for SDR/FDR and SDR/PRD probability ratio.

MARKER	**MEST001**	**JK-CAC15**	**3p11355960**	**mCrCIR07D06**	**5p22687304**	**CiC4356-06**	**mCrCIR03B07**	**8p18684429**	**9p4699283**		
LG	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		
Centromere Position (cM)	**0.607**	**0.569**	**0.906**	**0.161**	**0.231**	**0.064**	**0.964**	**0.542**	**0.522**		
Marker Position (cM)	**0.706**	**0.435**	**0.885**	**0.163**	**0.210**	**0.062**	**0.834**	**0.560**	**0.500**	**LOD (SDR/FDR)**	**LOD (SDR/PRD)**
Distance to the centromere (cM)	**0.099**	**0.134**	**0.021**	**0.002**	**0.021**	**0.002**	**0.130**	**0.018**	**0.022**		
Genotypes analyzed	**2 n gamete genetic configuration**										
EuIch 1	HE	HO	HO	HO	HO	HO	HE	HO	HE	8.69	0.55
EuIch 2	HO	HE	HO	HO	HO	HO	HO	HO	HO	13.28	3.12
EuIch 3	HO	HO	HO	HO	HO	HO	HO	HO	HO	14.53	3.86
EuIch 4	HO	HO	HO	HO	HO	HO	HO	HO	HE	11.53	2.21
EuIch 5	HO	HO	HO	HO	HO	HO	HO	HE	HO	11.36	2.13
EuIch 6	HE	HE	HO	HO	HO	HO	HO	HO	HO	11.72	2.21
EuIch 7	HO	HO	HO	HO	HO	HO	HO	HO	HO	14.53	3.86
EuIch 8	HE	HO	HO	HO	HO	HO	HO	HO	HO	12.97	2.95
EuIch 9	HE	HE	HE	HE	HE	HE	HO	HO	HE	-7.60	-8.19
EuIch 10	HE	HO	HE	HE	HE	HE	HE	HE	HE	-10.80	-9.93
	Population LODs									**80.21**	**2.77**

LODs > 2 are significant for SDR. LOD < -2 are significant for FDR or PRD. LODs between 2 and -2 are not significant.

HE: Heterozygous; HO: Homozygous

Table 2.5. Heterozygous and homozygous profiles for 2n gametes from FinMac hybridization analyzed using SSR and SNP markers close to the centromeres of seven LGs and the LOD score test for SDR/FDR and SDR/PRD probability ratio.

MARKER	**MEST001**	**JK-CAC15**	**CiC4240-04**	**LapXcF238**	**mCrCIR03B07**	**8P16570424**	**CiBE3966**		
LG	**1**	**2**	**4**	**6**	**7**	**8**	**9**		
Centromere Position (cM)	**0.607**	**0.569**	**0.161**	**0.064**	**0.964**	**0.542**	**0.522**		
Marker Position (cM)	**0.706**	**0.435**	**0.071**	**0.110**	**0.834**	**0.500**	**0.523**	**LOD (SDR/FDR)**	**LOD (SDR/PRD)**
Distance to the centromere (cM)	**0.099**	**0.134**	**0.091**	**0.046**	**0.130**	**0.042**	**0.001**		
Genotypes analyzed	**2 n gamete genetic configuration**								
FinMac 1	HO	HO	HO	HO	HO	HO	HO	8.93	2.81
FinMac 2	HO	HO	HO	HO	HO	HO	HO	8.93	2.81
FinMac 3	HO	HO	HO	HO	HO	HO	HO	8.93	2.81
FinMac 4	HO	HO	HO	HO	HO	HO	HO	8.93	2.81
FinMac 5	HO	HO	HO	HE	HO	HO	HO	6.62	1.52
FinMac 6	HE	HO	HO	HO	HO	HO	HO	7.37	1.91
FinMac 7	HO	HO	HE	HO	HO	HE	HO	4.88	0.52
FinMac 8	HO	HO	HE	HO	HO	HO	HO	7.28	1.86
FinMac 9	HO	HO	HE	HE	HO	HO	HO	4.97	0.56
FinMac 10	HO	HO	HE	HO	HE	HO	HO	6.00	1.10
FinMac 11	HO	HO	HE	HO	HE	HO	HO	6.00	1.10
	Population LODs							**78.84**	**19.81**

LODs > 2 are significant for SDR. LOD < -2 are significant for FDR or PRD. LODs between 2 and -2 are not significant.

HE: Heterozygous; HO: Homozygous

Pattern of heterozygosity restitution along LG 1 for 2n gametes with an identified SDR origin and undetermined SDR/PRD origin

To validate (at the population level) the finding that 38 2n gametes were derived by SDR (as determined by individual LOD analysis) and to distinguish between SDR and PRD for the eight gametes with inconclusive individual LODs, we compared the PHR patterns of the two sets of gametes in LG 1. For this analysis, we used four SSR markers (CIBE6126, mCrCIR06B05, MEST001, and MEST431) and two SNP markers (CiC2110-02 and CiC5950-02) (Figure 2.2) mapped in LG 1 (Figures 2.3 and 2.4).

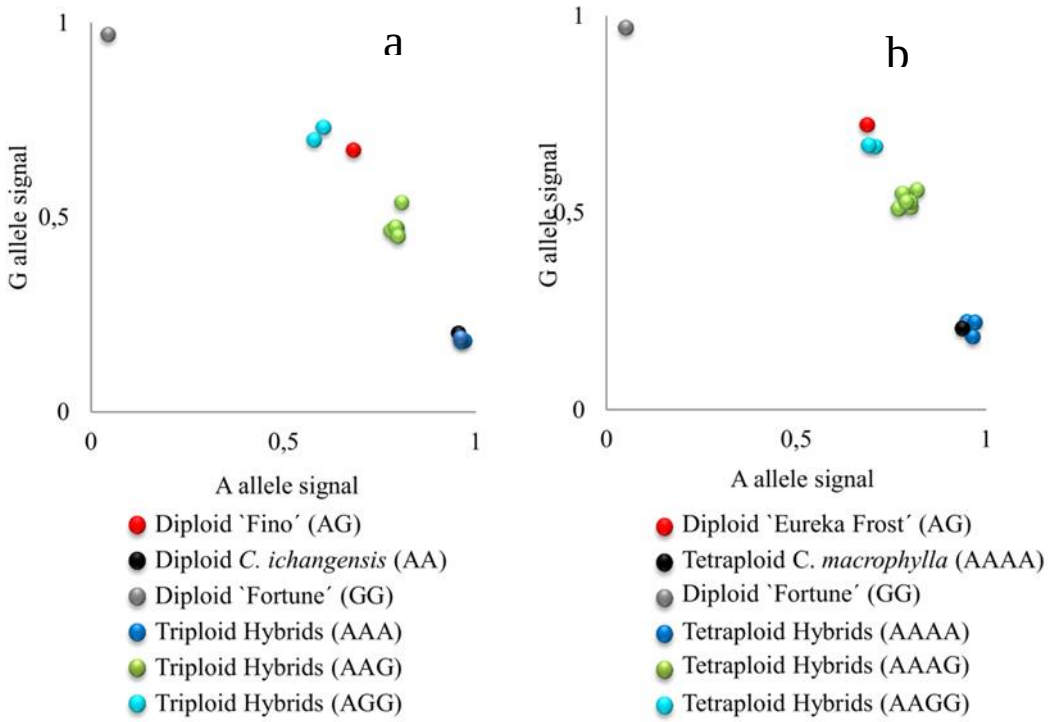

Figure 2.2. Plot of A, G allele signals of SNP marker CiC5950-02 representing triploid (**a**) and tetraploid (**b**) hybrids from EuIch and FinMac sexual hybridizations. Letters indicate the allelic configuration for each hybrid.

For the conclusive SDR gamete set, the PHR values in LG 1 (Figure 2.3) decreased from 67% for the telomeric marker CIBE6126 to 3% for the centromeric marker mCrCIR06B05 and progressively increased to 77% when moving towards the other telomeric marker, MEST431. The average PHR value was 42%. For the eight inconclusive 2n gametes, the same PHR pattern was observed: the lowest value was obtained for the centromeric marker mCrCIR06B05 (0%) and the highest for the telomeric markers (63% for CiC2110-02 in one telomere and 75% for MEST431 in the other). The average PHR for these eight gametes was 46% (Figure 2.3). These PHR patterns totally fit the profile for SDR. The average PHR value over the two sets of 2n gametes was 43%. Various studies have indicated that the global restitution of heterozygosity is expected to be near 80% for FDR and 40% for SDR, assuming a random distribution of heterozygous loci along the chromosomes (Peloquin, 1983; Hutten *et al*., 1994; Carputo *et al*., 2003). Both the patterns along LG 1 and the average PHR values comply with the SDR hypothesis. Therefore, we conclude that the eight 2n

gametes of indeterminate origin identified from the individual LOD (SDR/PRD) analysis also originated from SDR. Under this conclusion, the PHR pattern in LG 1 are very similar for `Eureka Frost´ and `Fino´ lemon SDR 2n gamete populations (Figure 2.4).

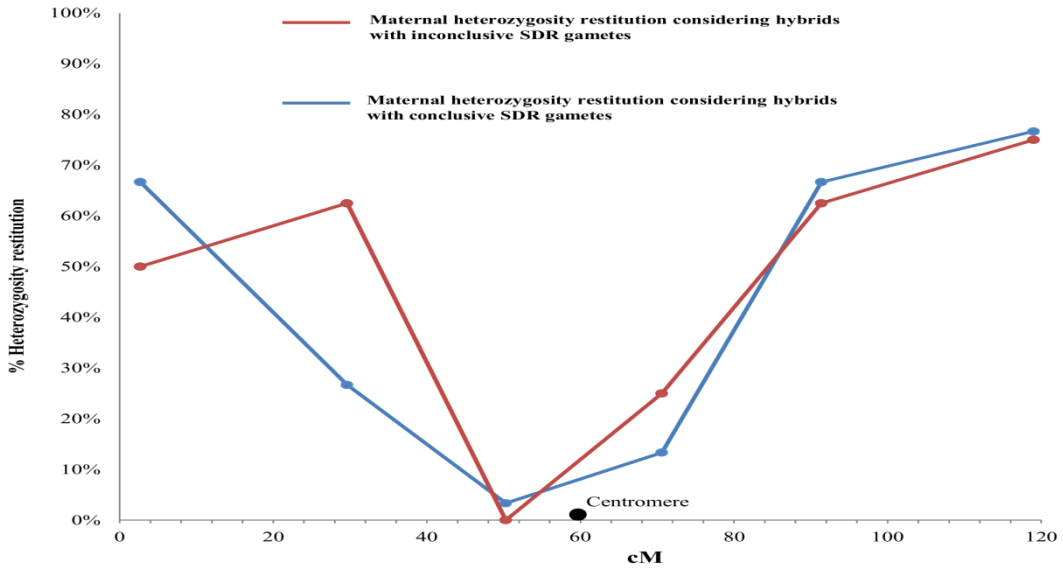

Figure 2.3. Evolution of maternal heterozygosity restitution values of the analyzed SSR and SNP markers in LG 1 considering the significance of the obtained LOD values for each hybrid from `Eureka Frost´ and `Fino´ lemons with conclusive and inconclusive SDR 2n gametes. Black dot indicates the centromere position on the reference clementine genetic map (Ollitrault *et al*., 2012a).

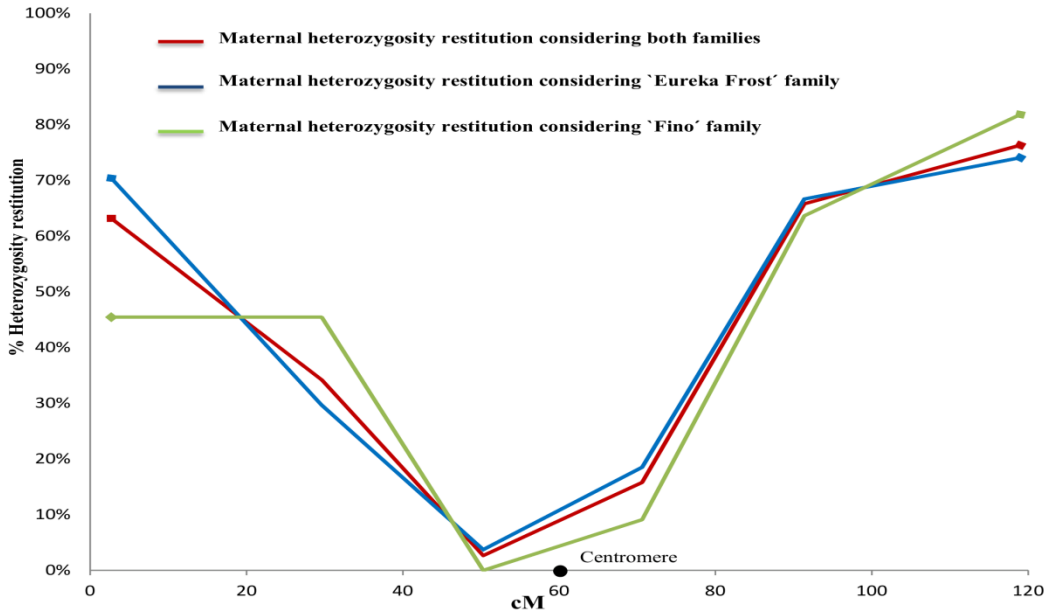

Figure 2.4. Evolution of maternal heterozygosity restitution values of the analyzed SSR and SNP markers in LG 1 considering both populations, `Eureka Frost´ and `Fino´ lemon SDR 2n gametes. Black dots indicate the centromere position on the reference clementine genetic map (Ollitrault *et al*., 2012a).

Distinction between SDR and PMD for fully homozygous 2n gametes

We performed additional analyses of the two inferred 2n gametes (FinMac 12 and FinMac 13 tetraploid plants) fully homozygous for the seven centromeric markers and the six markers of LG 1. Fully homozygous 2n female gametes for centromeric loci can originate through SDR or PMD, with different consequences for the genetic structures of 2n gametes. Bastiaanssen *et al*. (1998) defined two conditions that are necessary to conclude that PMD rather than SDR has occurred, i.e., 100% homozygosity for all genotyped loci and the occurrence of recombination between homozygous alleles in the same LG. Therefore, we genotyped FinMac 12 and FinMAc 13 using 11 telomeric loci found in different LGs to provide genetic evidence for a particular PMD mechanism. The average distance from these markers to their corresponding centromere is 53.22 cM (ranging from 25.32 to 89.59 cM). Both plants were homozygous for all molecular markers analyzed. Furthermore, *C. limon* is a direct hybrid between two genetically distant genotypes, *C. aurantium* and *C. medica* (Nicolosi *et al*., 2000; Curk *et al*., 2016), and the specific origins of the homozygous alleles can easily be distinguished. We found that some homozygous markers of the same LG were inherited from the *C. aurantium* ancestor and the others from *C. medica*. For example, multilocus analyses of the homozygous alleles in LG 1 (Figure 2.5) revealed interspecific recombination in the two plants with alternation of homozygosity originated from *C. aurantium* and *C. medica*. Consequently, according to Bastiaanssen *et al*. (1998), the observation of 100% homozygosity and recombination between *C. aurantium* and *C. medica* along the same LG provides evidence discarding the SDR mechanism and leads us to conclude that these two 2n gametes originated through PMD.

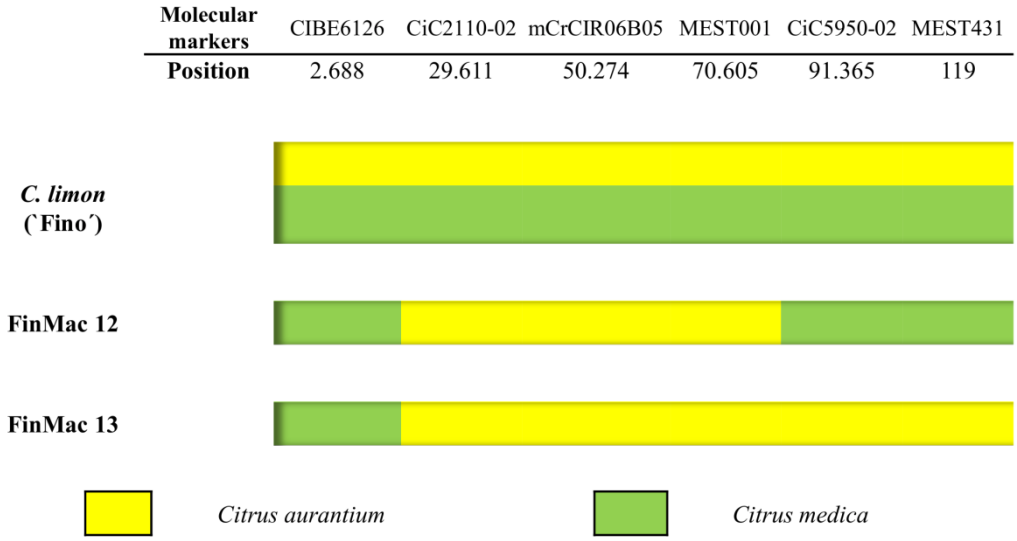

Figure 2.5. Multilocus configuration of the two fully homozygous plants recovered from FinMac hybridization with six molecular markers located on LG 1. Yellow indicates the presence of homozygous alleles inherited from *C. aurantium*, and green indicates those from *C. medica.*

Synthesis of different approaches

On the whole, we conclude that 38 (88%) of the 2n gametes analyzed had arisen from SDR, three (7%) from FDR or PRD, and two (5%) from PMD. At the population level, SDR appears to be by far the most common mechanism for 2n ovule formation in both *C. limon* genotypes, `Eureka Frost´ and `Fino´. Luro *et al*. (2004), Aleza *et al*. (2015) and Cuenca *et al*. (2015) also found that SDR was the predominant mechanism leading to 2n megagametophyte production in mandarins. Among the 19 mandarins investigated, the authors concluded that only 1.1% and 2.9% of plants were recovered from FDR in the `Ellendale´ and `Fortune´ genotypes, respectively. The coexistence of SDR and FDR has been observed in unreduced pollen (Rouiss *et al*., 2017a), but with FDR representing the main mechanism. In addition, FDR was the main mechanism for 2n female gamete production in `Femminello´ lemon (Ferrante *et al*., 2010). These results could be questionable because the authors used only a few molecular markers and lacked previous information about centromere location and the relative distances between the markers and the centromeres. With the recent location of centromeres in the citrus genetic map (Aleza *et al*., 2015; Ollitrault *et al*., 2012a), it appears that the markers used by Ferrante *et al*. (2010), JK-TAA1, JK-TAA15, JK-TAA41, and NB-GT03, are mostly telomeric, and therefore the high PHR values obtained in their study can fit both SDR or FDR mechanisms.

