How can we harness the genetic diversity for genetic improvement, taking into account reproductive traits acquired through the evolution of the citrus species?

François Luro

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INRAe-Cirad, Agap Corse, France

Seapag

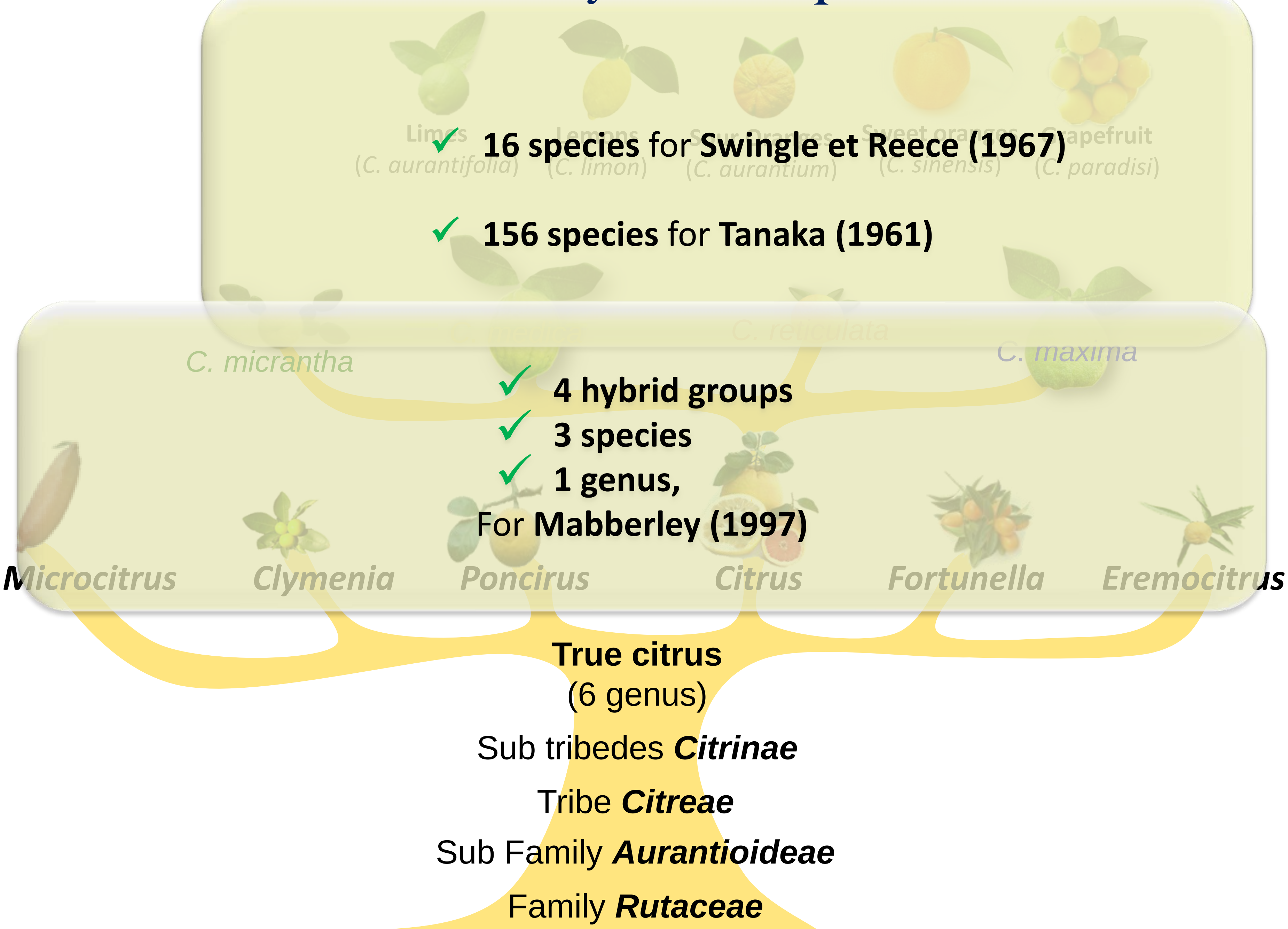
agAp INSTITUT
Amélioration génétique et adaptation des plantes méditerranéennes et tropicales

How to classify citrus species

- ✓ **16 species** for **Swingle et Reece (1967)**
- ✓ **156 species** for **Tanaka (1961)**

- ✓ **4 hybrid groups**
- ✓ **3 species**
- ✓ **1 genus,**

For **Mabberley (1997)**



True citrus
(6 genus)