At the methodological level, we demonstrated the power of using two complementary approaches, namely, analysis of the PHR pattern in one LG with the maximum-likelihood method proposed by Cuenca *et al*. (2015). Considering only centromeric loci, different PMD mechanisms can lead to the same homozygous patterns. Therefore, analyzing the heterozygosity restitution pattern along LGs at the individual level is a useful approach for distinguishing between SDR and PMD, since, under this mechanism, the heterozygosity restitution value is zero for all markers in all LGs. After LOD analysis at the individual level, this method is used to analyze PHR patterns at the population level to distinguish between SDR and PRD when individual LODs are under the threshold required to obtain conclusive results. When enough individuals are analyzed, this technique should also be utilized to distinguish between FDR and PRD. With FDR 2n gametes, heterozygosity restitution varies from 100% in centromeric loci to close to 66% in telomeric areas under the non-interference model (Cuenca *et al*., 2011), whereas, with PRD, heterozygosity restitution is expected to be very similar along the entire chromosome.

Crossovers and interference analysis

Crossover interference ensures the appropriate distribution of crossovers along the chromosome, since one crossover reduces the likelihood of other crossovers occurring nearby (Youds *et al*., 2010). The analysis of crossover rates (Table 2.6) for both arms of chromosome I revealed the presence of up to four crossovers on one arm and three on the other arm. In addition, three complementary crossovers (double crossing over involving four chromatids) were observed as a result of phase-changing between two homozygous markers. Similarly, Cuenca *et al*. (2011) and Aleza *et al*. (2015) detected up to four crossovers on one arm and complementary crossovers in `Fortune´ mandarin and *C. clementina*. We estimated the IC for each chromosome arm, finding partial interference in both arms (IC = 0.27 and 0.44). Such variation in interference values between both arms has also been observed in other citrus species, ranging from 0.82 to

0.48 for `Fina´ clementine on LG 1 (Aleza *et al*., 2015) and 0.73 to 0.53 for `Fortune´ mandarin on LG 2 (Cuenca *et al*., 2011). Variation in the level of interference between different parts of the genome has also been observed in Arabidopsis (Drouaud *et al*., 2007), humans (Lian *et al*., 2008), and mice (Broman *et al*., 2002).

Table 2.6. Number of observed crossover events on each arm of chromosome I based on analysis of 27 genotypes recovered from `Eureka Frost´ lemon pollinated with *C. ichangensis* and `Fortune´ mandarin using six molecular markers. Numbers between brackets indicate the number of complementary crossovers.

		Arm 1					
Number of crossovers		**0**	**1**	**2**	**3**	**4**	
Arm 2	**0**	2	2	1	0	0	**13%**
	1	7	17	3 (2)	0	1(1)	**74%**
	2	1	3	0	0	0	**11%**
	3	0	1	0	0	0	**3%**
		26%	**61%**	**11%**	**0%**	**3%**	

Implications of sexual polyploidization for breeding triploid lemon-like plants

Sexual polyploidization via 2n gametes and interploid sexual hybridizations using tetraploid parents (doubled diploids) are the main strategies used to produce triploid citrus hybrids (Ollitrault *et al*., 2008; Aleza *et al*., 2010b; 2012a, b; 2016a; Navarro *et al*., 2015). These different strategies and the different meiotic behaviors result in different genetic structures in the diploid gametes and, consequently, the resulting triploid progenies. The three hybrids obtained via FDR or PRD 2n gametes have a higher rate of heterozygosity than hybrids obtained via SDR. By contrast, the two plants obtained by PMD transmit 0% PHR (Bastiaanssen *et al*., 1998). Therefore, such a mechanism (PMD) generally promotes inbreeding in the hybrid progenies (Tai, 1986; Gallais, 2003). However, these lines constitute interesting parentals to be used as test lines in inheritance studies (Bastiaanssen *et al*., 1998).

In addition, the mechanism that generates the 2n gametes affects the breeding efficiency for a character in relation to the genetic distance to the centromeres of the major genes controlling this character. For instance, Cuenca *et al*. (2013b; 2016) found that Alternaria brown-spot fungal disease is a recessive trait controlled by a single locus located 10.5 cM from the centromere of chromosome III. Therefore, in crosses between a heterozygous parent producing diploid gametes and a resistant genotype, PMD is the most favorable mechanisms (50% of resistant hybrids), followed by SDR (40%). Under FDR, only 5% of the hybrids will be resistant. For diploid gametes produced by the DD genotype or resulting from PDR, the rates of resistant hybrids should vary from 16% (tetrasomic segregation) to 0% (disomic segregation) according to the preferential pairing behavior.

The aim of some lemon-breeding programs is to produce new lemon-like types of fruit, which essentially involves 2x x 4x crosses using diploid lemons as female parents and more or less complex hybrids as tetraploid parents (Viloria and Grosser, 2005; Recupero *et al*., 2005). This approach is used in an attempt to solve some of the problems caused by the low genetic variation of *C. limon*, although relatively few

tetraploids are available. This approach has allowed for the selection and protection of the triploid Lemox, a hybrid between a diploid female complex hybrid, and tetraploid lemon (Recupero *et al*., 2005). Lemox produces quality fruits resembling lemons with high tolerance to Mal secco. The 2n lemon gametes will be very useful for producing new lemon-like seedless citrus types via 2x x 2x hybridizations, thereby dramatically increasing the gene pool of genotypes that could be used as parents. Furthermore, the production of 2n gametes has been investigated in a small number of lemon genotypes. Evaluating the many existing lemon genotypes may result in the detection of specific genotypes that produce higher rates of 2n gametes and (eventually) genotypes with different ratios of FDR and SDR 2n gametes, which will increase the efficiency of breeding programs.

Conclusion

Genetic analysis with SSR and SNP markers revealed that two genotypes of *C. limon*, `Eureka Frost´ and `Fino´, produced 2n female gametes. The frequencies of 2n gametes were 4.9% and 8.3% for `Eureka Frost´ and `Fino´ lemons, respectively. The use of complementary methods, including individual LOD analysis from centromeric loci, telomeric loci genotyping, and the analysis of PHR patterns along a linkage group, allowed us to distinguish among the different mechanisms of 2n gamete formation. We detected three meiotic mechanisms in lemon, with 88% of 2n female gametes arising from SDR, 7% from FDR or PRD, and 5% from PMD. To our knowledge, this is the first report of the production of a large number of lemon progenies from 2n gametes and the identification of a new mechanism, PMD, that has never been observed in citrus and has rarely been described in other herbaceous or woody species. From the breeding point of view, the production of SDR 2n gametes would allow progenies with polymorphic genetic structures to be recovered, increasing the likelihood of obtaining new phenotypes by creating an increasing number of novel multilocus allelic combinations. The coexistence of different mechanisms for 2n gamete formation broadens the diversity of lemon 2n gametes and, therefore, their potential for breeding.

CHAPTER III

Doubled diploid `Mexican´ lime display preferential disomic segregation compatible with an interploid crosses origin of *C. latifolia* and *C. aurantifolia* triploid limes.

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Abstract

Triploid limes have an important worldwide fruit production, but it is based in a very narrow genetic basis. The `Tahiti´ lime type (*C. latifolia*) is predominant, while the `Tanepao´ type (*C. aurantifolia*) is less produced. Both types resulted from natural interspecific hybridization involving a diploid gamete of *C. aurantifolia* `Mexican´ lime type (a direct interspecific *C. micrantha* x *C. medica* hybrid). Doubled diploid (DD) `Mexican´ lime spontaneously occurs in seedlings. In a first step to implement a reconstruction breeding program of limes, based on phylogenomic data, we analysed the meiotic mechanisms of a DD `Mexican´ lime, the interspecific recombination and the resulting diploid gamete structures to evaluate the possibility that `Tahiti´ and `Tanepao´ varieties derived from interploid hybridization.

A population of 85 tetraploid hybrids was established by pollination of a DD clementine by a DD `Mexican´ lime and used to infer the genotypes of `Mexican´lime diploid gametes. Meiotic behaviour was studied combining segregation analysis of 35 SSRs and SNPs markers and cytogenetic studies. It was completed by a pollen viability evaluation. Genetic mapping allowed to evaluate the interspecific recombination rates and to compare them to diploid and tetraploid clementine ones.
Pollen viability of the DD `Mexican´ lime (64%) was much higher than the diploid one. In average, 65% of the chromosomes were in bivalent configuration and 31.4% in tetravalent ones. Parental heterozygosity restitution varied between 83% and 99%. Disomic inheritance with high preferential pairing values was deduced for three LGs. Intermediate inheritance with disomic tendency was found for five LGs and intermediate model was observed for one LG. The average effective interspecific recombination rate was 1.2 cM/Mb, three times lower than in diploid and tetraploid clementines.

The DD `Mexican´ lime had a predominantly disomic segregation producing interspecific diploid gamete structures with high *C. medica / C. micrantha* heterozygosity compatible with the phylogenomic structures of triploids *C. latifolia* and *C. aurantifolia* varieties. This disomic tendency limits the effective interspecific recombination and the diversity of the diploid gamete population. Interploid reconstruction breeding, using doubled diploid lime as one parent is a promising approach for the diversification of triploid limes.

Keywords

Citrus, DD `Mexican´ lime, *C. medica / C. micrantha*, Diploid gamete, Markers and Cytogenetic studies, Disomic tendency, Triploid limes.

Introduction

Limes and lemons are two closely related horticultural groups cultivated under all Mediterranean, sub–tropical and inter–tropical climates with around 15 million tons (Mt) produced worldwide (FAO, 2014). After expanding up to 2007, the lemon market is currently stagnating while the consumption of limes has increased dramatically since the 1980s (Duportal *et al*., 2013). Interestingly the triploid `Tahiti´ lime is one of the less susceptible citrus varieties for the main threats of citrus production in tropical and subtropical areas, the Huanglongbing disease (HLB) caused by the phloem limited bacteria *Candidatus Liberibacter* spp. However, the lime production is based on a very narrow genetic basis including a few diploid and triploid cultivars and varietal diversification is needed to promote sustainable lime production. At triploid level the seedless `Tahiti´ lime type is predominantly produced for the export market. The other major triploid variety, the `Tanepao´ lime type, produces seedy fruits and has only limited local areas of production.

The cultivated lime varieties are based on complex interspecific genomic structures as most cultivated citrus. Citrus, is a large genus that includes several major cultivated species. It is believed to be native to Southeast Asia (Webber *et al*., 1967), and its cultivation as a fruit crops occurred at least 4,000 years ago (Legge, 1865). Molecular markers and genomic studies identified four taxa, *C. reticulata*, *C. maxima*, *C. medica* and *C. micrantha* as the ancestors of all cultivated Citrus species (Nicolosi *et al*., 2000; Barkley *et al*., 2006; Ollitrault *et al*., 2012a; Garcia–Lor *et al*., 2013b; Curk *et al*., 2016). The differentiation between these ancestral taxa occurred through allopatric evolution and then the so called secondary species (*C. sinensis* –sweet oranges–, *C. aurantium* –sour oranges–, *C. paradisi* –grapefruits–, *C. limon* – lemons-) and particularly the limes (*C. aurantifolia* and C. *Latifolia*) were the result of reticulate evolution with a limited number of interspecific meiosis due to facultative apomixis (nucellar polyembryony).

While many citrus horticultural groups result only from *C. reticulata* and *C. maxima* gene pools at diploid level (Nicolosi *et al*., 2000; Barkley *et al*., 2006; Ollitrault *et al*., 2012a; Garcia–Lor *et al*., 2013b), the genomic structure of limes appeared more complex. Indeed, it involves the four ancestral taxa (Curk *et al*., 2016). Moreover lime is the only Citrus horticultural group that include triploid and tetraploid natural germplasm in addition to diploid one. Based on codominant markers various nuclear analysis revealed that diploid `Mexican´ lime *C. aurantifolia* results from a direct natural hybridization between *C. micrantha* as female parent and *C. medica* as male parent (Nicolosi *et al*., 2000; Ollitrault *et al*., 2012a; Garcia–Lor *et al*., 2013b; Curk *et al*., 2016). The tetraploid `Giant Key´ lime, also classified as *C. aurantifolia* by Tanaka (1961) was selected in a seedling of the diploid `Key´ lime (`Mexican´ lime type) in Florida (US Horticultural Research Laboratory, Orlando) by HC Barrett (Curk *et al*., 2016) .

Recently, Curk *et al*. (2016) demonstrated the contribution of the four ancestral taxa to the *C. latifolia* triploid varieties (`Tahiti´ lime type*)* genome and proposed that it resulted from the fertilization of a haploid ovule of *C. limon* by a diploid gamete of *C. aurantifolia*. Lemon is itself a complex genome issued from the hybridization of a citron (*C. medica*) and sour orange (a *C. maxima* x *C. reticulata* direct hybrid). The

same authors proposed that the *C. aurantifolia* triploid varieties (`Tanepao´ lime like), with only *C. medica* and *C. micrantha* contribution, probably resulted from an interspecific backcross of a diploid ovule of *C. aurantifolia* (*C. micrantha* x *C. medica*) fertilized by *C. medica*. The actual phenotypic diversity around the `Tahiti´ and `Tanepao´ lime types results from asexual variations (mutations or somaclonal variations).

Polyploidisation is a major mechanism of angiosperm evolution (Soltis and Soltis, 1993; Wendel and Doyle, 2005) and many authors consider that most polyploids arise from unreduced (2n) gametes (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998, 2002). Diploidy is the general rule in *Citrus* with a basic chromosome number of nine (X=9) (Krug 1943) and an estimated genome size of ~367 Mb (Terol *et al*., 2008). Only a few triploid and tetraploid genotypes have been found in the citrus germplasm (Longley, 1925; Lee, 1988). Despite this scarcity of polyploid germplasm, it appeared that polyploidisation events are relatively frequent in citrus seedling. Doubled diploids (DD) plants were observed early in seedling of diploid apomictic genotypes (Lapin, 1937; Russo and Torissi, 1951; Cameron and Frost, 1968) and their frequency depends on genotypes and environment (Aleza *et al*., 2011). They arise from spontaneous duplication of chromosomes in nucellar cells (Cameron and Frost, 1968; Aleza *et al*., 2011). The `Giant Key´ lime originated from this mechanism (Curk *et al*., 2016). Unreduced female and male gametes have also been described in citrus (Esen and Soost, 1971; Ollitrault *et al*., 2008; Cuenca *et al*., 2015; Rouiss *et al*., 2017a) and they can lead to the creation of triploid and tetraploid hybrids. Various mechanisms can produce 2n gametes in citrus. Second division restitution (SDR) is predominant for 2n megagametophytes (Esen *et al*., 1979; Cuenca *et al*., 2011, 2015; Aleza *et al*., 2015) while first meiotic restitution (FDR) was described as the major mechanism for the production of 2n pollen in a clementine x sweet orange hybrid (Rouiss *et al*., 2017a). Today there is no evidence on the polyploidisation mechanisms (interploid hybridization or 2n gametes) that produced triploid *C. latifolia* and *C. aurantifolia* limes.

The genetic structure of diploid gamete populations and particularly the parental heterozygosity restitution (PHR) is driven by their origin. In case of 2n gametes PHR is a function of the genetic distance to the centromere (Cuenca *et al*., 2015). In centromeric area PHR is respectively null and total for SDR and FDR respectively, increasing and decreasing with the genetic distance to centromere. For diploid gametes produced by tetraploid plants there are two extreme models, disomic in allotetraploids and tetrasomic in autotetraploids (Stebbins, 1947; Stift *et al*., 2008; Sybenga, 2012). In allotetraploids, resulting from the merger of two specie's genomes, there are two sets of homologous chromosomes and during meiosis, each chromosome pairs only with its homologous (Sybenga, 2012), and only bivalents are formed (Stebbins, 1947). It results in a disomic inheritance with 100% of the interspecific heterozygosity transmitted by each gamete (Stift *et al*., 2008). In autotetraploids, the presence of four homologous chromosomes instead of two, results in equal opportunities to pair at meiosis leading to multivalent formation and tetrasomic inheritance (Jackson and Jackson, 1996; Sybenga, 1996). For doubled diploids, it leads, hypothetically, to 66% of restitution of the heterozygosity of the diploid that originated the tetraploid (Sanford *et al.*, 1983; Aleza *et al*., 2016a). Allo and autotetraploids (with disomic and tetrasomic inheritance, respectively) are the extremes of a range. In cases where parents are divergent but have retained enough homology to prevent exclusive preferential pairing, inheritance patterns

intermediate `segmental patter´ between di and tetrasomic can be expected (Stebbins, 1947; Sybenga, 1996; Stift *et al*., 2008; Jeridi *et al*., 2012). Many polyploid taxa display a combination of autopolyploid and allopolyploid pairing behavior (Allendorf and Danzmann 1997; Fjellstrom, *et al*., 2001; Jackson and Jackson 1996) and several studies presented inheritance patterns intermediate among disomic and tetrasomic (Danzmann and Bogart 1983; Hickok 1978; Marsden *et al*., 1987; Stift *et al*., 2008). Stift *et al*. (2008) developed a likelihood–based approach to evaluate whether disomic, intermediate or tetrasomic inheritances best fitted the segregation of genetic markers and to estimate preferential pairing and double reduction (DR) rates. DR can occur for tetravalents and increases the homozygosity of diploid gametes (Stift *et al*., 2008; Ronfort *et al*., 1998; Sybenga, 1995; Aleza *et al*., 2016a). This method was simplified for doubled–diploids by Aleza *et al*. (2016a).