Sub tribedes ***Citrinae***

Tribe ***Citreae***

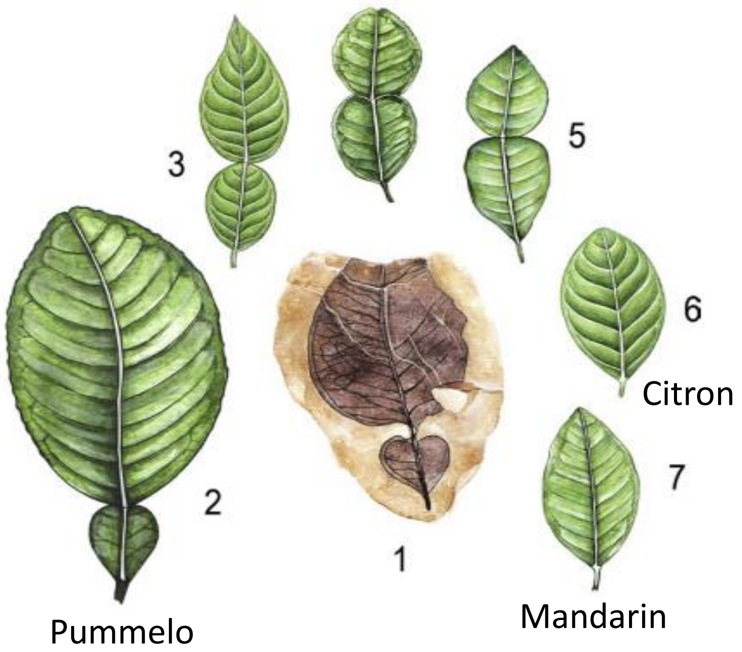
Sub Family ***Aurantioideae***

Family ***Rutaceae***

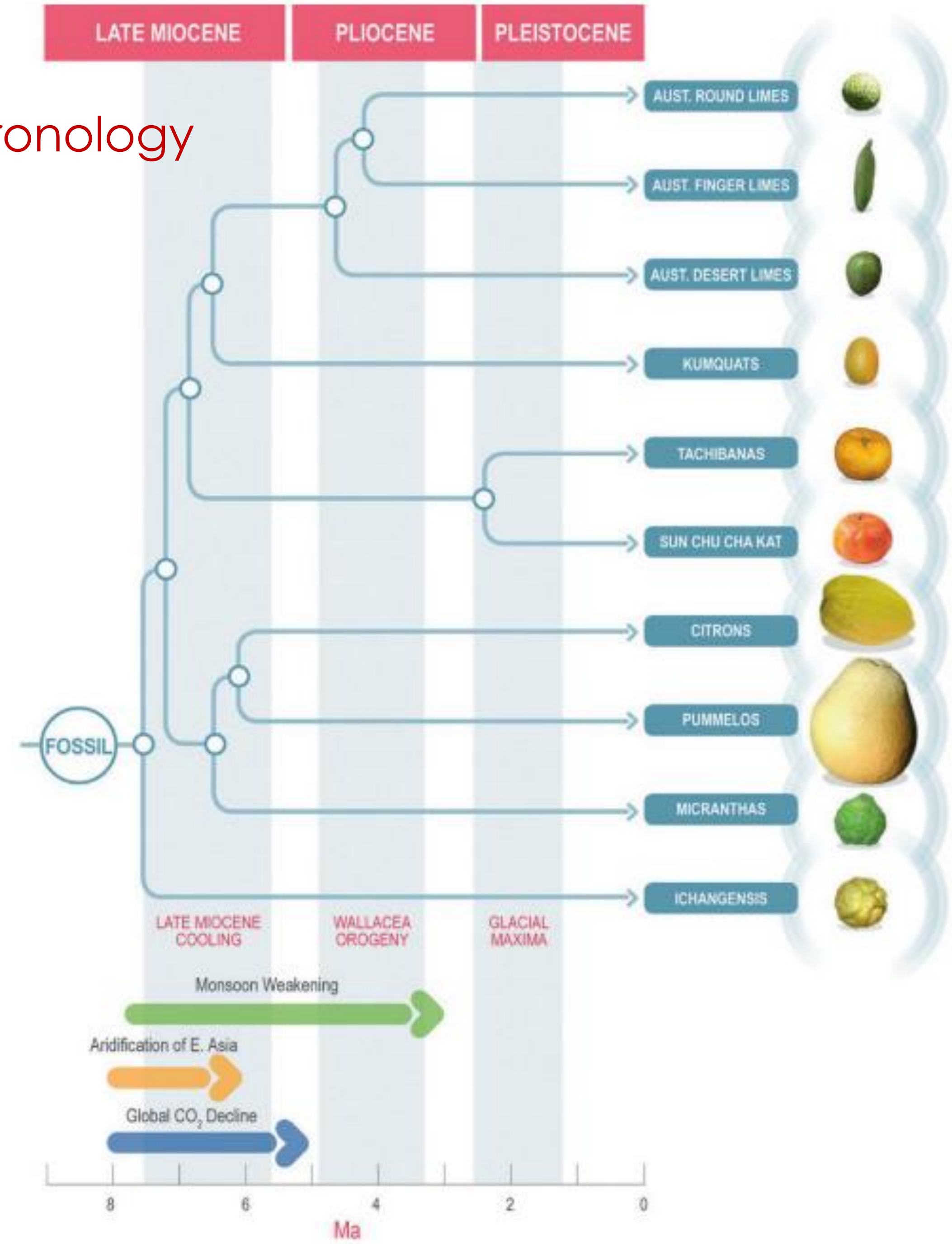
Evolution of citrus ancestors

Speciation chronology

Characteristics of a fossil leaf of *C. linczangensis* (1) compared with those of present-day species

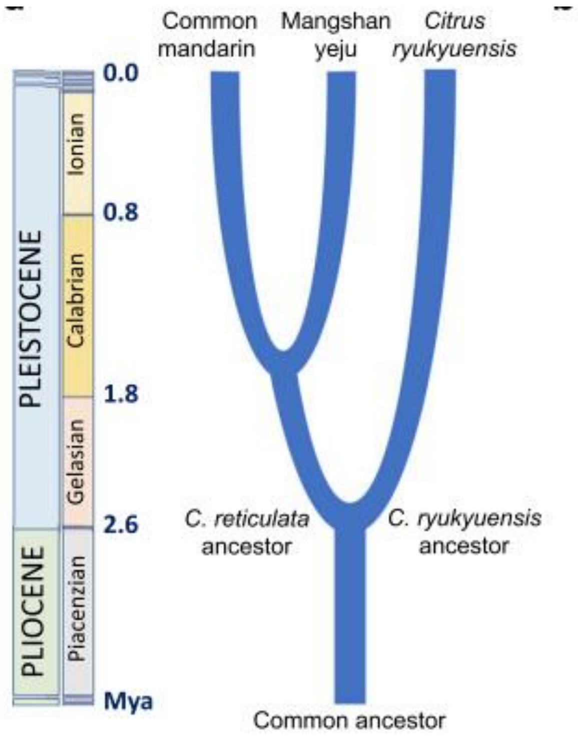


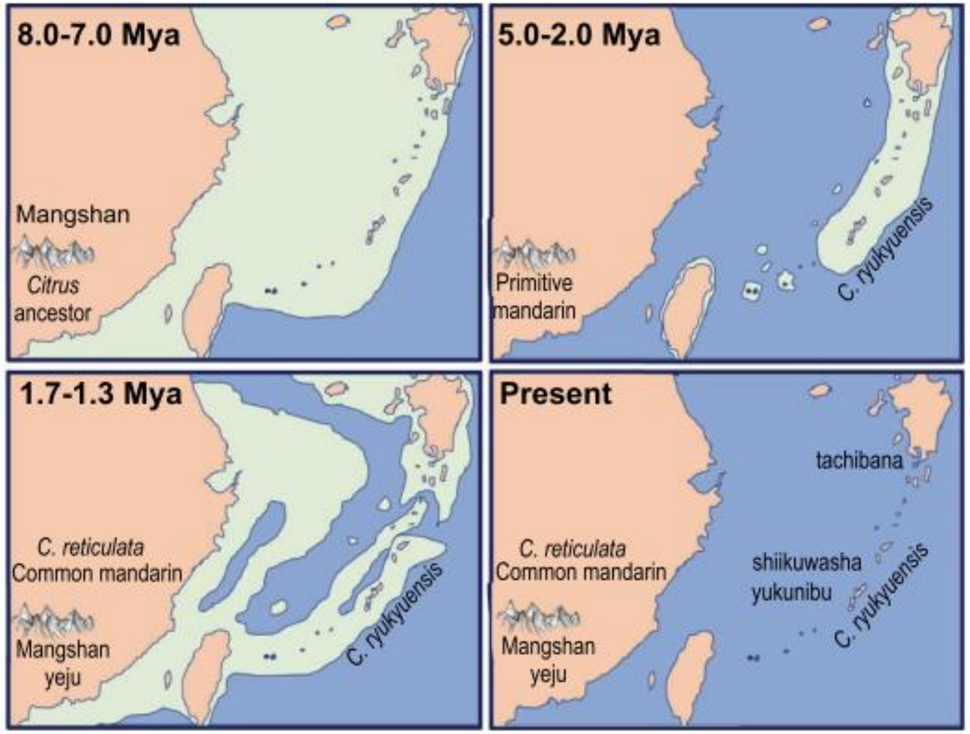
Xie et al . 2013

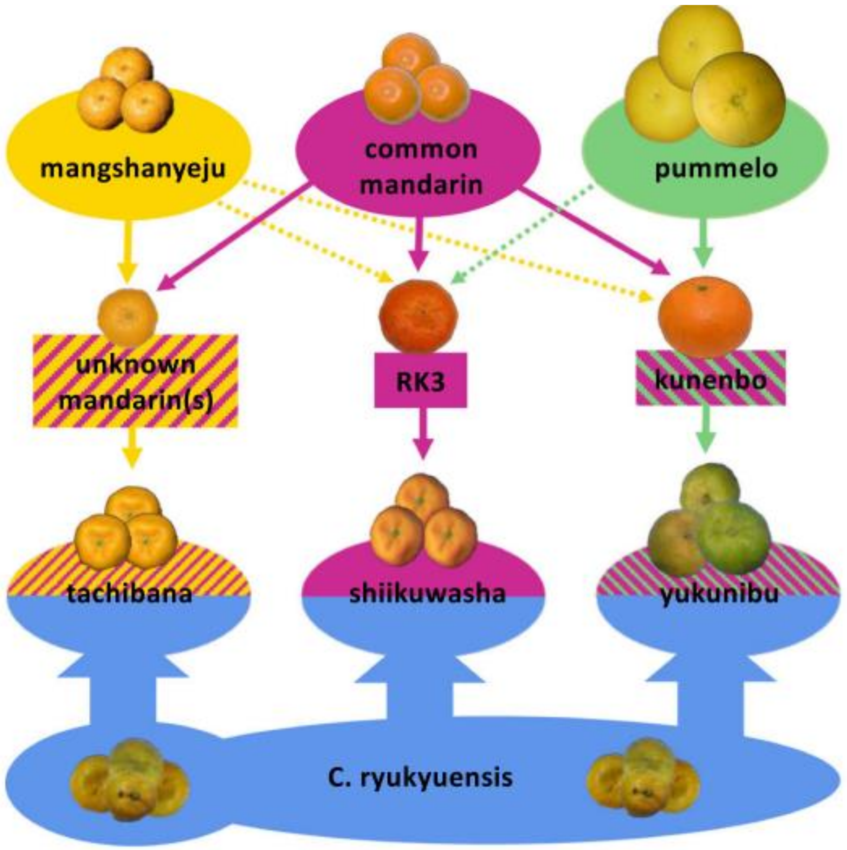


Mandarin evolution: allopatric evolution and interspecific crosses

Chronogram of speciation and biogeography of the East Asian mandarin tree in the Ryukyu arc and mainland Japan