Large structural rearrangements (inversions, translocations and deletions), sequences divergence but also genetic control (Jenczewski *et al*., 2003; Griffiths *et al*., 2006; Qi *et al*., 2007 Cifuentes *et al*. , 2010) and meiosis mutation (such asynapsis or desynapsis) can affect chromosome pairing. Inversion and asynapsis, were described in diploid `Mexican´ lime (Iwamasa *et al*., 1962; Iwamasa and Nito, 1988) resulting in partial sterility.

In the present work we analyzed the preferential chromosome pairing and inheritance of the interspecific (*C. medica / C. micrantha*) doubled diploid `Mexican´ lime. It was performed by combining a meiotic cytogenetic study and the analysis of single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers segregations. The interspecific recombination and interspecific structures of diploid `Mexican´ lime gametes where then analyzed from the genetic markers data and their compatibility with *C. aurantifolia* and *C. latifolia* triploid lime phylogenomic structure was evaluated. The implications for lime breeding programs are discussed.

Materials and methods

Plant materials

Sexual hybridization between doubled–diploid `Clemenules´ clementine (*C. clementina* Hort. Ex Tan.) as female parent and doubled–diploid `Mexican´ lime as male parent (Cl4x x ML4x hybridization) was performed in order to obtain tetraploid hybrids (named ClemMex) to study the segregation model of the doubled–diploid `Mexican´ lime. Doubled–diploid `Clemenules´ clementine was recovered by shoot–tip grafting *in vitro* and colchicine treatment (Aleza *et al*., 2009b) whereas tetraploid `Mexican´ lime was identified by flow cytometry in seedlings of diploid `Mexican´ lime and proved to be a doubled–diploid by molecular marker analysis (Aleza *et al*., 2011). The hybrids were recovered from normal seeds since the pollination of a tetraploid plant with diploid pollen provides the correct embryo/endosperm ploidy level ratio (4/6 = 2/3), leading to normal seed development. The ploidy level was verified by flow cytometry as described in Aleza *et al*. (2010a). Eighty five tetraploid hybrids were obtained.

Pollen viability

Pollen viability was estimated using aceto–carmine colorimetric tests (Stanley and Linskens, 1947). The stain was added on pollen grains and observed under photonic microscope (Leica DM LB). The pollen viability was scored according to staining level; pollen with bold red colour are viable and colourless are unviable. The percentage of pollen viability was determined as the ratio of the number of viable grains to the total grain number.

Meiotic chromosome preparation

Fifty one pollen mother cells (PMC) of different anthers were observed and analysed for this work. Basic cytogenetic protocols that have been described for meiotic chromosomes pairing for the genus *Musa* (Shepherd, 1999) were used. Anthers at the appropriate stage of meiosis were selected by aceto–carmine test preparation of two opposite anthers of each flower harvested from the IVIA Citrus Germplasm Bank of pathogen–free plants (Navarro *et al*., 2002). They were fixed in a mixture of absolute ethanol, chloroform and acetic acid (6,3,1) for 24 hours. Later they were transferred to 70% alcohol for storage. The next steps of the protocol were done directly in the slides. The anthers were dissected and stained in a drop of 1% carmine in 45% acetic acid. A cover glass was placed, and the slides were warmed to well short of boiling point. The anthers were lightly pressed under the cover glass. Finally the cover glass edges were sealed with nail varnish to avoid drying out of the smear.

Genotyping of progenies using Simple Sequence Repeat (SSR) and Single Nucleotide Polymorphism (SNP) markers

Genomic DNA of the hybrids and their parents was isolated using the Plant DNAeasy kit from Qiagen Inc. (Valencia, CA, USA), following the manufacturer´s protocol. For SSRs, PCR amplifications were performed using a Thermocycle rep gradient S (Eppendorf®) in 10 µL final volume containing 0.8 U of Taq DNA polymerase (Fermentas®), 2 ng/mL of citrus DNA, 0.2 mM of wellRED (Sigma®) dye–labelled forward primer, 0.2 mM of non dye–labelled reverse primer, 0.2 mM of each dNTP,

10X PCR buffer and 1.5 mM MgCl2. The PCR protocol was as follows, denaturation at 94°C for 5 min followed by 40 repeats of 30 s at 94°C, 1 min at 50°C or 55°C, 45 s at 72°C; and a final elongation step of 4 min at 72°C. Capillary electrophoresis was carried out using a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc.). PCR products were initially denatured at 90°C for 2 min, injected at 2 kV for 30 s and subsequently separated at 6 kV for 35 min. Alleles were sized, based on a DNA size standard (400 bp). The GenomeLab™ GeXP v.10.0 genetic analysis software was used for data collection. Allele dosage was calculated using the MAC–PR (microsatellite DNA allele counting–peak ratio) method (Esselink *et al*., 2004), validated in citrus by Cuenca *et al*. (2011).

Progenies were also genotyped with SNP markers using KASPar technology. 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 Company based on the SNP locus flanking sequence (approximately 50 nt on each side of the SNP). SNP genotyping was performed using the KASPar technique. Detailed explanation on specific conditions and reagents can be found in Cuppen (2007). Identification of allele doses in heterozygous tetraploid hybrids has been carried out from the relative allele signals as described by Cuenca *et al*. (2013).

Control of the hybrid origin of tetraploid plants and inference of the diploid gamete genotype

Confirmation of hybrids origin was performed using two SSRs (mCrCIR07F11 and MEST001) with total differentiation between the parents (A_1A_2 x A_3A_4).
To study the genetic structure of the diploid gametes produced from the doublesd-diploid `Mexican´ lime, the male and female parents and 85 hybrids were genotyped using a total of 35 molecular markers (27 SSRs and 8 SNPs) heterozygote for the `Mexican´ lime and polymorphic with `Clemenules´ clementine (Table 3.1). They are distributed across all LGs of the clementine genetic map (Ollitrault *et al*., 2012b) with a minimum of three molecular markers in LG07 and a maximum of five molecular markers in LG06 and LG09. Distances of the markers to the centromere were estimated from the clementine genetic map (Ollitrault *et al*., 2012b) and the estimated centromere genetic mapping (Aleza *et al*., 2015). In case that the markers were not in the clementine genetic map, their position was inferred from their physical position and local correlations between physical and genetic positions. The marker closer to a centromere was 6P7496245 at 0.10 cM from the centromere of LG06 and the furthest was MEST131 at 88.74 cM from the centromere of LG03. In total, 18 markers are considered as centromeric markers (15 SSR and 3 SNP), the rest are either telomeric or intermediate markers. In all LGs, at least one centromeric marker (less than 20 cM from the centromere) and one telomeric marker have been used. For `Mexican´ lime the allele inherited from *C. medica* and *C. micrantha* ancestors are respectively in first and second positions in table 3.1.

Table 3.1. Molecular markers used to study the genetic structure of the diploid gametes produced from the tetraploid `Mexican´ lime, with their gene bank accesion, genetic position, noted alleles and bibliographic reference

Locus	Gene Bank Accesion	LG	Genetic position (cM)	Distance to centromere	Alleles [1]		Bibliographic reference
					Clementine	`Mexican´ lime [2]	
1P199494	Ciclev10010680m.g	1	1.00	59.66	C-C	T-C	Curk *et al*. (2015)
CIBE5720	ET082224	1	58.45	2.21	325-337	320-308	Ollitrault *et al*. (2010)
MEST001	DY262452	1	70.61	9.95	170-174	186-190	Luro *et al*. (2008)
JK-taa15	none	1	119.73	59.07	188-192	164-168	Kijas *et al*. (1997)
mCrCIR02D09	FR677569	2	11.37	45.50	230-238	233-248	Cuenca *et al*. (2011)
2P25198777	Ciclev10015267m.g	2	67.6	10.73	A-A	G-A	Curk *et al*. (2015)
JK-TAA41	none	2	131.86	74.99	147-154	132-170	Kijas *et al*. (1997)
mCrCIR04F12	FR692369	3	29.66	60.93	261-263	263-259	Ollitrault *et al*. (2012)
CIBE1644	ET097780	3	70.23	20.36	346-364	350-368	Ollitrault *et al*. (2010)
JITC01	CK934237	3	109.68	19.09	333-347	349-335	(Ollitrault *et al*., 2012b)
MEST131	DY276912	3	179.4	88.81	141-147	141-124	Garcia-Lor *et al*. (2012a)
MEST070	DY268779	4	4.25	11.89	218-220	224-193	in preparation
CID6458	ET086604	4	15.88	0.259	385-397	397-388	Ollitrault *et al*. (2012a)
mCrCIR07D06	FR677581	4	16.33	0.19	165-188	172-167	Cuenca *et al*. (2011)
mCrCIR03G05	FR677578	4	75.06	58.92	226-228	219-215	Cuenca *et al*. (2011)
mCrCIR07G11	AM489751	5	20.2	2.92	202-210	208-145	Froelicher *et al*. (2008)
cms30	none	5	36.84	13.72	152-156	150-154	Ahmad *et al*. (2003)
mCrCIR01F08	AM489737	5	54	30.88	118-118	131-128	Froelicher *et al*. (2008)
mCrCIR04H12	FR692371	6	0	6.4	160-160	166-178	Ollitrault *et al*. (2012)
6P7496245	Ciclev10013603m.g	6	6.3	0.1	C-C	G-C	Curk *et al*. (2015)
MEST488	DY297637	6	68.48	62.08	126-130	120-128	Garcia-Lor *et al*. (2012a)
PSY-C461	AB037975	6	69.72	63.32	A-A	T-A	Ollitrault *et al*. (2012b)
AOC-C593	DY293375	6	89.88	83.48	T-T	A-T	Ollitrault *et al*. (2012b)
MEST107	DY274062	7	8.899	87.531	175-183	175-181	Garcia-Lor *et al*. (2012a)
DXS-C545	Ciclev10024949m.g	7	40	56.43	G-G	C-G	Garcia-Lor *et al*. (2013b)
mCrCIR03B07	FR677573	7	83.39	13.04	263-265-	273-277	Cuenca *et al*. (2011)
mCrCIR01F04	AM489736	8	5.92	48.29	186-202	202-171	Froelicher *et al*. (2008)
CiBE0214	ET088913	8	40.41	13.8	313-324	316-307	Ollitrault *et al*. (2010)
8P18684429	Ciclev10028449m.g	8	55	0.79	C-C	T-C	Curk *et al*. (2015)
mCrCIR02A09	FR677568	8	98.63	44.42	160-163	163-178	Cuenca *et al*. (2011)
Ci02B07	AJ567403	9	0	52.16	163-165	165-154	Froelicher *et al*. (2008)
mCrCIR07F11	FR677567	9	49.57	2.59	152-160	168-158	Kamiri *et al*. (2011)
JI-TCT01	CV704385	9	52.8	0.64	148-154	145-148	in preparation
Ci08C05	AJ567415	9	55.14	2.98	154-175	135-152	Froelicher *et al*. (2008)
9P31143176	Ciclev10006644m.g	9	88	35.84	A-A	A-G	Curk *et al*. (2015)

1: Alleles. The numbers indicate the size of alleles in nucleotides for SSR markers and letters correspond to SNP markers alleles.

2: The first allele is the one inherited from *C. medica* and the second one from *C. micrantha*

For markers with total allelic differentiation between parents ($A_1A_1A_2A_2$ x $A_3A_3A_4A_4$ and $A_1A_1A_1A_1$ x $A_2A_2A_3A_3$), the genotype of the diploid gamete from `Mexican´ lime was inferred directly from the presence/absence of the specific alleles of the

`Mexican´ lime in the hybrid. When the male and female genitor shared one allele ($A_1A_1A_1A_1$ x $A_1A_1A_2A_2$ and $A_1A_1A_2A_2$ x $A_2A_2A_3A_3$), the inference of the diploid male gamete structure was carried out from the estimated allele dosage in the tetraploid hybrid. For markers with $A_1A_1A_1A_1$ x $A_1A_1A_2A_2$ configuration, A_1A_1, A_1A_2 and A_2A_2 male gametes were inferred respectively from $A_1A_1A_1A_1$, $A_1A_1A_2A_2$ and $A_1A_1A_2A_2$ hybrid genotypes. For markers with $A_1A_1A_2A_2$ x $A_2A_2A_3A_3$ allelic configuration, the potential nine combinations of the two parental diploid gametes produce nine tetraploid hybrids genotypes totally differentiated by allele doses; $A_1A_1A_2A_2$, $A_1A_1A_2A_3$, $A_1A_1A_3A_3$, $A_1A_2A_2A_2$, $A_1A_2A_2A_3$, $A_1A_2A_3A_3$, $A_2A_2A_2A_2$, $A_2A_2A_2A_3$ and $A_2A_2A_3A_3$. The male diploid gametes inferred from these tetraploid hybrid genotypes were respectively, A_2A_2, A_2A_3, A_3A_3, A_2A_2, A_2A_3, A_3A_3 A_2A_2, A_2A_3 and A_3A_3.

Statistical analysis of preferential pairing

Stift *et al*. (2008) proposed a segregation model to interpret the inheritance model in allotetraploid citrus. Aleza *et al*. (2016a) simplified it for the doubled diploid, considering that the expected gamete frequencies only depends of the `tetrasomic´ parameter (τ) corresponding to the proportion of gametes formed by random meiotic chromosome associations (random bivalent or tetravalent pairing) taking values from zero (full disomic) to one (full tetrasomic). τ was estimated by a maximum likelihood approach as proposed by Aleza *et al*. (2016a), from the analysis of the closet marker to the centromere for each chromosome. Having τ value estimation for each chromosome, the preferential pairing (PP) was calculated as 1–τ. Parental heterozygosity restitution (PHR), was calculated for each marker as the percentage of inferred heterozygous diploid gametes.

Genetic mapping

With the objective to study the interspecific recombination (*C. medica* / *C. micrantha*) at tetraploid level we anchored the LGs inferred from the `Mexican´ lime diploid gametes on the reference clementine genome sequence (https://phytozome.jgi.doe.gov/pz/portal.html) and compare it with genetic maps of clementine at diploid and tetraploid level, also anchored in the reference sequence. The diploid clementine genetic map (Ollitrault *et al*., 2012b) was used as reference map for *Citrus* for the reference sequence assembly (Wu *et al*., 2014). For the tetraploid clementine map we used the 57 molecular markers segregation analysis published by Aleza *et al*. (2016a). For tetraploid `Mexican´ lime 34 out of the 35 markers analysed for this study were used. Indeed, the SNP marker (8P18684429) showed no segregation with 100% of heterozygosity. Each tetraploid progeny was analysed with Tetraploid Map Software (Hackett *et al*., 2007) using the default parameters to establish the different map distances in cM.
Then, the genetic and physical maps were drawn using the MapChart program (Voorrips, 2002). To anchor the genetic maps in the physical sequence, we divided the physical position by 300.000 in order to obtain comparable drawing sizes.

Results

Pollen viability and Cytogenetic analysis

A total of 1.179 pollen grain were observed and 64% of pollen viability was recorded (Figure 3.1).

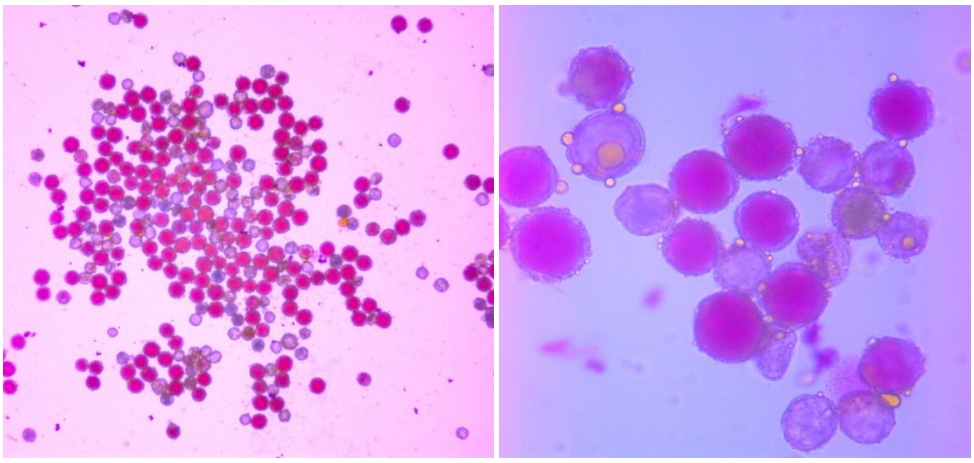

Figure 3.1. Pollen grains of tetraploid `Mexican´ lime stained with aceto-carmine. Bold red color are viable and colorless (blue) are non-viable.

As for the cytogenetic observations, two thirds of the chromosomes paired in bivalent (Figures 3.2a and 3.2b and Table 3.2). The majority of the other chromosomes paired in tetravalents.

Table 3.2. Chromosome configuration at meiosis in pollen mother cells (PMC) of the tetraploid `Mexican´ lime

	Univalents	Bivalents	Trivalents	Tetravalents	
				Ring	Chain
Number of association structures	21	597	15	29	115
Percentage of involved chromosomes	1.14	65.03	2.45	6.32	25.05
Average number of configuration by PMC	0.41	11.71	0.29	0.57	2.25

Two types of tetravalents, closed and chain, were distinguishable. Chain tetravalent configuration concerned 25.05% of the chromosomes and closed tetravalents 6.32% (Figure 3.2, c, d). The average number of bivalent and tetravalent configurations by PMC was respectively 11.71 and 2.82. In contrast, occurrence of monovalent and trivalent configuration by PMC was very low (mean of 0.41 and 0.29 respectively per PMC).