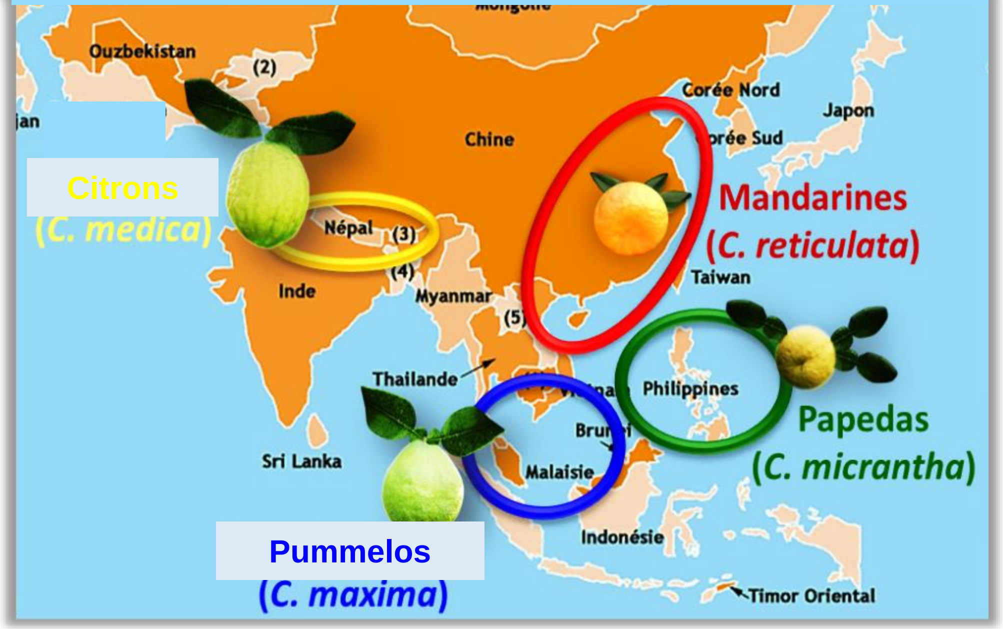


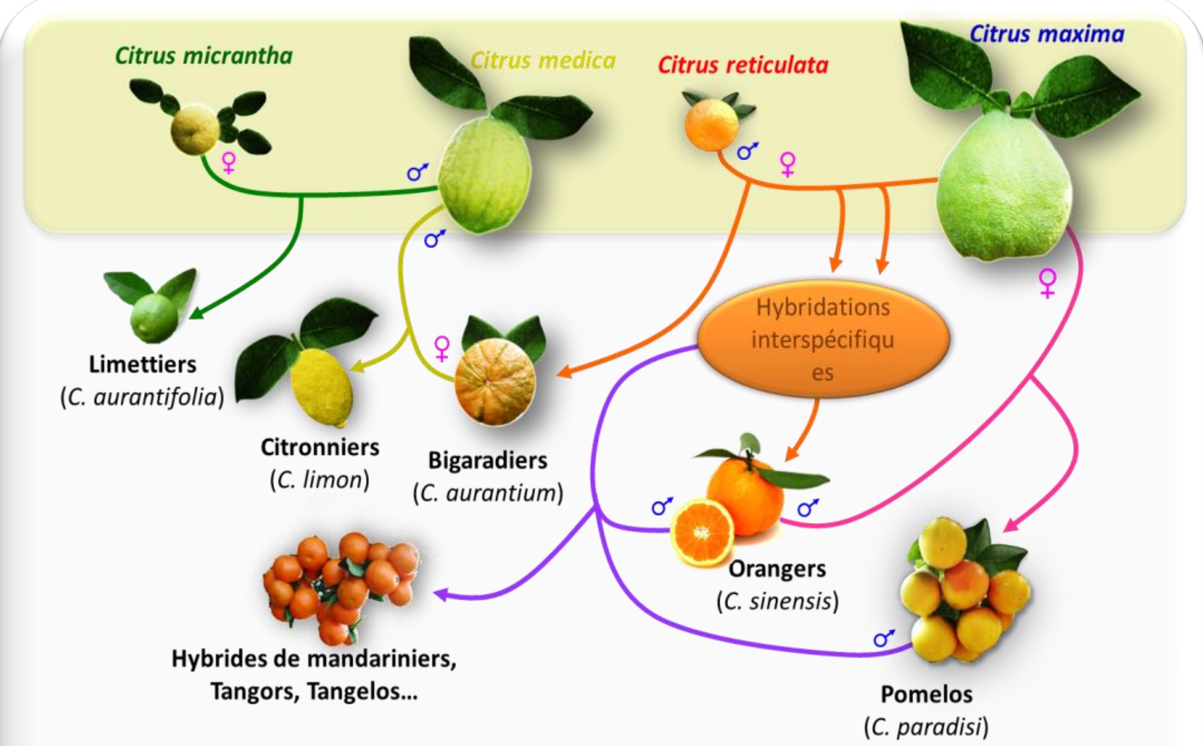


Crosses between mandarin subpopulations and with *C. maxima* are the source of *C. reticulata* diversification

Reticulate evolution of citrus species

Differentiation between basic taxa
initial allopatric evolution



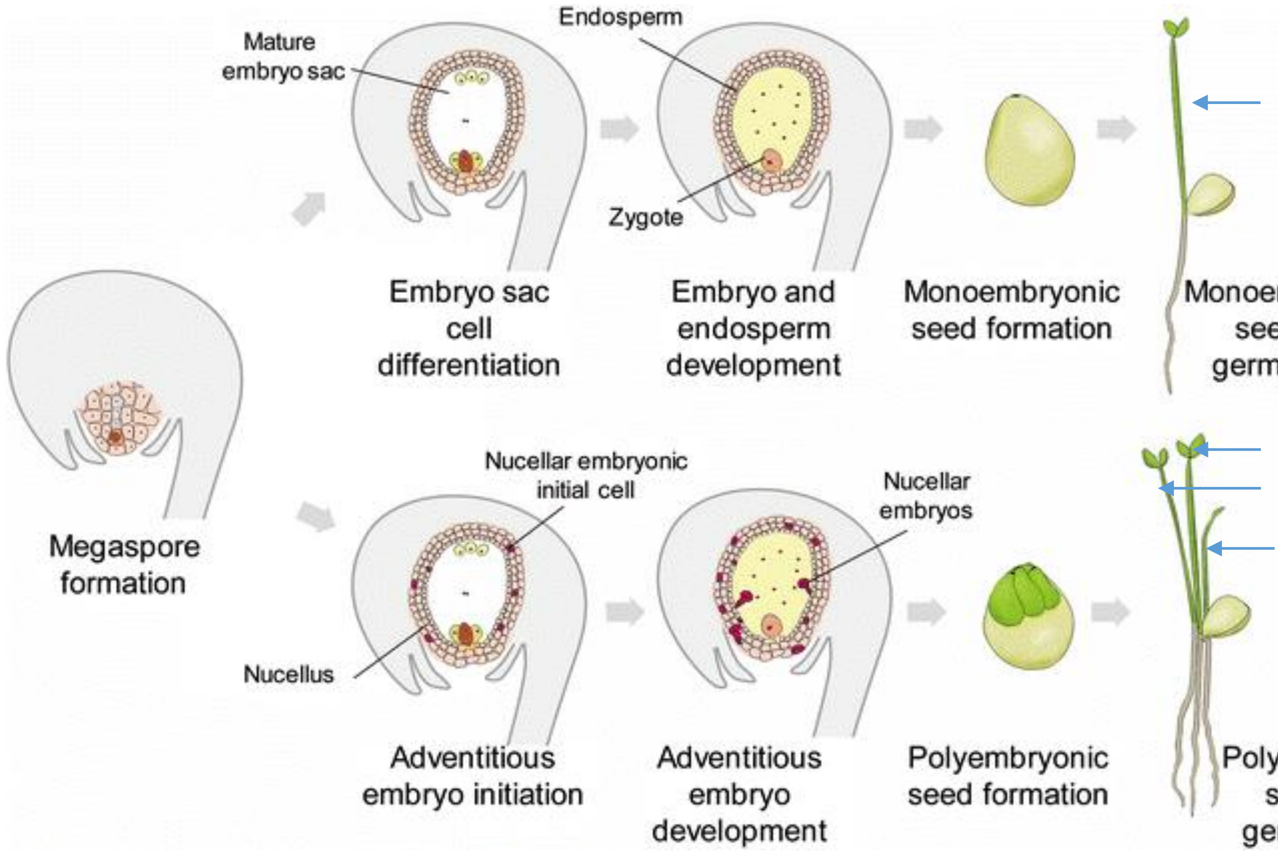


Reticulation and limited number of interspecific meiosis events

The genomes of cultivated *Citrus* are mosaics of large chromosome fragments from the basic taxa in frequent interspecific heterozygosity

Structures maintained by apomixis or horticultural vegetative propagation practices

Apomictic reproduction in citrus



Zygote formation is necessary for adventitious embryogenesis

Variation of development step between embryos

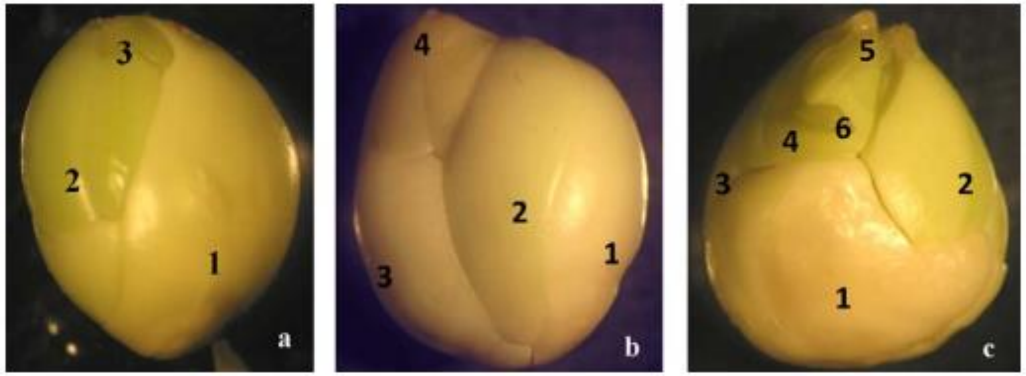


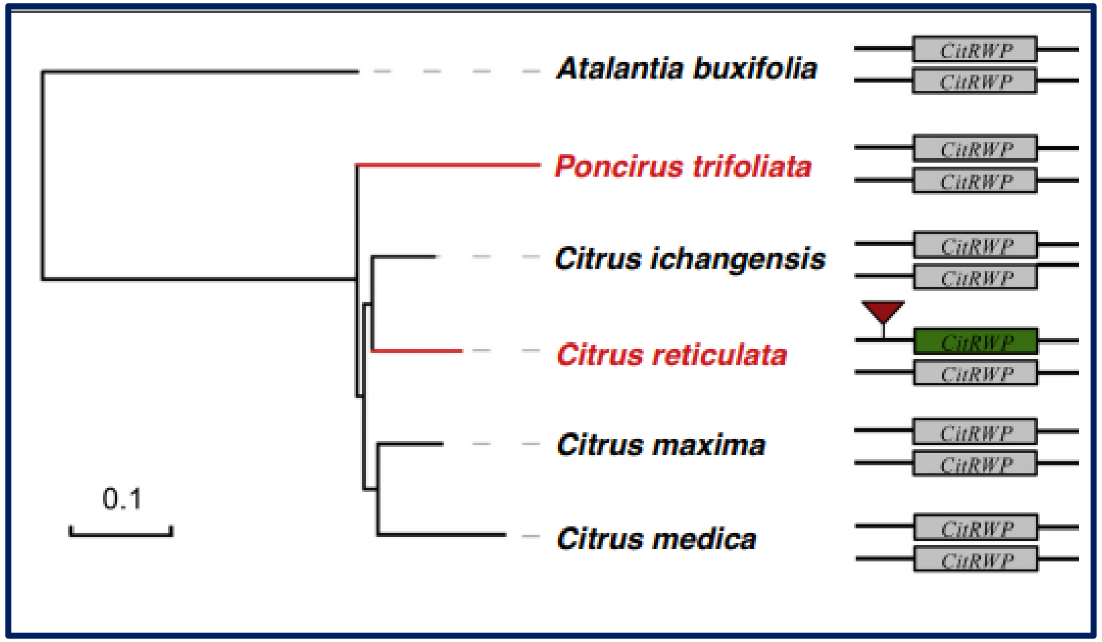
Fig. 4. Variation in biomass partitioning among embryos. (a) Triplet; (b) quadruplet; and (c) sextuplet.

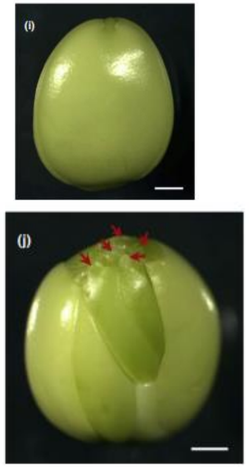
Emergence

asynchrone synchrone

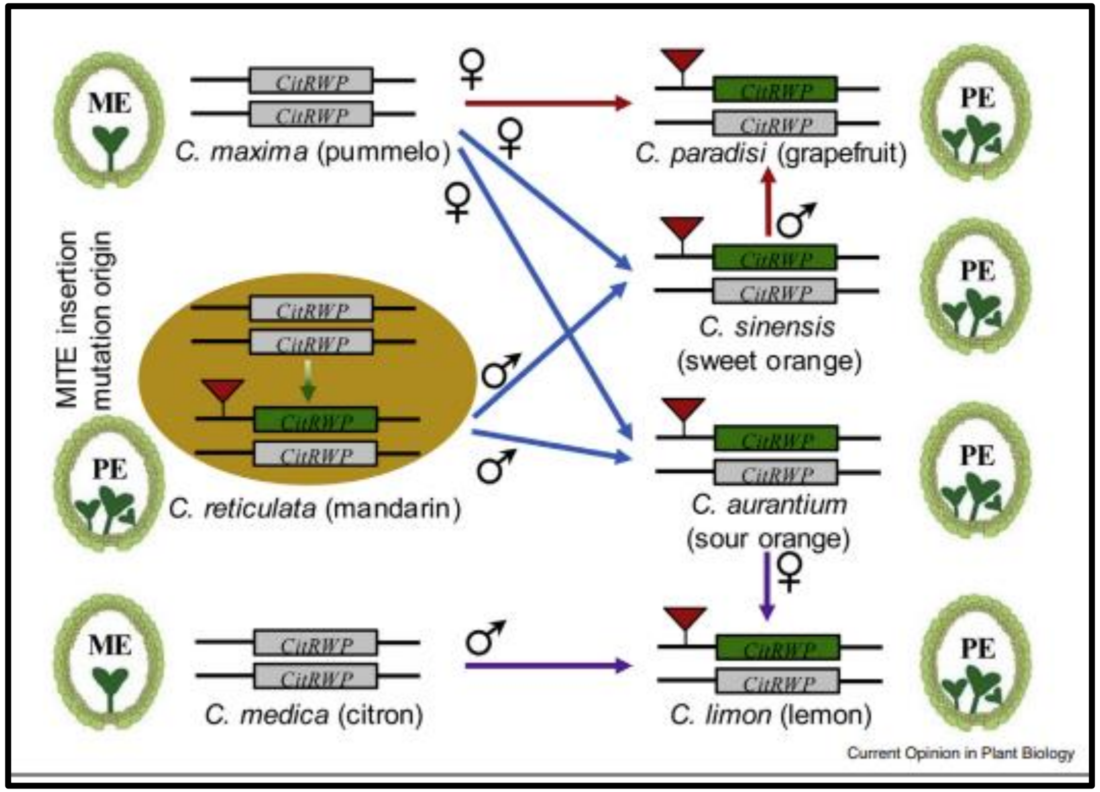
Competition between embryos

Origin and heredity of somatic embryogenesis





(*Zhang et al. 2018*)



- Nucellar embryony has appeared in mandarins after insertion of a transposable element (MITE) upstream of the CitRWP gene.
- The mechanism of nucellar embryony is unknown in *Poncirus trifoliata*
- All cultivated taxa have inherited the apomictic reproduction from mandarin.
- Apomixis contributed to the selection of descendants who inherited this trait

The citrus genome


A « small » genome (**360** Mpb. - **398** Mpb.) [Ollitrault *et al.* **1994**]