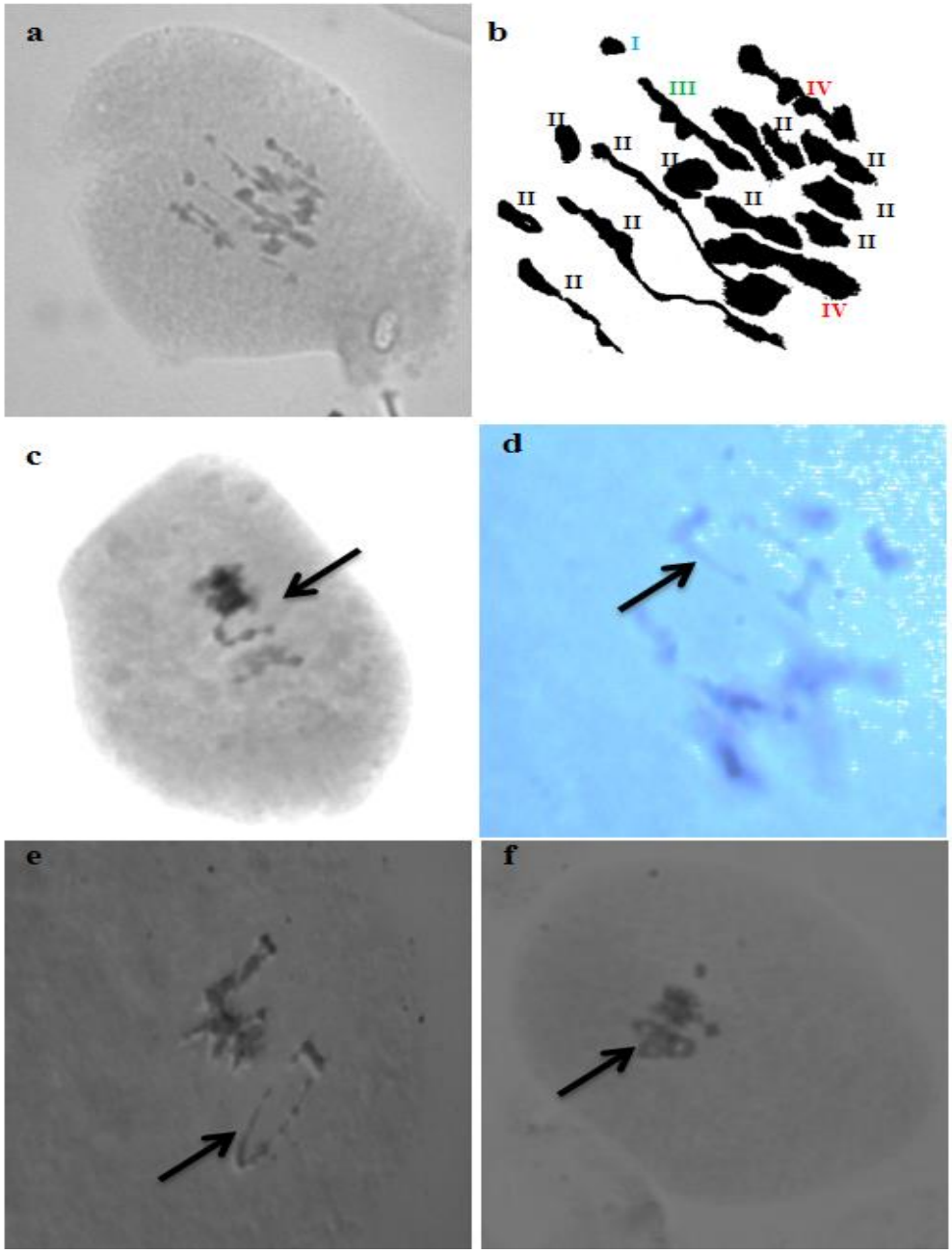

Figure 3.2. Chromosome pairing configuration, **a:** Pollen Mother Cells (PMC) of the tetraploid `Mexican´ lime**. b**, Schematic interpretation of (a) 1 univalent (blue colour) + 12 bivalents (black colour) + 1 trivalent (green colour) + 2 tetravalents (red colour). c, Open tetravalent (arrow). d, e, f closed (ring) tetravalents (arrows).

The analysis of configuration at individual PMC level (Figure 3.3) revealed at least eight bivalents and two tetravalents for each PMC. The occurrence of twelve bivalents / PMC, was the most frequent situation (19 PMC) with a maximum of 14 bivalents observed in 13 PMC. Up to four tetravelents /PMC were found in 14 PMC. Two PMC exhibited only two monovalents and 15 PMC showed at least one monovalent and one trivalent.

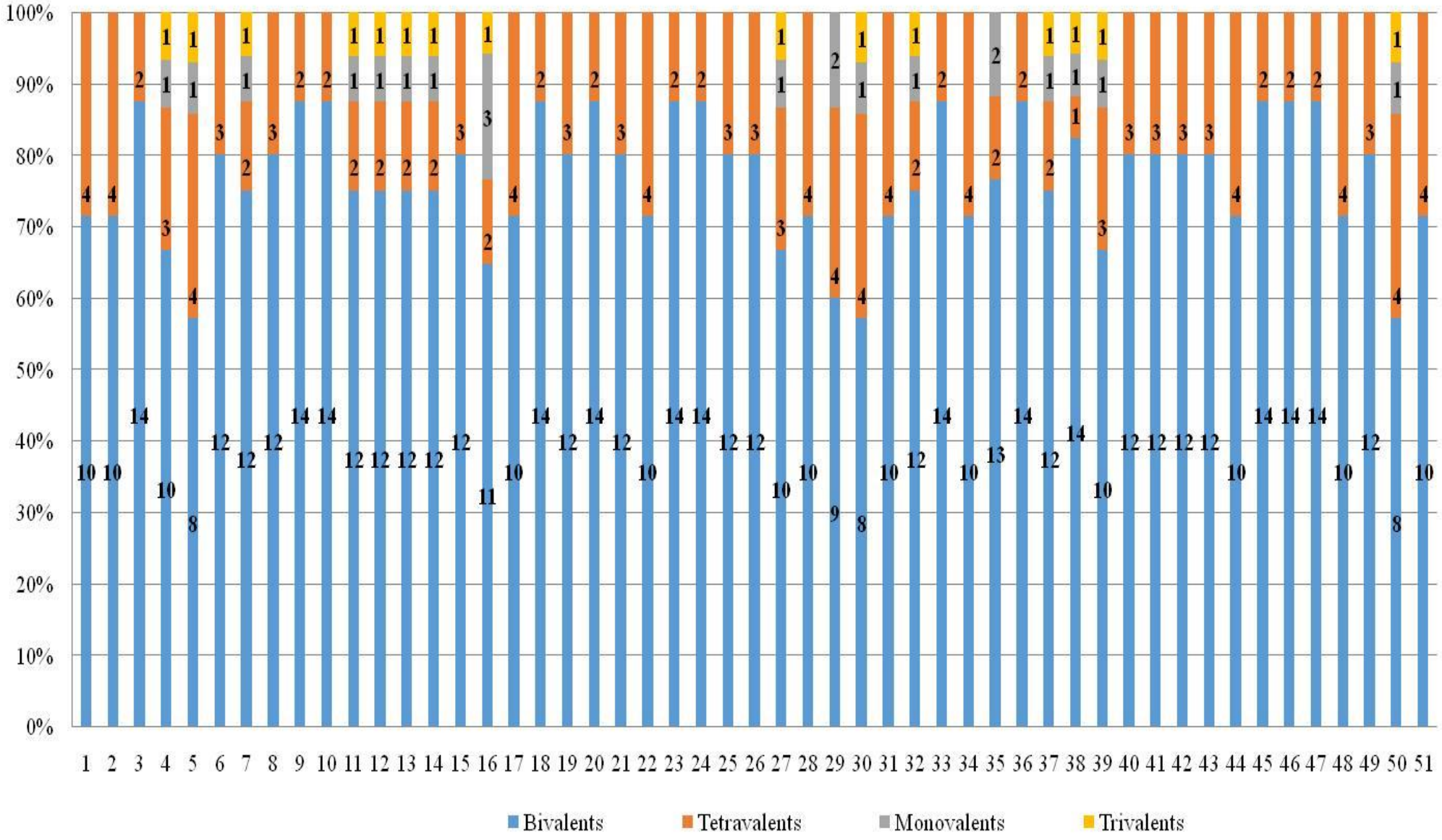

Figure 3.3. Individual meiotic configuration of each observed PMC for tetraploid `Mexican´ lime.

Monovalents and trivalents may be interpreted as a broken tetravalent or incomplete tetravalent pairing by the absence of chiasma on its two arms (Jeredi *et al*., 2012). Under this hypothesis three configurations were predominant, 12 bivalents / 3 tetravalents (18 PMC; 35%), 14 bivalents / 2 tetravalents (13 PMC; 25%) and 10 bivalents /4 tetravalents (13 PMC; 25%).

Molecular markers analysis

Eighty five plants were obtained from the Clementine 4x x `Mexican´ lime hybridization. Ploidy analysis by flow cytometry demonstrated that all were tetraploids. They were analysed with two SSR markers, mCrCIR07F11 and MEST001, displaying a total differentiation between the parents, to study their genetic origin. For each marker, at least one specific allele of `Mexican´ lime was observed in all plants. Moreover, in many plants the two specific alleles of `Mexican´ lime were observed in combination with clementine alleles (Figure 3.4a). The Cl4x x ML4x hybrid origin of all analyzed plants was thus confirmed.

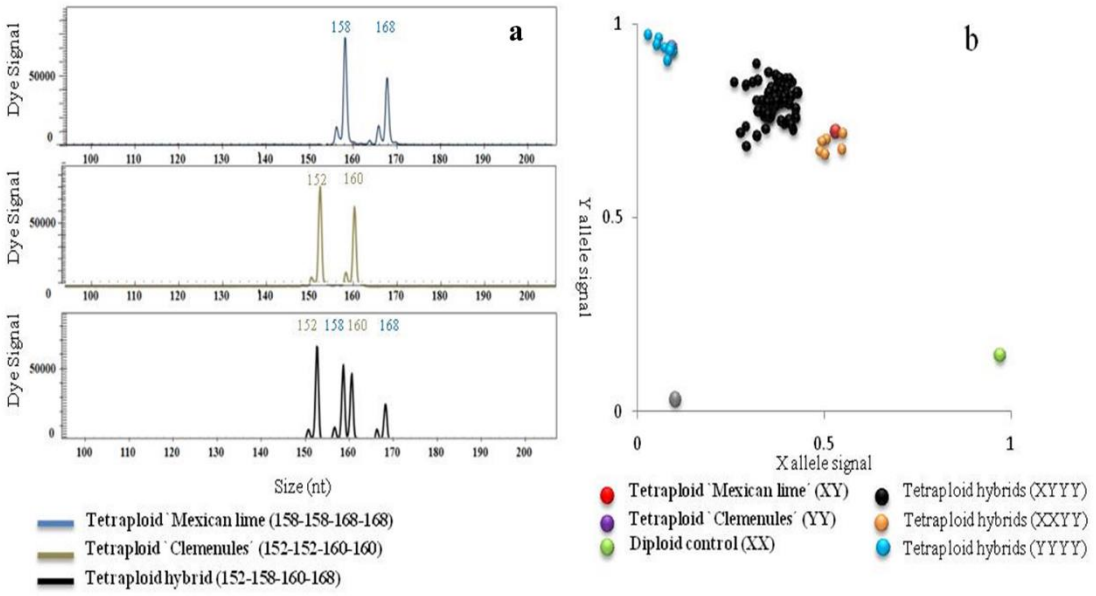

Figure 3.4. Illustration of tetraploid hybrids genotyping. **a.** Electroferogram of a tetraploid hybrid recovered from hybridization between tetraploid `Clemenules ´clementine and tetraploid `Mexican´ lime with mCrCIR07F11 SSR marker. nt : nucleotides. **b.** Plot of X and Y allele signals of the 1P199494 SNP marker representing tetraploid hybrids from the same hybridization. Letters indicate the allelic configuration for each genotype.

These 85 hybrids were analysed with 35 codominant markers and the `Mexican´ lime diploid gamete genotypes and their phylogenomic structure (*C. micrantha* or *C. medica* homozygosity and interspecific heterozygosity) were inferred (Additional Table 3.1). On average, for all the loci, a 90.2% of PHR has been observed (Table 3.3; Additional Table 3.1) and varied between 82.7% for the LG05 and 95.6 % for LG08 (Table 3.3). At individual marker level (Figure 3.5.a; Additional Table 3.1), PHR varied between

74.1% and 100% for mCrCIR04F12 (LG03) and 8P18684429 (LG08) markers respectively.

A slight diminution of PHR is observed in most LGs from centromeric to telomeric markers. As an example, for LG01, the PHR values were 92.9% and 94.1% for the two centromeric SSR markers, CIBE5720 and MEST001 respectively, and only decrease to 85.9% and 88.2% for the telomeric markers 1P199494 and JK–TAA15 respectively. This reduction could be associated with DR in case of tetravalent associations.
At individual gamete level PHR displayed a unimodal distribution and varied between 0.66 and 1; six gametes were fully heterozygous and 58.8% of diploid gametes displayed a PHR value over 90%. (Figure 3.5.b; Additional Table 3.1).

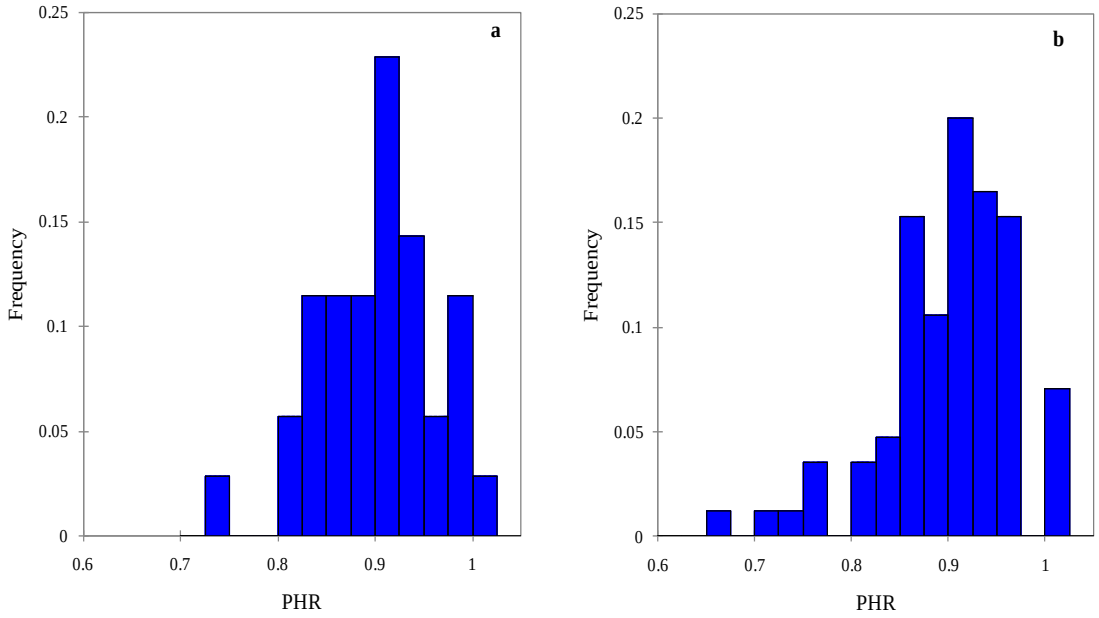

Figure 3.5. Distribution of PHR values among markers (a) and gametes (b) for diploid gametes obtained from tetraploid `Mexican´ lime.

Over all loci and gametes, the percentages of *C. micrantha* and *C. medica* homozygosity were 4.7% and 5.1% respectively. When analysing more deeply the data at gamete level (Table 3.3; Additional Table 3.1) it appeared that the majority (77.8%) of individual LGs of the different hybrids were fully heterozygous. For the considered markers six hybrids resulted from fully heterozygous gametes. At the opposite, only nine (1.2%) and six (0.8%) fully homozygous LGs for *C. micrantha* and *C. medica* respectively were observed. 20.26% of the individual LGs displayed mixed structure with homozygosity and heterozygosity and all the nine citrus LGs were concerned. Homozygous and mixed LGs reveal pairing of *C. micrantha* and *C. medica* chromosomes and mixed LGs testify for interspecific recombination. The number of LGs with homozygosity for both markers (if available) flanking the centromere was low (5.23%) and varied between chromosomes, from 12.9% in LG05 to lower value than 0.01% for LG03, LG07 and LG08.

Table 3.3. Interspecific structures of the `Mexican´ lime diploid gametes for the nine LGs

	PHR	FH	Fmed	Fmic	Mixed
LG1	90.3	76.5	2.4	1.2	20.0
LG2	95.3	90.6	0.0	0.0	9.4
LG3	85.0	57.6	1.2	0.0	41.2
LG4	89.7	77.6	1.2	1.2	20.0
LG5	82.7	72.9	2.4	4.7	20.0
LG6	88.0	80.0	0.0	3.5	16.5
LG7	92.9	82.4	0.0	0.0	17.6
LG8	95.6	82.4	0.0	0.0	17.6
LG9	92.0	80.0	0.0	0.0	20.0
Total	**90.2**	**77.8**	**0.8**	**1.2**	**20.3**

FH: percentage of fully heterozygous gametes for the LG; Fmed: percentage of fully *C. medica* homozygous gametes for the LG; Fmic: percentage of fully *C. micrantha* homozygous gametes for the LG; Mixed: percentage of gametes with mixed heterozygosity and homozygosity for the LG.

Estimation of preferential association frequency

τ and PP were estimated from the likelihood models (Table 3.4), for each LG. Disomic inheritance with high preferential pairing values was observed for LG07 and LG08 (PP=0.965) and LG02 (PP=0.86). Tendency for preferential pairing was found for LG01, LG03, LG04, LG06, and LG09 (0.68< PP < 0.79). For LG05 the intermediate model fitted better than disomic or tetrasomic models (PP = 0.50).

Table 3.4. Estimation of τ and PP from centromeric loci of the nine LG of 'Mexican' lime 4x

LG	Locus	DC	Mic/Mic	Med/Mic	Med/Med	τ	PP
1	Cibe5720	2.21	1	79	5	0.210	0.790
2	2P25198777	10.73	1	81	3	0.140	0.860
3	JITC01	19.09	1	78	6	0.245	0.755
4	mCrCIR07D06	0.19	3	79	3	0.210	0.790
5	mCrCIR07G11	2.93	7	71	7	0.495	0.505
6	6P7496245	0.10	4	76	5	0.320	0.680
7	mCrCIR03B07	13.04	0	84	1	0.035	0.965
8	CiBE0214	13.80	0	84	1	0.035	0.965
9	JI-TCT01	0.64	0	78	7	0.245	0.755

LG: Linkage Group; DC: Distance to the centromere (from reference genetic map data - Ollitrault *et al*. 2012b - and location of centromere - Aleza *et al*. 2015). Med/Med, Med/Mic and Mic/mic: Number of individuals with such allelic configuration; τ: Tetrasomic rate; PP: Preferential Pairing

Genetic mapping and recombination rate analysis

The genetic maps were established from SSR and SNP marker segregations and then compared using the physical positions as common references (Figure 3.6). The average recombination rates by Mb were estimated for each LG and each population considering the extreme marker positions in the genetics maps and the physical one (Table 3.5).

Table 3.5. Average recombination rates per LG (cM/Mb) for three segregating progenies

	2x Clementine	4x Clementine	4x `Mexican´ lime
LG1	3.53	2.99	0.82
LG2	3.97	4.65	0.31
LG3	2.79	3.46	1.88
LG4	3.37	3.53	1.28
LG5	2.64	2.42	1.68
LG6	3.51	3.78	1.07
LG7	5.49	3.49	2.12
LG8	4.12	3.40	1.16
LG9	2.63	3.30	0.94
Total	3.29	3.41	1.21

LG: Linkage Group

For the diploid clementine genetic and physical maps, the considered positions were the ones published respectively by Ollitrault *et al*. (2012b) and Wu *et al*. (2014). Those maps where used to compare the results of both tetraploid genetic maps. For the genetic maps, only the markers common with the tetraploid clementine mapping were selected. For the physical map we retained the previous markers plus the ones of the tetraploid `Mexican´ lime map.

For tetraploid clementine map, the positions of 57 molecular markers of tetraploid clementine published previously by Aleza *et al*. (2016a) were inferred. The map size was 864 cM. Compared with the clementine genetic map, the synteny was complete and the order of markers conserved except for very close telomeric markers in LG02 and LG06. Genetic distances of the two clementine maps were very similar with average rates of 3.29 and 3.41 cM/Mb for the diploid and tetraploid clementine and limited variations between linkage groups.

For tetraploid `Mexican´ lime map, the 34 segregating molecular markers of the present study (8P18684429 marker was heterozygous for the 85 analyzed hybrids) where mapped. The map spanned only 272 cM. The majority of the markers conserved the same order than in the clementine physical map, although three inversions were observed on LG02, LG04 and LG06. The distances between markers were considerably lower than in the diploid and tetraploid clementine maps. Indeed, the average rate of recombination of the tetraploid `Mexican´ lime was 1.21 cM/Mb, a third than the one observed for the diploid and tetraploid clementine.

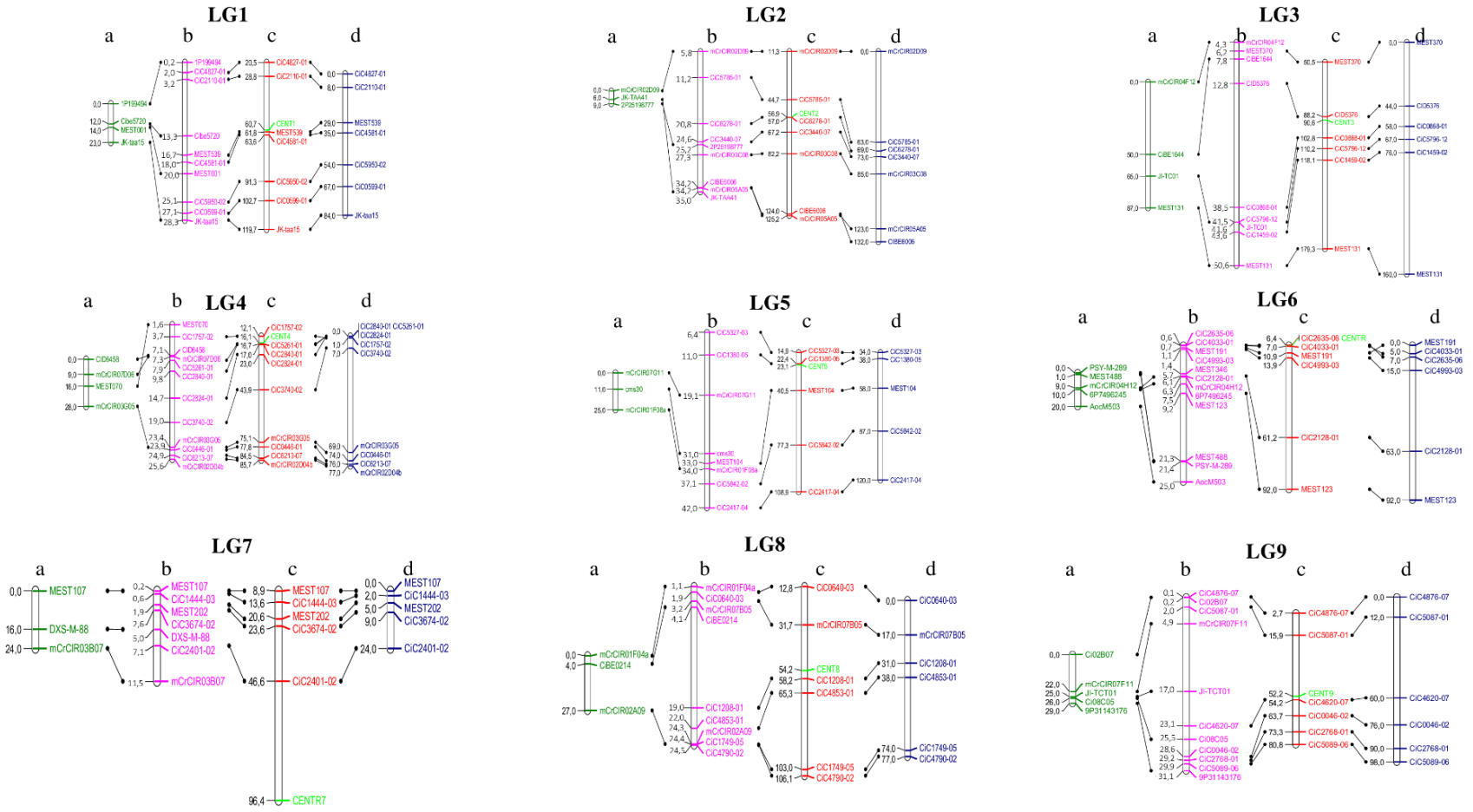

Figure 3.6. Comparative mapping between tetraploid `Mexican´ lime and diploid and tetraploid `Clemenules´ a: Tetraploid `Mexican´ lime (cM); b: diploid Clementine (physical; Mb); c: diploid Clementine (genetics; cM); d: tetraploid Clementine (cM). The centromere of each linkage group is indicated in green in the diploid Clementine map.

Discussion

Meiotic behavior of the doubled diploid `Mexican´ lime revealed by cytogenetics

Asynapsis, dependent to low temperature, has been described in diploid `Mexican´ lime (Iwamasa *et al*., 1962). No evidence of such configuration was observed for the tetraploid `Mexican´ lime. Indeed, the very low rate of monovalents observed during the microsporogenesis indicates that such meiosis abnormality was not induced in the tetraploid lime cultivated in Spain. The asynaptic behavior of chromosomes was recorded at temperatures lower than 10°C (Iwamasa and Iwasaki, 1963) while our sampling was made at temperature over 16 °C.

A common approach to distinguish autotetraploids from allotetraploids is to evaluate the frequency of tetravalent formation. In genuine autotetraploids, about two third of the chromosomes are usually involved in tetravalent configurations (Morrison Rajhathy, 1960). However, it has to be used with caution since genetic systems of diploidization or preferential pairing could exist.

The predominance of bivalents (65%) in the meiosis of tetraploid `Mexican´ lime is similar with the observations made in several allotetraploid somatic hybrids, like *C. deliciosa* + *C. limon* (Kamiri *et al*., 2011), *C. sinensis* + *C. limon* (Del Bosco *et al*., 1999; Chen *et al*., 2004), and Tangelo (*C. reticulata* x *C. paradisi*) + *C. grandis* (Xie *et al*., 2015). They also revealed tetravalent formation and a low percentage of monovalents and trivalents. In some species, homoeologous pairing can be under genetic control (Cifuentes *et al*., 2010). However, multivalent frequency in tetraploids is usually related with the pairing affinity (Jeredi *et al*., 2012). Length of chromosomes and position of the centromere may also influence the multivalent frequencies (McCollum, 1958).

Structural variations strongly affect chromosome pairing. A large heterozygous inversion was described in diploid `Mexican´ lime (Iwamasa and Nito 1988). Its frequency attained 44%, indicating large inverted segment resulting in partial sterility of gametophytes (Iwamasa, 1966). In Valencia (Spain), the pollen viability of the diploid `Mexican´ lime was estimated to be less than 10% (Pons *et al*., 2011). Interestingly we observed 64% of pollen viability for the DD `Mexican´ lime, higher than the rates ranging from 31% to 41%, reported by Aleza *et al*. (2012a) and Del Bosco *et al*. (1999) for different DD and somatic hybrids. In cases in which sterility of interspecific diploid hybrids is due to improper chromosome pairing, the generation of allotetraploids by chromosome doubling provides a homolog for each chromosome to pair with during meiosis and can allow for the development of fertile gametes (Zadoo *et al*., 1975; Lu and Bridgen, 1997; van Tuyl and De Jeu, 1997; Contreras *et al*., 2007).

We observed that 6.3% of the chromosomes were involved in closed tetravalents. Moreover in some PMC more than one closed tetravalent has been observed. In diploid species, the observation of closed tetravalents is considered an evidence for the presence of heterozygous reciprocal translocation (Sybenga, 1975). Reciprocal translocation is defined as the interchange of part of a chromosome with part of another (Sybenga, 1995) and results in alterations of the meiotic configurations. The affected chromosomes may form a ring or a chain tetravalent structure depending in the rate of the chiasmata (Sybenga, 1975; 2012). In citrus, a reciprocal translocation was described in diploid `Valencia´ and `Lue Gin Gong´ sweet oranges (*C. sinensis*), for which tetravalents were frequently observed (Iwamasa, 1963). Del Bosco *et al*. (1999) studied the meiosis of allotetraploid somatic hybrid between `Valencia´ sweet orange and `Femminello´ lemon and revealed that the reciprocal translocation still exist in the

somatic hybrid. Closed tetravalents were not observed in cytogenetic study of diploid `Mexican´ lime (Iwamasa and Nito, 1988) while inversion(s) were evidenced. A reconciliation between diploid and doubled diploid microsporogenesis data should be the presence of a double inversion affecting the two arms of a same chromosome (Figure 3.7). This double inversion pattern may results from chromosome structural variation between *C. medica* and *C. micrantha*, the two parents of the diploid `Mexican´ lime (Curk *et al*., 2016).

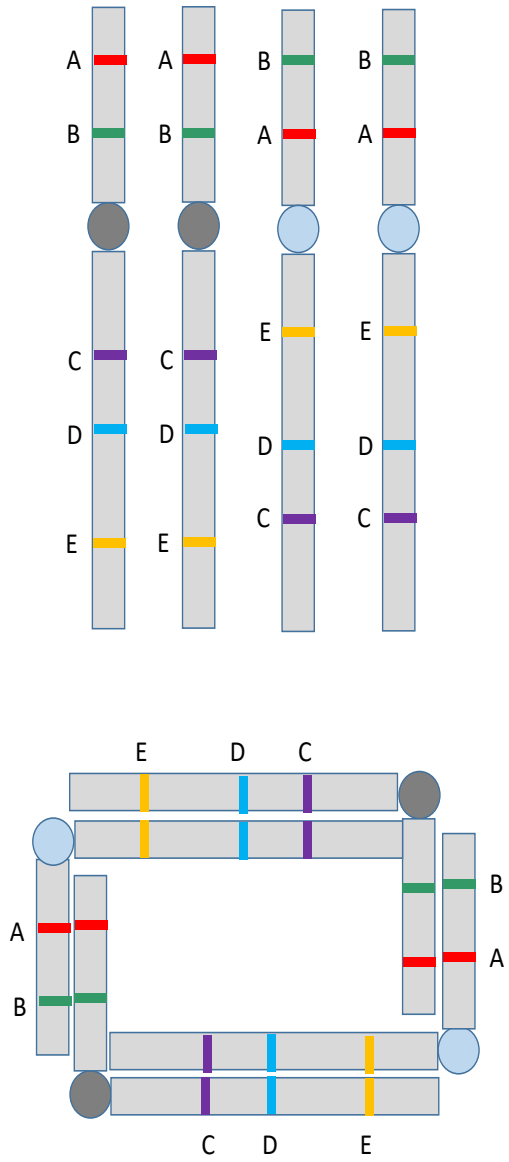

Figure 3.7. Interpretation for reconciliation of microsporogenesis observations in diploid and doubled diploid `Mexican´ lime: double inversion can produce closed tetravalent during doubled diploid meiosis.

Doubled diploid `Mexican´ lime has an intermediary preferential disomic inheritance

Froelicher *et al*. (2000) were the first to analyse the inheritance of a tetraploid Aurantioideae, *Clausena excavata* using molecular markers. They revealed a strict disomic inheritance. Later, the meiosis behaviour of interspecific somatic hybrids within the *Citrus* genus has been studied combining cytogenetics and molecular markers analysis (Kamiri *et al*., 2011; Xie *et al*., 2015); depending on the parents and chromosomes, intermediate to tetrasomic inheritance were observed. For the DD clementine obtained from colchicine treatment, Aleza *et al*. (2016a), using molecular markers analysis, observed multivalent pairing and preferential tetrasomic inheritance tendency testifying for non–preferential pairing.

For the DD `Mexican´ lime, the average PHR values reported in this work (90%) are higher than those observed by Kamiri *et al*. (2011) who reported PHR values ranging from 54% to 79%, for a *C. deliciosa* + *C. limon* tetraploid somatic hybrids. Xie *et al*. (2015) reported 76.2% of PHR for a somatic hybrid between Tangelo and a pummelo while it was 65% for a DD clementine (Aleza *et al*., 2016a). In direct relation with PHR, the preferential pairing rate was high for most LGs for the tetraploid `Mexican´ lime. Disomic inheritance with high preferential pairing values was observed for LG02, LG07 and LG08. Tendency for preferential pairing was found for five LGs (LG01, LG03, LG04, LG06, and LG09). For LG05 the intermediate models fitted better than disomic or tetrasomic model (PP = 0.50). Lower values were estimated by Kamiri *et al*. (2011) and Aleza *et al*. (2016a). For instance, for the tetraploid clementine, Aleza *et al*. (2016a) concluded for non-preferential pairing (PP=0) for five LGs.

The high fertility of most interspecific hybrids within the citrus genus, excepted `Mexican´ lime at diploid level (Ollitrault and Navarro, 2012) testify for a good pairing affinity and therefore limited chromosomes variations between species. However tetraploid genotypes offer a choice for chromosome partners, not available at diploid level, and therefore can reveal chromosomal variations between ancestral species. The different meiotic behavior observed by Kamiri *et al*. (2011), Aleza *et al*. (2016a) and our study can be due to the phylogenomic structure of the different genotype. In case of the *C. reticulata* + *C. limon* studied by Kamiri *et al*. (2011), it is highly complex as *C. limon* results from (*C. maxima* x *C. reticulata*) x *C. medica* natural hybridization (Curk *et al*., 2016). Therefore it harbors a two ancestor heterozygosity (*C.ret/C.ret/C.ret/C.med*) or three ancestor heterozygosity (*C.ret/C.ret/C.max/C.med*) at each locus. The DD clementine studied by Aleza *et al*. (2016a) had a more simple structure with a predominant *C. reticulata* genomic constitution with some genomic segments in *C.ret/C.ret/C.max/C.max* heterozygosity. *C. aurantifolia* is a hybrid of two distant species (*C. micrantha* x C. *medica*). Therefore, each locus displays *C.mic/C.mic/C.med/C.med* heterozygosity. Molecular studies (García *et al*., 2013; Curk *et al*., 2014; 2015; Carbonell-Caballero *et al*., 2015) have shown that *C. medica* is the cultivated citrus ancestor most distant to the three other ones. It can be suspected that both sequence divergence and structural variations between *C. medica* and *C. micrantha* drive the preferential pairing and intermediary preferential disomic inheritance observed for the DD `Mexican´ lime. As stated by Stebbins, (1950), the importance of the differentiation may vary between the different sets of chromosomes and should explain the difference of PP rates between the chromosomes. Interestingly in the tetraploid `Mexican´ lime, none of the 9 chromosomes display a tetrasomic inheritance,

suggesting that pairing is affected by a global differentiation rather than discrete and local large structural variations, as the inversion described in diploid `Mexican´ lime.