Arabidobsis taliana **119** Mpb

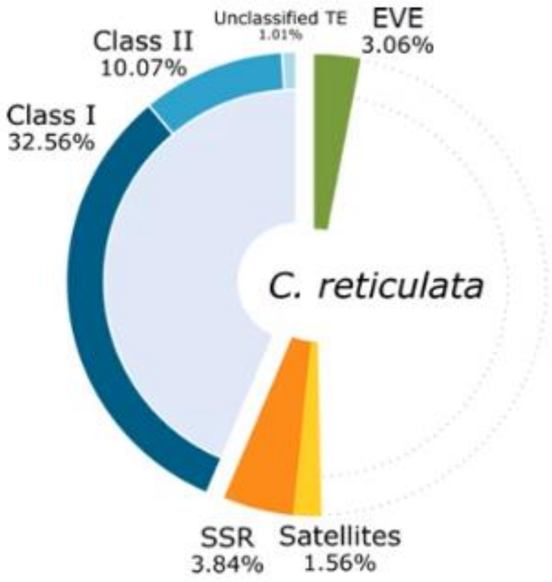
Populus trichocarpa **485** Mbp

Zea mays **5.000** Mbp

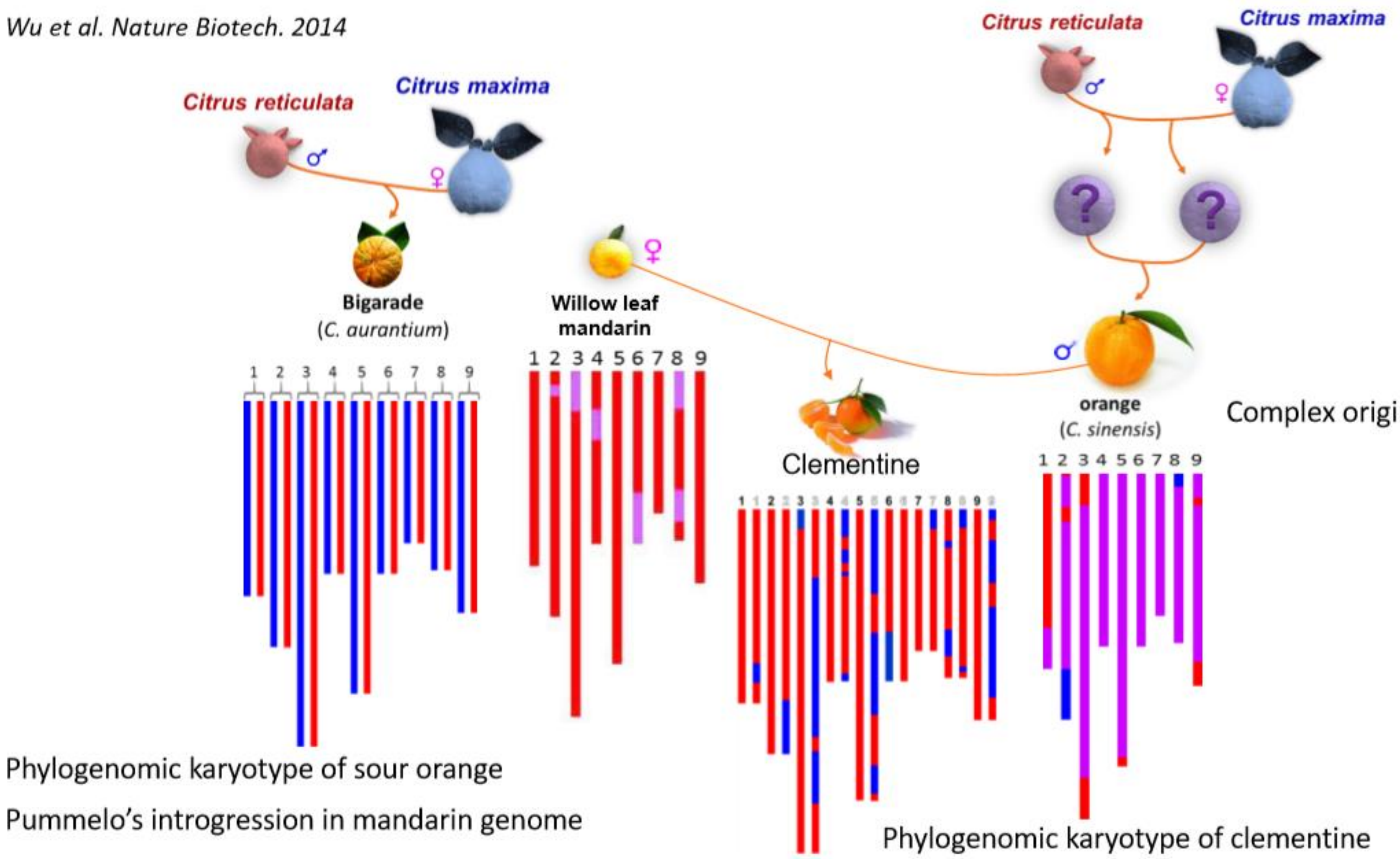
Triticum **17.000** Mbp

n = 9 chromosomes

About 37 000 genes
Repeated sequences ~50% genome
with 42% of transposable elements



Genomic structures of cultivated citrus by Genome Sequencing (WGS)