Interspecific recombination occurs in each LGs but is strongly lower compared with recombination rates in doubled–diploid clementine

Despite the disomic tendency, mixed heterozygous/homozygous structures were observed for the nine citrus LGs revealing interspecific recombination between *C. medica* and *C. micrantha*, the parents of the `Mexican´ lime (Nicolosi *et al*., 2000; Curk *et al*., 2016) for the nine citrus chromosomes. For LG05, 12.9% of the gametes displayed homozygosity both side of the centromere, suggesting higher homology between *C. micrantha* and *C. medica* for the corresponding chromosome than for the others (5.3% in average). This is confirmed by the conclusion for intermediate preferential pairing for chromosome 5 while the other chromosomes displayed intermediate with disomic tendency inheritance or disomic inheritance. For most chromosomes, interspecific recombination was observed in distal areas. It appeared however very limited for LG08 with only 2.4% of identified interspecific recombined gametes in one of the two chromosome arms.
The genetic mapping reveals effective recombination rates per Mb (1.2 cM/Mb) strongly lower when compared with diploid (3.3 cM/Mb) and tetraploid clementine (3.4 cM/Mb). It is, at least in part, a direct consequence of medium to high preferential pairing preventing interspecific chiasmata and thus interspecific recombination. It is also possible that sequence divergence between *C. medica* and *C. micrantha* decrease the recombination frequency when interspecific pairing is effective. Interspecificity is well known to decrease recombination rates (Manrique–Carpintero; 2016). The impact of structural heterozygosity on recombination frequency is variable as discussed by Parker *et al*. (1982). It is however well established that sequence divergence at the interspecific level has an inhibitory effect on sexual recombination (Chambers *et al*., 1996; Opperman *et al*., 2004; Li *et al*., 2006; Chetelat *et al*., 2000). For citrus, variations of recombination rates were observed between clementine and sweet orange (Ollitrault *et al*., 2012b). The authors proposed that this may be related with the higher *C. reticulata / C. maxima* heterozygosity in sweet orange than in clementine. A comparative mapping analysis between diploid and tetraploid `Mexican´ lime would enlighten the relative impacts of preferential pairing at tetraploid level and sequence divergence when interspecific pairing is effective, on effective interspecific recombination during tetraploid `Mexican´ lime meiosis.
Synteny was observed on the diploid clementine, DD clementine and `Mexican´ lime. Collinearity was high between diploid and tetraploid clementine maps, while the alignment of the genetic maps of the tetraploid `Mexican´ lime with the diploid clementine revealed three inversions in LG02, LG04 and LG06. However the low number of observed recombinations and analyzed markers made the liability of these observations questionable (a few genotyping errors can lead to erroneous ordering). Saturate mapping of larger populations should be necessary to be able to associate the inversion concluded from cytogenetic studies (Iwamasa, 1970) and inverted linkage groups.

Diploid gametes structures of the doubled diploid `Mexican´ lime are compatible with the origin of the triploid *C. aurantifolia* and *C. latifolia* limes

`Persian´,`Tahiti´ and `Bears´ limes are different varieties classified as *C. latifolia*, representing a same ideotype, producing big seedless lime fruits. It is believed that they are a group of clones deriving from a same ancestral hybrid (Morton, 1987). `Tahiti´ lime like was introduced into the Mediterranean region through Iran (where it is called `Persian´ lime), while it reached California from `Tahiti´ between 1850 and 1880 and was introduced in Florida by 1883. The genetic origin of `Tahiti´ lime was unclear until a recent publication of Curk *et al*. (2016). Previous cytoplasmic studies showed that `Tahiti´ lime shared the same cytoplasm than *C. limon* and *C. aurantium* (Bayer *et al*., 2009; Froelicher *et al*., 2011). Reece and Childs (1962) proposed from morphological trait segregation studies in `Tahiti´ lime seedlings that this variety may result from lime by citron or lemon hybridization but did not recognize the triploid status of `Tahiti´ lime. Another ideotype of triploid lime producing seedy big lime fruits, represented by several cultivars (such as `Tanepao´, `Coppenrhad´, `Ambilobe´ and `Mothasseb´ limes and `Madagascar´ lemon) is cultivated at lower extent. Curk *et al*. (2016) demonstrated that these varieties, classified as *C. aurantifolia*, are genetically very close and probably derive from a same ancestral hybrid by mutation or epigenetic variations. They share the same cytoplasm than the `Mexican´ lime (Curk *et al*., 206). From nuclear molecular study, Curk *et al*. (2016) proposed that the two main types of triploid limes, `Tahiti´ lime type and `Tanepao´ lime type, were interspecific hybrids involving a diploid gamete of *C. aurantifolia* combined respectively with an haploid ovule of *C. limon* and an haploid pollen of *C. medica*. Moreover, their data suggest that the PHRs of the concerned diploid gamete were respectively 88% and 95%. Curk *et al*. (2016) hypothesized that these diploid gametes should be originated from a natural DD of `Mexican´ lime like, such as the `Giant key´ lime selected in a seedling of diploid `Key´ lime (a `Mexican´ lime clone) or should be unreduced gametes from a diploid `Mexican´ lime like variety. The average PHR value (90.2%) and range (between 65.7% and 100%) observed in the present work for the DD `Mexican´ lime are compatible with the one estimated by Curk *et al*. (2016) for `Tahiti´ and `Tanepao´ types. At the opposite, secondary division restitution (SDR) described as the main mechanism of unreduced mega–gametophyte production in citrus (Luro *et al*., 2004; Cuenca *et al*., 2011; 2015; Aleza *et al*., 2016a) results on lower value of PHR (40% in average) (Peloquin, 1983; Hutten *et al*., 1994; Carputo *et al*., 2003). Therefore SDR 2n gametes are not compatible with the interspecific genetic structure of `Tahiti´ and `Tanepao´ limes. First division restitution (FDR) identified as the predominant mechanism for diploid pollen formation in a clementine x sweet orange hybrid (Rouiss *et al*., 2017a) and secondary mechanisms in lemon 2n gamete ovule production (Rouiss *et al*., 2017b) should produce diploid gamete with high PHR (80 % in average; Peloquin, 1983; Hutten *et al*., 1994; Carputo *et al*., 2003) particularly if it is coupled with asynapsis described in diploid `Mexican´ lime (Iwamasa *et al*., 1966). Indeed FDR associated with strict asynapsis for all chromosomes would result in 100% of PHR. The study of the mechanisms and structure of unreduced gamete of diploid `Mexican´ lime will be necessary to definitively conclude on the origin of the *C. latifolia* and *C. aurantifolia* triploid limes. However the interploid hybridisation hypothesis fits well with the actual molecular data on these two types of triploid limes, the natural occurrence of tetraploid `Mexican´ limes and our present results on the phylogenetic diploid gamete structure produced by the doubled diploid `Mexican´ lime.

Implications for `Tahiti ´ and `Tanepao´ lime like breeding

As discussed before, doubled diploid `Mexican´ lime can produce diploid gametes with genetic structure similar to the ones that originated the `Tahiti´ and `Tanepao´ lime types. Therefore this opens the possibility to develop a reconstruction breeding strategy for these limes using a doubled diploid `Mexican´ lime like parent. It should be based on the selection of breeding parents with interesting variations (disease resistance, improved phenology, primary and secondary metabolites contents, etc).

Chromosome doubling of the diploid `Mexican´ lime restored good pollen viability, so it could be used for extensive breeding programs to produce `Tahiti ´ and `Tanepao´ lime like hybrids. It should have much more efficiency than the search for triploid hybrids resulting from unreduced gametes of `Mexican´ limes. Indeed the partial apomixes of parents, limiting strongly hybrid recovery, coupled with the relatively low frequency of 2n gametes described in citrus (Esen and Soost, 1971; Geraci *et al*., 1977; Cuenca *et al*., 2016) should be a real impediment for efficient triploid lime breeding from unreduced gametes, as it was developed using non apomictic female parent for mandarin breeding (Ollitrault *et al*., 2008; Aleza *et al*., 2010b).

The predominant intermediate segregation with tendency for disomic inheritance observed for the different LGs of the DD `Mexican´ lime results in highly heterozygous gametes. Indeed, about 90 % of the `Mexican´ lime heterozygosity would be transmitted to triploid progenies via diploid gametes. It should avoid inbreeding depression in triploid hybrids that can occur when using doubled diploid parents with tetrasomic inheritance (Gallais, 2003). The lower diversity of diploid gametes produced by such meiotic mechanisms is also favorable to reconstruct phylogenomic structures similar to the two triploid limes ideotypes, optimizing the probability to select new variety phenotypically close to the ideotypes. The development and application of molecular markers diagnosis of the four ancestral taxa of cultivated citrus (Curk *et al*., 2015) will allow improving the efficiency of such reconstruction breeding strategies.

On the other hand, the limitation of the effective interspecific recombination associated with predominant disomic inheritance, as illustrated by the decrease of the genetic length of the different genetic LGs for the tetraploid `Mexican´ lime, when compared with diploid and teraploid clementine maps, should impact breeding efficiency due to an increased linkage drag. It should require developing large progenies if it is needed to separate a given locus of genetic importance from another linked undesired locus. However, even limited, interspecific recombination has been observed for each chromosome, opening large possibilities for lime breeding considering the high pollen viability of the DD `Mexican´ lime and thus the capacity to generate large triploid progenies.

Conclusion

The doubled–diploid `Mexican´ lime had a predominantly disomic segregation. Preferential pairing varies between chromosomes. Disomic inheritance with high preferential pairing values was observed for three LGs (LG02, LG07 and LG08), intermediate segregation with tendency for preferential pairing was found for five LGs (LG01, LG03, LG04, LG06, and LG09) and intermediate segregation for LG05. The cytogenetic observations are compatible with the interspecific (*C. medica / C. micrantha*) chromosome pairing and recombination revealed for each LGs by molecular marker study. The disomic tendency limits the effective interspecific recombination and the diversity of the diploid gamete population. The interspecific phylogenetic structures of the produced diploid gametes with high *C. medica / C. micrantha* heterozygosity are compatible with the ones that generate the triploids *C. latifolia* and *C. aurantifolia* varieties that may therefore results from interploid hybridization. The restored pollen fertility of the doubled diploid `Mexican´ lime compared with the diploid and the genetic structures of the diploid gametes fitting with the origin of *C. aurantifolia* and *C. latifolia* triploid limes open the way for efficient reconstruction breeding programs based on interploid hybridization for the diversification of triploid lime germplasm.

Supplementary information

Additional table 3.1. Phylogenomic structure of the 'Mexican' lime diploid gametes for the nine LGs

	LG1					LG2				LG3				
Position	**1.00**	**58.45**	**60.66**	**70.61**	**119.73**	**11.37**	**56.87**	**67.60**	**131.86**	**29.66**	**70.23**	**90.59**	**109.68**	**179.33**
Marker/Genotype	**1P199494**	**CIBE5720**		**MEST001**	**JK-taa15**	**mCrCIR02D09**		**2P25198777**	**JK-TAA41**	**mCrCIR04F12**	**CiBE1644**		**JITC01**	**MEST131**
ClemMex1														
ClemMex2														
ClemMex3														
ClemMex4														
ClemMex5														
ClemMex6														
ClemMex7														
ClemMex8														
ClemMex9														
ClemMex10														
ClemMex11														
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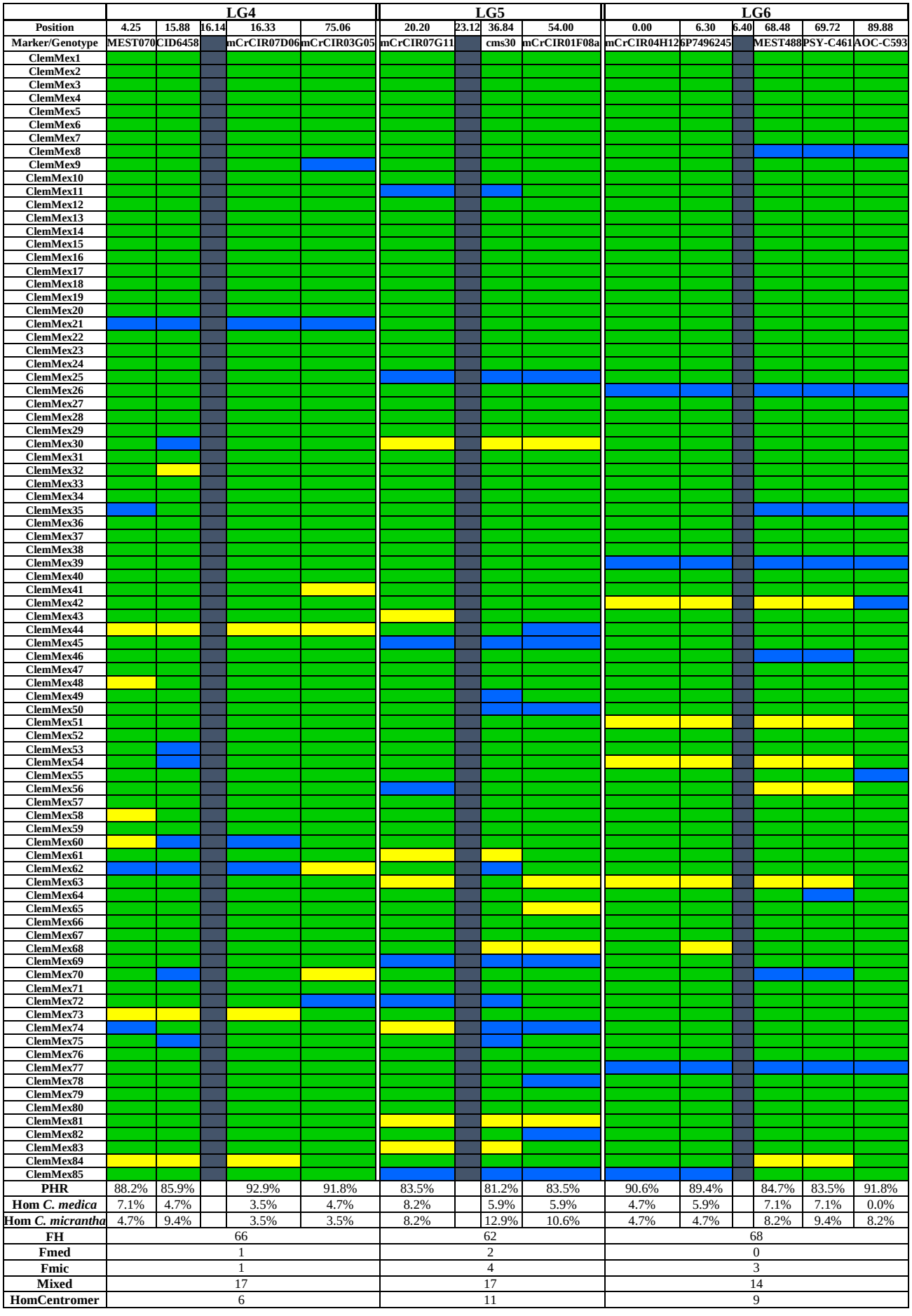
ClemMex79														
ClemMex80														
ClemMex81														
ClemMex82														
ClemMex83														
ClemMex84														
ClemMex85														
PHR	85.9%	92.9%		94.1%	88.2%	91.8%		95.3%	98.8%	74.1%	91.8%		91.8%	82.4%
Hom *C. medica*	4.7%	5.9%		4.7%	8.2%	5.9%		3.5%	1.2%	11.8%	5.9%		1.2%	12.9%
Hom *C. micrantha*	9.4%	1.2%		1.2%	3.5%	2.4%		1.2%	0.0%	14.1%	2.4%		7.1%	4.7%
FH	65					77				49				
Fmed	2					0				1				
Fmic	1					0				0				
Mixed	17					8				35				
HomCentromer	4					4				1				

Additional table 3.1. –cont. Phylogenomic structure of the 'Mexican' lime diploid gametes for the nine LGs



	LG4					LG5				LG6					
Position	4.25	15.88	16.14	16.33	75.06	20.20	23.12	36.84	54.00	0.00	6.30	6.40	68.48	69.72	89.88
Marker/Genotype	MEST070	CID6458		mCrCIR07D06	mCrCIR03G05	mCrCIR07G11		cms30	mCrCIR01F08a	mCrCIR04H12	6P7496245		MEST488	PSY-C461	AOC-C593
ClemMex1															
ClemMex2															
ClemMex3															
ClemMex4															
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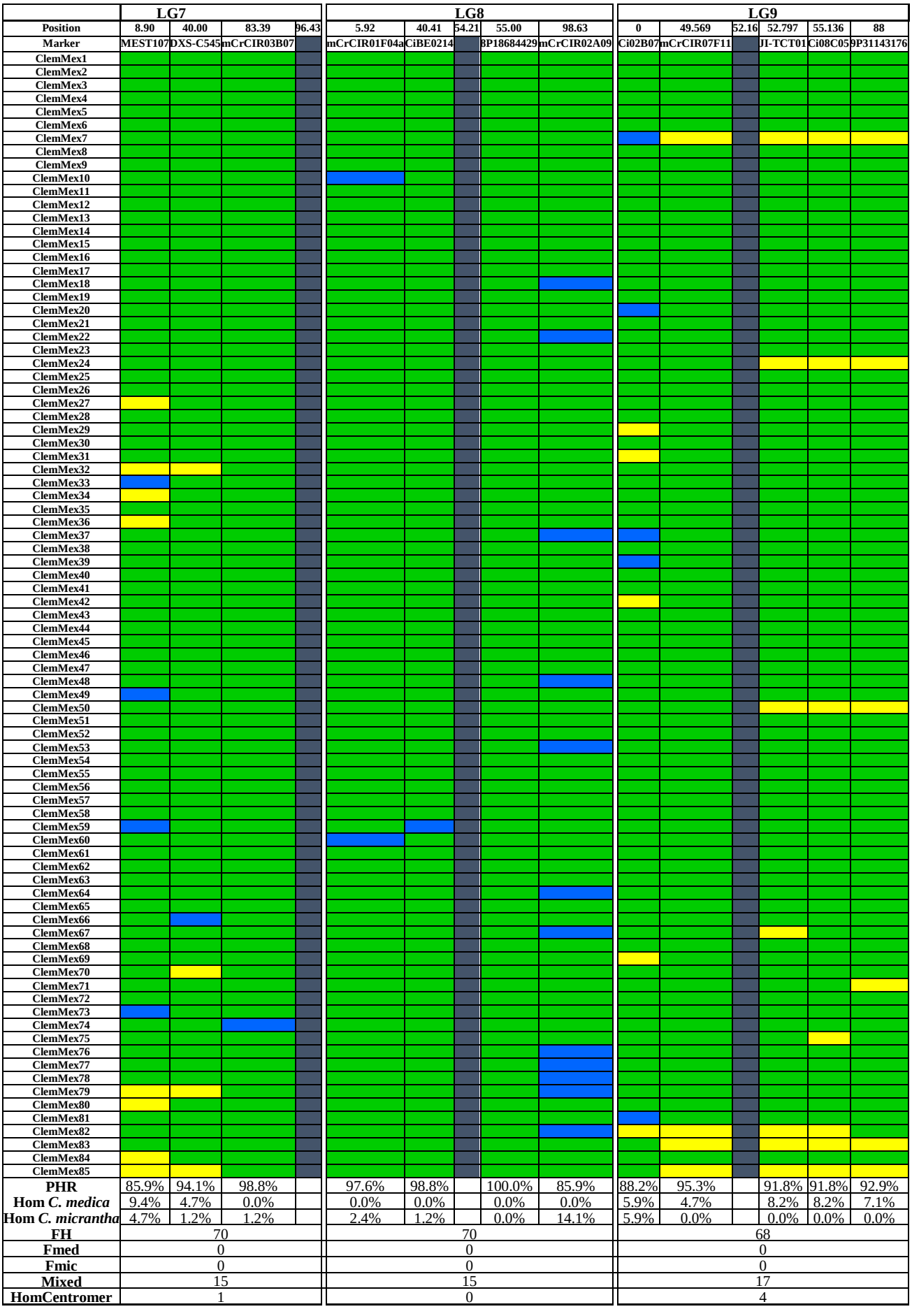
PHR	88.2%	85.9%		92.9%	91.8%	83.5%		81.2%	83.5%	90.6%	89.4%		84.7%	83.5%	91.8%
Hom *C. medica*	7.1%	4.7%		3.5%	4.7%	8.2%		5.9%	5.9%	4.7%	5.9%		7.1%	7.1%	0.0%
Hom *C. micrantha*	4.7%	9.4%		3.5%	3.5%	8.2%		12.9%	10.6%	4.7%	4.7%		8.2%	9.4%	8.2%
FH	66					62				68					
Fmed	1					2				0					
Fmic	1					4				3					
Mixed	17					17				14					
HomCentromer	6					11				9					

Additional table 3.1. –cont. Phylogenomic structure of the 'Mexican' lime diploid gametes for the nine LGs



	LG7				LG8					LG9					
Position	**8.90**	**40.00**	**83.39**	**96.43**	**5.92**	**40.41**	**54.21**	**55.00**	**98.63**	**0**	**49.569**	**52.16**	**52.797**	**55.136**	**88**
Marker	**MEST107**	**DXS-C545**	**mCrCIR03B07**		**mCrCIR01F04a**	**CiBE0214**		**8P18684429**	**mCrCIR02A09**	**Ci02B07**	**mCrCIR07F11**		**JI-TCT01**	**Ci08C05**	**9P31143176**
PHR	85.9%	94.1%	98.8%		97.6%	98.8%		100.0%	85.9%	88.2%	95.3%		91.8%	91.8%	92.9%
Hom *C. medica*	9.4%	4.7%	0.0%		0.0%	0.0%		0.0%	0.0%	5.9%	4.7%		8.2%	8.2%	7.1%
Hom *C. micrantha*	4.7%	1.2%	1.2%		2.4%	1.2%		0.0%	14.1%	5.9%	0.0%		0.0%	0.0%	0.0%
FH	70				70					68					
Fmed	0				0					0					
Fmic	0				0					0					
Mixed	15				15					17					
HomCentromer	1				0					4					

Additional table 3.1. –cont. Phylogenomic structure of the 'Mexican' lime diploid gametes for the nine LGs

Position	PHR	Hom Med	Hom Mic	FH	Fmed	Fmic	Mixed	HomCentromer
ClemMex1	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex2	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex3	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex4	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex5	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex6	100.0%	0.0%	0.0%	9	0	0	0	0
ClemMex7	85.7%	11.4%	2.9%	8	0	0	1	1
ClemMex8	91.4%	0.0%	8.6%	8	0	0	1	0
ClemMex9	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex10	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex11	94.3%	0.0%	5.7%	8	0	0	1	1
ClemMex12	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex13	97.1%	2.9%	0.0%	8	0	0	1	0
ClemMex14	94.3%	5.7%	0.0%	8	0	0	1	1
ClemMex15	97.1%	2.9%	0.0%	8	0	0	1	0
ClemMex16	94.3%	2.9%	2.9%	8	0	0	1	0
ClemMex17	91.4%	8.6%	0.0%	8	0	0	1	0
ClemMex18	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex19	91.4%	8.6%	0.0%	8	0	0	1	0
ClemMex20	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex21	88.6%	0.0%	11.4%	8	0	1	0	1
ClemMex22	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex23	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex24	91.4%	8.6%	0.0%	8	0	0	1	0
ClemMex25	91.4%	0.0%	8.6%	8	0	1	0	1
ClemMex26	85.7%	0.0%	14.3%	8	0	1	0	1
ClemMex27	97.1%	2.9%	0.0%	8	0	0	1	0
ClemMex28	97.1%	2.9%	0.0%	8	0	0	1	0
ClemMex29	97.1%	2.9%	0.0%	8	0	0	1	0
ClemMex30	88.6%	8.6%	2.9%	7	1	0	1	1
ClemMex31	94.3%	2.9%	2.9%	7	0	0	2	0
ClemMex32	91.4%	8.6%	0.0%	7	0	0	2	0
ClemMex33	91.4%	0.0%	8.6%	7	0	0	2	0
ClemMex34	94.3%	5.7%	0.0%	7	0	0	2	0
ClemMex35	88.6%	0.0%	11.4%	7	0	0	2	0
ClemMex36	94.3%	2.9%	2.9%	7	0	0	2	0
ClemMex37	94.3%	0.0%	5.7%	7	0	0	2	0
ClemMex38	91.4%	5.7%	2.9%	7	0	0	2	0
ClemMex39	82.9%	0.0%	17.1%	7	0	1	1	1
ClemMex40	91.4%	5.7%	2.9%	7	0	0	2	0
ClemMex41	94.3%	2.9%	2.9%	7	0	0	2	0
ClemMex42	82.9%	14.3%	2.9%	7	0	0	2	1
ClemMex43	91.4%	2.9%	5.7%	7	0	0	2	0
ClemMex44	85.7%	11.4%	2.9%	7	1	0	1	1
ClemMex45	85.7%	5.7%	8.6%	7	0	1	1	1
ClemMex46	88.6%	5.7%	5.7%	7	0	0	2	1
ClemMex47	94.3%	5.7%	0.0%	7	0	0	2	0
ClemMex48	94.3%	2.9%	2.9%	7	0	0	2	0
ClemMex49	94.3%	0.0%	5.7%	7	0	0	2	0
ClemMex50	85.7%	8.6%	5.7%	7	0	0	2	0
ClemMex51	85.7%	14.3%	0.0%	7	0	0	2	2
ClemMex52	97.1%	0.0%	2.9%	8	0	0	1	0
ClemMex53	94.3%	0.0%	5.7%	7	0	0	2	0
ClemMex54	85.7%	11.4%	2.9%	7	0	0	2	1
ClemMex55	94.3%	0.0%	5.7%	7	0	0	2	0
ClemMex56	91.4%	5.7%	2.9%	7	0	0	2	0
ClemMex57	94.3%	2.9%	2.9%	7	0	0	2	0
ClemMex58	91.4%	5.7%	2.9%	6	0	0	3	0
ClemMex59	91.4%	2.9%	5.7%	6	0	0	3	0
ClemMex60	85.7%	5.7%	8.6%	6	0	0	3	1
ClemMex61	85.7%	11.4%	2.9%	6	0	0	3	2
ClemMex62	80.0%	5.7%	14.3%	6	0	0	3	1
ClemMex63	80.0%	17.1%	2.9%	6	0	0	3	1
ClemMex64	91.4%	0.0%	8.6%	6	0	0	3	0
ClemMex65	82.9%	5.7%	11.4%	6	0	1	2	1
ClemMex66	88.6%	8.6%	2.9%	6	0	0	3	0
ClemMex67	88.6%	2.9%	8.6%	6	0	0	3	0
ClemMex68	85.7%	14.3%	0.0%	6	0	0	3	1
ClemMex69	77.1%	14.3%	8.6%	6	1	1	1	2
ClemMex70	85.7%	5.7%	8.6%	6	0	0	3	0
ClemMex71	88.6%	8.6%	2.9%	6	0	0	3	0
ClemMex72	88.6%	0.0%	11.4%	6	0	0	3	1
ClemMex73	85.7%	8.6%	5.7%	6	0	0	3	1
ClemMex74	85.7%	2.9%	11.4%	6	0	0	3	2
ClemMex75	91.4%	2.9%	5.7%	6	0	0	3	0
ClemMex76	91.4%	2.9%	5.7%	6	0	0	3	0
ClemMex77	71.4%	11.4%	17.1%	6	1	1	1	2
ClemMex78	91.4%	0.0%	8.6%	6	0	0	3	0
ClemMex79	88.6%	5.7%	5.7%	6	0	0	3	0
ClemMex80	82.9%	8.6%	8.6%	5	0	0	4	1
ClemMex81	74.3%	20.0%	5.7%	5	2	0	2	2
ClemMex82	80.0%	11.4%	8.6%	5	0	0	4	1
ClemMex83	77.1%	20.0%	2.9%	5	0	0	4	2
ClemMex84	77.1%	17.1%	5.7%	4	0	0	5	1
ClemMex85	65.7%	20.0%	14.3%	4	0	1	4	3

Interspecific heterozygosity *(C. micrantha* X *C. medica*)
***C. micrantha* homozygosity**
***C. medica* homozygosity**
Centromere position

FH: percentage of fully heterozygous gametes for the LG; Fmed: fully *C. medica* homozygous gametes for the LG; Fmic: fully *C. micrantha* homozygous gametes for the LG; Mixed: gametes with mixed heterozygosity and homozygosity for the LG. HomCentromer: gametes with fully homozygous centromere

General discussion

Background

Polyploidy represents a major evolutionary step toward speciation and diversification (Grant, 1981; Soltis *et al*., 1993; Otto and Whitton, 2000; Wendel and Doyle, 2005). Its origin, evolutionary significance and interest for breeding programs are still under study and discussion (Bretagnolle and Thompson, 1995; Ortiz, 1997; Ramsey and Schemske, 1998; Otto and Whitton, 2000; Gallais, 2003; Ollitrault *et al*., 2008).

Sexual polyploidization through unreduced gametes (2n gametes) is considered as the major mechanism leading to polyploidy (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). Up to seven major mechanisms of 2n gamete formation have been characterized. However, De Storme and Geelen (2013a) reduced them in two major mechanisms: genomic duplication and meiotic restitution. For the genomic doubling, we can distinguish two variants depending when it occurs. If it is before the meiosis, it is called the pre-meiotic doubling (PRD), and it was observed in *Solanum lycopersicum* (De Storme and Geelen, 2013b). Otherwise, if the meiosis precedes the doubling, it is called the post-meiotic restitution (PMD), characterized by the formation of fully homozygous 2n gametes. This was observed in *S. tuberosum* (Bastiaanssen *et al*., 1998), in some *Rubus* species (Dowrick, 1966), and in *Alstroemeria* (Ramanna and Jacobsen, 2003). For the meiotic restitution, two main mechanisms have been widely described: first-division restitution (FDR) and second-division restitution (SDR). They occur, respectively, as a result of an abnormal development of the first and second meiotic division. FDR have been observed in potato, alfalfa, *Vaccinium* spp. and some of grasses (Ramanna and Jacobsen, 2003).

These different mechanisms result in very different genetic structures of the 2n gamete populations. PRD produces 2n gametes equivalent to the meiosis of doubled diploid (DD) genotypes. Therefore, PHR depends mainly on the chromosomal preferential pairing rate and varies between 66% for fully tetrasomic meiosis to 100% for fully disomic meiosis (Stift *et al*., 2008). For PMD, the duplication affects the haploid gametes, leading to the formation of fully homozygous 2n gametes. Thus, 100% homozygosity for all LGs is expected among the 2n gametes (Ramanna and Jacobsen, 2003). FDR 2n gametes contain non-sister chromatids, which in the absence of crossover maintain the parental heterozygosity. For SDR, the 2n gametes contain two sister chromatids, which reduces the parental heterozygosity level (Cuenca *et al*., 2011; De Storme and Geelen, 2013a). When crossover occurs, the parental heterozygosity restitution (PHR) rate for FDR varies from 100% for loci close to the centromere to 60–70% for loci far from the centromere. For SDR, it varies from 0% for loci close to the centromere to 60–75% for loci far from the centromere depending on the level of chromosome interference (Cuenca *et al*., 2011).

Citrus species produce a, relatively, high percentage of unreduced ovules, reaching 20% in some genotypes (Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Cuenca *et al*., 2011). Before undertaking the present study, only FDR and SDR had been reported in citrus, being SDR the main mechanism of 2n megaspore formation in mandarins (Esen *et al*., 1979; Luro *et al*., 2000; Cuenca *et al*., 2011; 2015; Aleza *et al*., 2016a). Not really conclusive results were published for other citrus species due to insufficient numbers of individuals or markers under analysis. Although there is no reason to discard the possibilities to observe/interpret other mechanisms resulting in unreduced gametes in citrus mainly the PMD and PRD mechanisms described in other species.

Harlan and deWet (1975) argued that chromosome doubling of somatic cells must be relatively rare in nature compared to the occurrence of unreduced gametes. If it affects only some cells of a meristem it should result in ploidy chimeras or cytochimeras (Zonneveld, 2007). However, Citrus is an interesting example were chromosome doubling appears to be frequent in nucellar cells and results in non-chimeric doubled-diploid genotypes (Aleza *et al*., 2012a, b; Guerra *et al*., 2016) in polyembryonic citrus (facultative apomixes). It also was observed in apomictic mangos (Saúco *et al*., 2001). Curiously, despite this relatively high rate of chromosome doubling most of the natural citrus germplasm is diploid (Ollitrault *et al*., 2008). Several breeding programs over the word (Florida, Brazil, Italy, Spain, France, etc…) have taken advantage of this mechanism to diversify the citrus tetraploid gene pool. Recently, chemical treatments with colchicine and oryzalin were used to obtain double-diploid of mono-embryonic varieties such as clementine or Fortune mandarin (Aleza *et al*., 2009b). Therefore, this new citrus tetraploid gene pool is mostly constituted by natural and induced doubled-diploid accessions, but also include somatic hybrids (Grosser *et al.,* 2000; Dambier *et al*., 2011) and a few sexual tetraploid hybrids.

The inheritance of tetraploids can be schematically considered around two extreme models, disomic and tetrasomic (Gallais, 2003; Stift *et al*., 2008; Jeridi *et al*., 2012). Tetrasomic inheritance is observed in autotetraploids, where each chromosome has three other homologous copies and has equal opportunity to pair with other homologs, leading to multivalent formation and random pairing of chromosomes. At the opposite, allotetraploids resulting from the merging of two different genomes, display disomic inheritance; each chromosome has only one homolog to pair, resulting in strictly preferential pairing during meiosis (Sybenga, 2012). However, the meiotic inheritance in tetraploids may be affected by the chromosomes pairing affinity (Sybenga, 1996). Both bivalent and multivalent pairing behavior can be observed simultaneously (Wu *et al*., 2001), which may result in an intermediate chromosome pairing between strictly preferential and non-preferential pairing and intermediate segregation (Stift *et al*., 2008). Strict disomic inheritance results in the complete restitution of the interspecific heterozygosity. In the case of allotetraploids each diploid gamete is the equivalent of the parental interspecific diploid hybrid (PHR= 100%). For autotetraploids with no double reduction, the expected percentage is PHR=66%. Intermediate pairing results in PHR between these two values.

Cytological techniques, especially genomic in situ hybridization-GISH or/and fluorescent in situ hybridization-FISH (Lim *et al*., 2001; Crespel and Gudin, 2003; Dewitte *et al*., 2012; Jeridi *et al*., 2012; Silkova and Loginova, 2016) have been used to analyze the origin of 2n gametes and the meiosis of tetraploids. For unreduced gametes, half tetrad analysis approaches (Mendiburu and Peloquin, 1979) with a predefined order of markers (Tavoletti *et al*., 1996) or without any previous information about marker position (Da *et al*., 1995) have been developed and applied in different species. These approaches required working at population level with numerous markers. More recently, Cuenca *et al*. (2015) proposed a maximum likelihood method based on PHR of centromeric markers to differentiate between FDR and SDR mechanisms at individual and population level. It was successfully applied to Citrus, taking advantage of the centromeres location (Aleza *et al*., 2015) in the reference genetic map (Ollitrault *et al*., 2012a). Although this method allows comparing a large range of partial interference

model functions (Cuenca *et al*., 2015), its limitation is that it does not take in consideration other mechanisms of 2n gamete formation, such as PRD or PMD.

Ploidy manipulation is an integrated component of several citrus breeding projects around the world. It is applied with two main objectives: (i) the development of triploid seedless cultivars mainly in the mandarin group, and to a lesser extend in the acid citrus group (limes, lemons) and (ii) the creation of new tetraploid rootstocks with a better adaptation to biotic and abiotic stresses. Spontaneous unreduced gametes have been widely exploited for triploid citrus breeding from diploid x diploid crosses (Esen and Soost, 1971; 1973a; Ollitrault *et al*., 2008; Aleza *et al*., 2010b; Cuenca *et al*., 2015; Navarro *et al*., 2015) and the development of the tetraploid gene-pool also allowed to produce triploid hybrids by interploid crosses 2x x 4x and 4x x 2x (Esen and Soost, 1973b; Cameron and Burnett, 1978; Starrantino and Recupero, 1981; Ollitrault *et al*., 2008; Grosser and Gmitter, 2011; Aleza *et al*., 2012a, b; Navarro *et al*., 2015). Most tetraploid parents are doubled-diploids, but also allotetraploid parents are used). Sexual hybridization at tetraploid level is a relatively recent strategy for rootstock breeding. The so called tetrazyg breeding strategy was initiated in Florida (Grosser *et al*., 2003) and it is also developed in France (Ollitrault Pers. Com.) and Italy (Caruso Pers. Com.). For tetrazyg rootstock development, the parents are either somatic hybrids or DD of interspecific or intergeneric origins. A clear understanding of the meiotic mechanisms producing the diploid gametes (unreduced from diploid parents and reduced from tetraploid parents) and their implication in the genetic structures of gamete populations is fundamental to optimize the breeding strategies based on ploidy manipulation in citrus.