Complex origin of orange

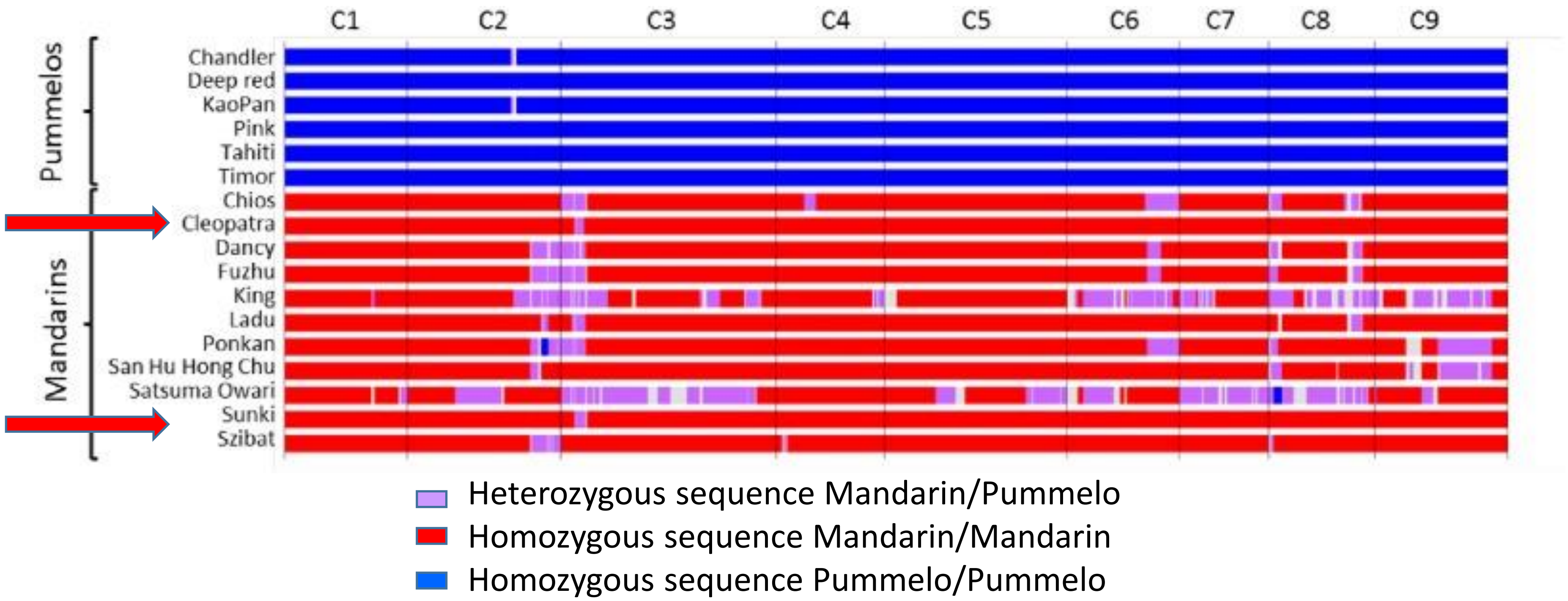
Phylogenomic karyotype of sour orange

Pummelo's introgression in mandarin genome

Phylogenomic karyotype of clementine

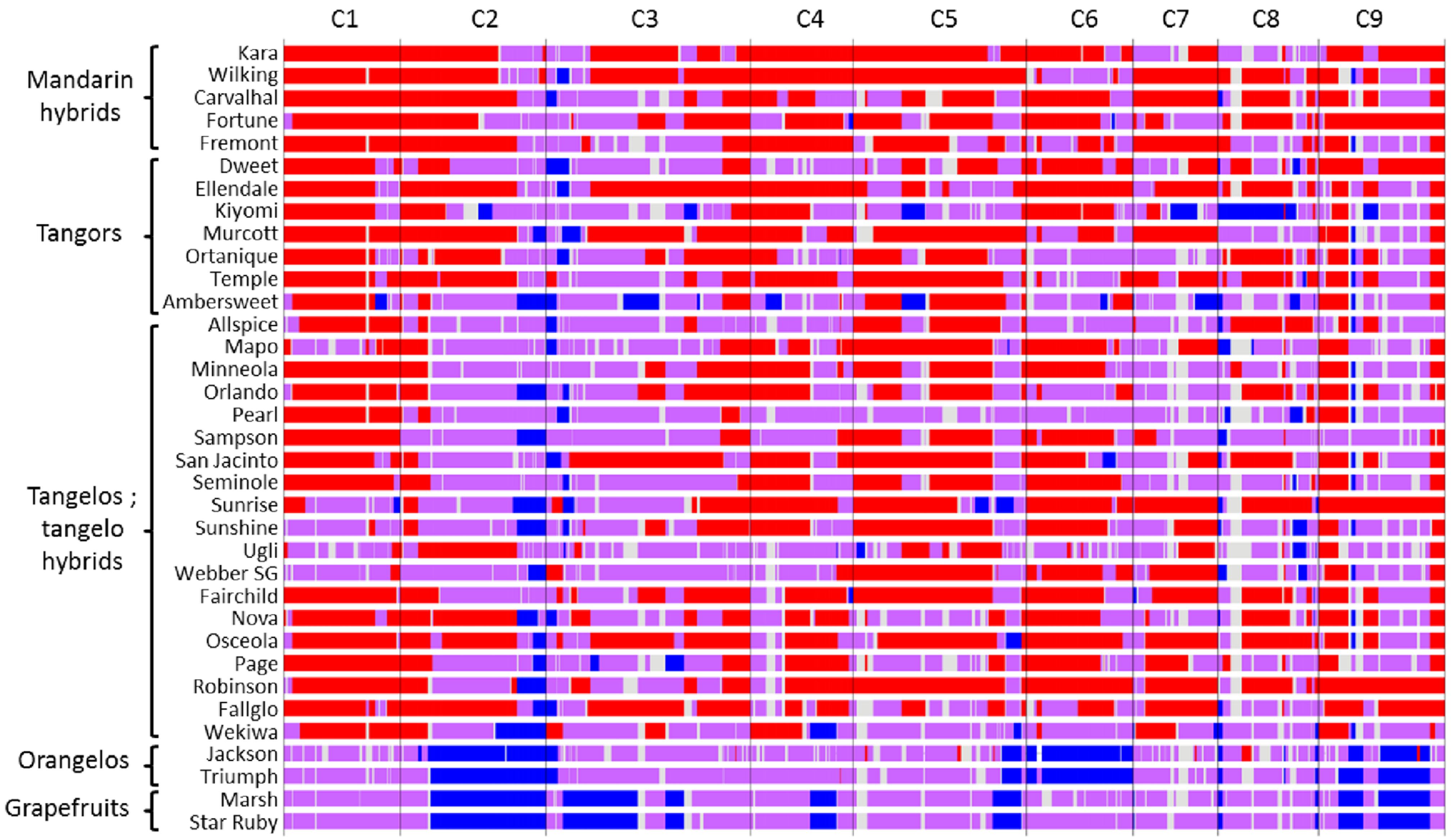
Introgression of pummelo into modern cultivars of mandarin genome

Are representatives of *C. reticulata* ancestral taxa existing ?
Phylogenomic karyotype based on GBS data of varieties used as references of pummelos and mandarins



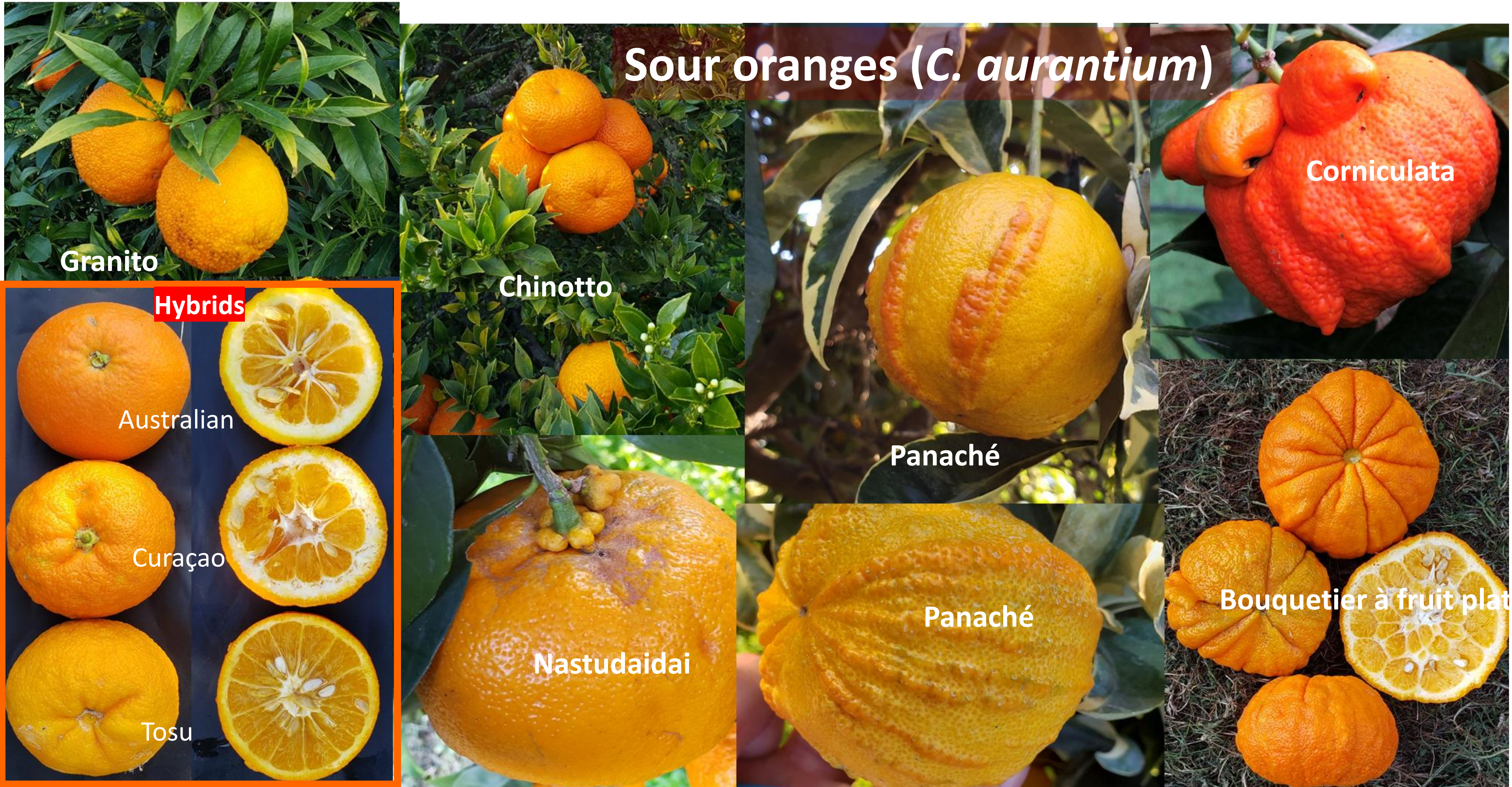
Cleopatra and Sunki are the closest to wild mandarins
Other mandarins share more or less introgressions of pummelo genome