In this thesis, we have extended the knowledge on the formation of unreduced gametes to the male gametophyte by the analysis of the genetic structure of diploid pollen population produced by the diploid `CSO´ tangor, inferred from tetraploid hybrids in tetraploid x diploid `CSO´ cross and to the megagametophytes of *C. limon* species based on the inference of the diploid ovules of `Eureka Frost´ and `Fino´ lemons from triploid and tetraploid progenies arising respectively from diploid x diploid and diploid x tetraploid crosses. We have also analyzed the meiotic behavior of a DD `Mexican´ lime to evaluate the possibility that triploid `Tahiti´ (*C. latifolia*) and `Tanepao´ (*C. aurantifolia*) limes were originated by interploid hybridization involving a DD `Mexican´ lime. The results are discussed with the perspective of triploid mandarin and acid citrus breeding.

The predominant mechanisms of 2n gamete formation is SDR for megagametophytes and FDR for pollen

The frequency of 2n megagametophytes, for `Fino´ and `Eureka Frost´ lemons were 4.9% and 8.3% respectively. These frequencies are in agreement with 1% to 5% reported by Geraci *et al.* (1975) for `Lisbon´ and `Eureka Frost´ lemons respectively. More variable range were observed in mandarins, (1% to 20%) for clementines, `Sukega´ and `Ortanique´ tangor (Ollitrault *et al.*, 2008; Aleza *et al.*, 2010b; Wakana *et al.*, 1982; Esen and Soost, 1971; Xie *et al.*, 2014).

The frequency of 2n gametes that we obtained, confirm the genotype effect for unreduced gamete frequencies. This was observed in citrus and in other herbaceous and woody plants such as *Brassica*, potato, and peach (Dermen, 1938; Mok and Peloquin,

1975; Parrott and Smith, 1986; Ollitrault *et al.,* 2008; Aleza *et al.,* 2010b; Mason *et al.,* 2011; Younis *et al.,* 2014) either in unreduced pollen or ovule gametes (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Otto and Whitton, 2000). This information could be used for the genetic improvement of unreduced gamete rates, as already attained for Trifolium (frequencies increased from 0.04% to 47%) and *Medicago sativa* (from 9% to 78%) in only three generations of recurrent selection (Gallais, 2003).

The 4x x 2x and 2x x 4x crosses of our studies were favorable to reveal 2n pollen and egg gamete occurrences. Indeed the embryo/endosperm ploidy ratio 4/6 (=2/3) is equivalent to the one produced in 2x x 2x hybridization with reduced male and female gametes, allowing a normal development of the seeds (Esen and Soost, 1971)(the ratio is different in the two types of crosses).

We analyzed the genetic structures of the inferred unreduced gametes of `CSO´ tangor and `Eureka Frost´ and `Fino´ lemons, using the maximum-likelihood method based on parental heterozygosity restitution (PHR) of centromeric loci and the PHR pattern for one linkage group. Our results showed that FDR is the predominant mechanism producing unreduced pollen for `CSO´, while SDR is the main mechanism that produces 2n ovules in `Eureka Frost´ and `Fino´ lemons. However, we also found that 18.8% of 2n `CSO´ pollen arisen from SDR and 7% of 2n megagametophytes of `Eureka frost´ and `Fino´ lemons arisen from (FDR) or (PRD). Honsho *et al*. (2016) also concluded for the occurrence of FDR unreduced pollen from a molecular marker study performed at individual pollen grain level. However no plant resulting from such FDR pollen where described prior our work. SDR was previously proved to be the main restitution mechanism for female gametes in mandarins (Esen *et al*., 1979; Luro *et al*., 2000; Cuenca *et al*., 2011; 2015; Aleza *et al*., 2015). We demonstrated that it is also the main mechanism in two lemon cultivars. We also revealed the simultaneous occurrence of FDR and SDR unreduced gametes both in `CSO´ pollen and lemons gametophytes. Such observation was previously made in other plant species and particularly potatoes (Conicella *et al*., 1991) and the predominance of SDR for 2n megagametophytes and FDR for pollen was also described for other plants (Bretagnolle and Thompson, 1995; d´Erfurth *et al*., 2008).

The combination of the analysis of the PHR pattern in one LG and the maximum-likelihood method based on centromeric markers revealed a new mechanisms of 2n gamete formation in citrus: the post meiotic chromosome doubling

At methodological level, we demonstrated the complementarity between the analysis of PHR pattern in one LG and the maximum-likelihood method proposed by Cuenca *et al*. (2015) at individual level. Indeed, considering only centromeric *loci*, the PMD can lead to the same homozygous patterns than SDR. Therefore the analysis of heterozygosity restitution pattern along LGs at individual level is a useful approach to distinguish between SDR and PMD. Indeed, under PMD, heterozygosity restitution is zero for all markers in all LGs, while heterozygosity may be found for telomeric loci for SDR. The analysis of PHR pattern at population level is also useful to distinguish between SDR and PRD when individual LODs are under the threshold for conclusive results. With enough individuals, it should also be applied to distinguish between FDR and PRD. With FDR-2n gametes, heterozygosity restitution vary from 100% in centromeric *loci* to close to 66% in telomeric areas under non-interference model (Cuenca *et al*., 2011),

whereas with pre-meiotic doubling, heterozygosity restitution is expected to be very constant along all the chromosome. Coupling the two approaches, we have revealed for the first time in citrus the occurrence of post-meiotic genome doubling (PMD) in lemons, originating 5% of the unreduced ovules and therefore the coexistence of at least three mechanisms producing unreduced ovules in lemons.

Doubled diploid `Mexican´ lime display preferential disomic segregation

In previous studies to evaluate the paring model and the meiotic behavior of a given citrus tetraploid genotype, two methodologies has been used individually or combined: cytogenetic observation through chromosome-squashing techniques (Kamiri *et al*., 2011; Xie *et al*., 2015) or/and molecular markers analysis (Kamiri *et al*., 2011; Xie *et al*., 2015; Aleza *et al*., 2016a) to estimate preferential paring from the PHR values in different LGs. To analyze the meiotic behavior of `Mexican´ lime diploid gametes, we produced a populations of 85 tetraploid hybrids between a DD clementine and a DD `Mexican´ lime. We combined pollen viability evaluation, cytogenetic study and segregation analysis of 35 SSR and SNPs markers. The last analysis also allowed evaluating the interspecific recombination rates and comparing them with diploid and tetraploid Clementine. The predominance of bivalents (65%) in the meiosis of the tetraploid `Mexican´ lime is similar to the observations made in several allotetraploid somatic hybrids (Del Bosco *et al*., 1999; Chen *et al*., 2004; Kamiri *et al*., 2011; Xie *et al*., 2015). Moreover, we observed that 6.3% of the chromosomes were involved in closed tetravalents. At diploid level, closed tetravalents are usually considered as an evidence for the presence of heterozygous reciprocal translocation (Sybenga, 1975). However there is no indication for such structure in the diploid `Mexican´ lime while typical figures for inversion were observed (Iwamasa and Nito, 1988). A reconciliation between diploid and doubled diploid microsporogenesis cytogenetic observations showed the presence of a double inversion affecting the two arms of a same chromosome. This double inversion pattern may results from chromosome structural variation between *C. medica* and *C. micrantha*, the two parents of the diploid `Mexican´ lime (Nicolosi *et al*., 2000; Curk *et al*., 2016). The average PHR value (90.2%) reported in this work is higher than the ones observed by Kamiri *et al*. (2011), Xie *et al*. (2015) and Aleza *et al*. (2016a). Disomic inheritance with high preferential pairing values was deduced for three LGs (LG2, LG7 and LG8). Intermediate inheritance with disomic tendency was found for five LGs (LG1, LG3, LG4, LG6, and LG9) and intermediate models for LG5. Tetrasomic inheritance was not observed for any chromosome, suggesting that chromosome pairing was affected by a global differentiation rather than discrete and local large structural variations, as the inversion described in diploid `Mexican´ lime. This high preferential pairing could explain the low recombination rates per Mb (1.2 cM/Mb), three times lower than in diploid and tetraploid Clementine due to the impediment of the interspecific recombination.

The meiotic behaviour of the DD `Mexican´ lime is compatible with interploid crosses as origin of *C. latifolia* and *C. aurantifolia* triploid limes

Curk *et al*. (2016) proposed that the triploid *C. latifolia* (Tahiti type) and *C. aurantifolia* (`Tanepao´ type) triploid limes, arisen respectively from a diploid gamete of *C. aurantifolia* (`Mexican´ lime type) pollinating an ovule of lemon or pollinated by a haploid pollen of citron. For both triploid lime types, considering a `Mexican´ lime diploid and DD parent, the PHR of the gametes involved in the origin of these two lime

types was evaluated to be between 88% and 95%. The same authors proposed two alternative hypotheses for the origin of this diploid gamete: (i) a diploid gamete originated from natural DD of `Mexican´ like lime, such as the `Giant key´ or (ii) an unreduced gamete from a diploid `Mexican´ lime like variety. The average PHR rate we observed for the DD `Mexican´ lime (90.2%) and the natural occurrence of DD `Mexican´ limes make the interploid hybridisation hypothesis compatible with the origin of `Tahiti´ and `Tanepao´ types. An SDR unreduced gamete hypothesis is not compatible with the PHR values estimated by Curk *et al*. (2016). An FDR unreduced gamete hypothesis could not be discarded. FDR gametes transmit in average 80 % of the parental heterozygosity (Peloquin, 1983; Hutten *et al*., 1994; Carputo *et al*., 2003), lower than the one estimated by Curk *et al*. (2016). However when coupled with asynapsis, described in diploid `Mexican´ lime (Iwamasa and Iwasaki, 1963), it could reach 100%. The analysis of the genetic structures of unreduced gametes of `Mexican´ lime is still necessary to definitively conclude on the origin of the natural *C. latifolia* and *C. aurantifolia* triploid limes.

Implications for citrus breeding

For breeding programs based on ploidy manipulation aiming the production of triploid or tetraploid hybrids, the determination of mechanisms underlying 2n pollen formation is a key information to model the genetic structure of triploid progenies, to develop association studies in polyploid progenies and to optimize breeding strategies. Several previous publications discussed the relative advantages of SDR and FDR gametes in polyploid breeding (Mendiburu and Peloquin, 1977a, b; Hutten *et al*., 1994; Aleza *et al*., 2015). If the objective is to create progenies more similar to the parent producing the unreduced gamete, FDR and PRD -2n gametes will be a better strategy because the resulting 2n gametes will be heterozygous as their parent from the centromere to the first crossing over in case of FDR and highly heterozygous for all the LGs in the case of PRD (heterozygosity values depending on the pairing model). At the opposite, SDR-2n gametes provide the opportunity to create a larger number of new multilocus genotypic combinations and a higher number of polymorphic progenies, providing new products to meet commercial market segmentation strategies (Cuenca *et al*., 2011; Aleza *et al*., 2016a). The PMD mechanism leads to the formation of fully homozygous gametes (Bastiaanssen *et al*., 1998). Therefore, this mechanism generally promotes inbreeding in the produced hybrids (Tai, 1986; Gallais, 2003).

In addition, the mechanism that generates the 2n gametes, in relation with the genetic distance to the centromeres of the major genes controlling a selected trait, affects the breeding efficiency. For instance, Cuenca *et al*. (2013b; 2016) showed that, the Alternaria brown-spot fungal disease was controlled as a recessive trait by a single locus located at 10.5 cM from the centromere of chromosome III. Therefore in crosses between a heterozygous parent producing diploid gametes and a resistant genotype, PMD is the most favorable mechanisms (50% of resistant hybrids) followed by SDR mechanisms (40%). Under FDR mechanism only 5% of the hybrids will be resistant. For diploid gametes produced by a DD genotype or resulting of PRD the rates of resistant hybrids should vary from 16% (tetrasomic segregation) to 0% (disomic segregation) according to the preferential pairing behavior. For major breeding traits, such *Alternaria* resistance, this knowledge will strongly drive the choice of the polyploid breeding strategy.

The coexistence of several mechanism of unreduced gamete production in a same genotype and the observed differences for the predominant mechanism between unreduced pollen (FDR) and ovules (SDR) open the way to develop oriented triploid breeding strategies in mandarin types and lemons. It is also the opportunity to develop progenies with increased phenotypic diversity. The tetraploid plants obtained in 4x x 2x and 2x x 4x hybridizations for `CSO´ and the `Eureka frost´ and `Fino´ lemon will be integrated in the IVIA tetraploid germplasm. They may be used as parents for further triploid breeding. The plants arising from FDR should be more interesting to provide increased gametic diversity and heterosis in the triploid progenies due to their higher level of heterozygosity, particularly in centromeric regions.

Our work also enlighten the possibility to develop a reconstruction breeding program of the two main ideotypes of triploid limes (`Tahiti´ and `Tanepao´ types) by an interploid breeding strategy using a DD `Mexican´ lime. Although the disomic tendency of this DD genotype limits the effective interspecific recombination and the diversity of the diploid gamete population, the restored pollen fertility of the doubled diploid `Mexican´ lime and the consistency of the diploid gametes genetic structures with the ones that originated `Tahiti´ and `Tanepao´ limes open the way for intensive and efficient breeding programs.

Perspectives

Our results confirm that the *Citrus* genus is an interesting model for tetraploid meiosis and unreduced gamete mechanism studies and polyploid research (Ollitrault *et al*., 2008; Cuenca *et al*., 2015; Aleza *et al*., 2009b; 2016a). The associated development of molecular, genetic, and cytogenetic techniques will lead to rapid advancements in the field in coming years.
We have extended to lemon the demonstration that SDR is the main mechanisms of 2n ovule formation and demonstrated that FDR was predominant for a tangor 2n pollen. Further studies on other ancestral and secondary species, such as citron, pummelo, grapefruit, sweet orange, and lime could determine whether the mechanisms found in mandarin, tangor and lemons are representative for the whole *Citrus* genus. Moreover, further studies on environment influence and genetic control of unreduced gamete formation would pave the way for improved frequencies of 2n gametes in triploid breeding programs.

Predominant disomic inheritance was found for a DD `Mexican´ lime, while previous studies of tetraploid citrus (several somatic hybrids and DD clementine) concluded for tetrasomic and intermediate predominance. The meiotic behavior of DDs of citrus secondary species and direct interspecific hybrids between the ancestral taxa will provide information about the genomic differentiation between the basic taxa and its implication in the recombination and segregation of genome fragments. It will be a key to develop further QTLs analysis in polyploid progenies involving tetraploid interspecific parents. Accurate QTL analysis on polyploid progenies will require the development of genotyping methods coupling a pangenomic coverage and the ability for allele doses estimation. Methods based on next-generation sequencing such as Genotyping-by-sequencing (GBS) are promising.

Already developed and future knowledge on the origin and genetics of diploid gametes will strongly improve the efficiency of citrus polyploidy breeding and will result in an increasing position of triploid varieties and tetraploid rootstock in the citrus industry worldwide.

Conclusions

First Division Restitution (FDR) and Second Division Restitution (SDR) mechanisms are involved in the unreduced pollen gametes formation

Tetraploid plants arising from crosses between tetraploid clementine as female parent and `CSO´ tangor as male parent were analyzed with molecular markers (SSRs and SNPs). The results showed that the obtained hybrids resulted from unreduced 2n pollen. The Maximum likelihood method based on PHR of centromeric loci and analysis of PHR patterns along LG2 were used at individual and population levels to determine whether FDR or/and SDR was the mechanism underlying the production of unreduced gametes.

FDR was the conclusive mechanism for 64.1% of the analyzed plants and SDR for 18.8%. No conclusive results were found for the remaining plants. For citrus, it is the first report of tetraploid progenies obtained from unreduced pollen and the first observation of SDR and FDR leading, in a same genotype, to unreduced microsporogenesis.

Coexistence of different mechanisms of unreduced ovule gametes in lemon

Unreduced ovule gamete production was evidenced by SSR and SNP markers analysis in two lemon genotypes `Eureka Frost´ and `Fino´ at frequencies of 4.9% and 8.3% respectively. Individual and population LOD analysis of centromeric loci, telomeric loci genotyping, and the analysis of PHR patterns along LG1 allowed us to determine three different meiotic mechanisms for formation of 2n female gametes in lemon, 88% arised from SDR, 7% from FDR or PRD, and 5% from PMD.

This is the first recovery of large lemon progenies through unreduced 2n gametes and the first identification of a new mechanism, PMD that has never been observed previously in citrus.

PHR pattern in one LG is an essential complementary analysis to the maximum-likelihood method to detect the unreduced gametes mechanisms

The analysis of PHR pattern in one LG helped to distinguish between PMD and SDR mechanisms and between FDR and PRD mechanisms at population level. Therefore the analysis of heterozygosity restitution pattern along LGs is an essential approach to detect the mechanism producing unreduced gametes.

Doubled diploid `Mexican´ lime display preferential disomic segregation, and could be useful for triploid lime breeding programs

The molecular marker analysis of a population of tetraploid hybrids obtained from a doubled diploid (DD) clementine x DD `Mexican´ lime cross proved that DD `Mexican´ lime has a predominantly disomic segregation producing interspecific diploid gamete structures with high *C. medica*/*C. micrantha* heterozygosity. This disomic tendency limits the recombination and the diversity of the diploid gamete population. However, the relatively high pollen viability compared with the diploid `Mexican´ lime parent is an advantage to develop efficient triploid lime breeding programs.

The meiotic behavior of the doubled-diploid `Mexican´ lime is compatible with interploid crosses as origin of *C. latifolia* and *C. aurantifolia* triploid limes

The high level of PHR observed for the diploid pollen of `Mexican´ lime is compatible with the phylogenomic structures of triploids *C. latifolia* and *C. aurantifolia* varieties. This conclusion could support the hypothesis of the interploid cross (diploid by tetraploid) origin of both triploid varieties.

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