Analysis of phylogenomic structures and interspecific recombination for a population derived from the *C. reticulata* / *C. maxima* gene pools…



Oueslati et al. (Plos One, 2017)

Mutation as source of diversity in secondary species



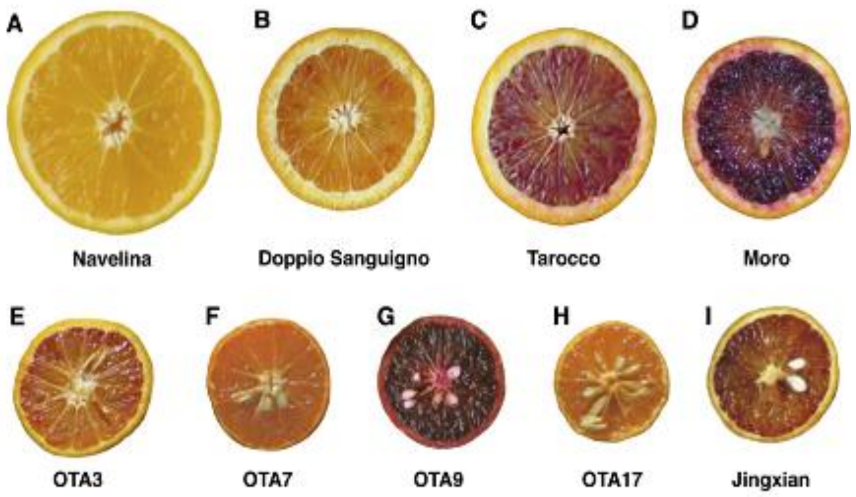
Mutation as source of diversity in secondary species

Anthocyanin synthesis in bloody oranges is induced by an insertion of a transposable element in *Ruby* promoter region

The Plant Cell, Vol. 24: 1242–1255, March 2012, www.plantcell.org

Retrotransposons Control Fruit-Specific, Cold-Dependent Accumulation of Anthocyanins in Blood Oranges [W][OA]

Eugenio Butelli,[a] Concetta Licciardello,[b] Yang Zhang,[a] Jianjun Liu,[c] Steve Mackay,[a] Paul Bailey,[a] Giuseppe Reforgiato-Recupero,[b] and Cathie Martin[a,1]
[a] John Innes Centre, Norwich NR4 7UH, United Kingdom
[b] Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee, 95024 Acireale, Italy
[c] Sichuan Academy of Agricultural Sciences, Chengdu City, Sichuan 610066, China



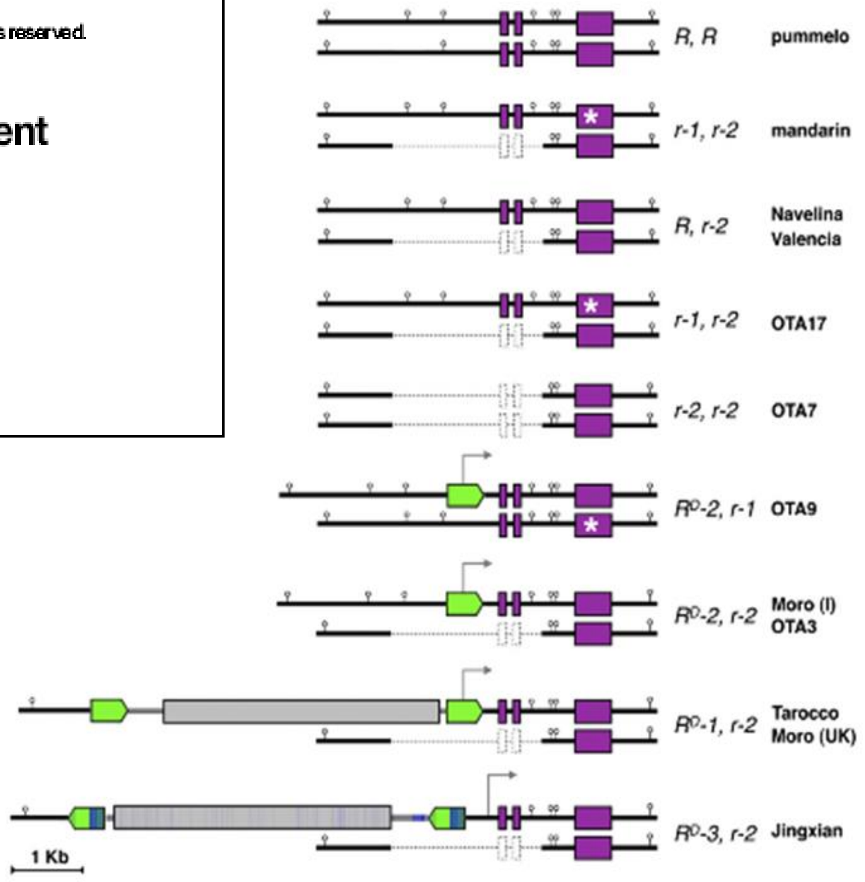
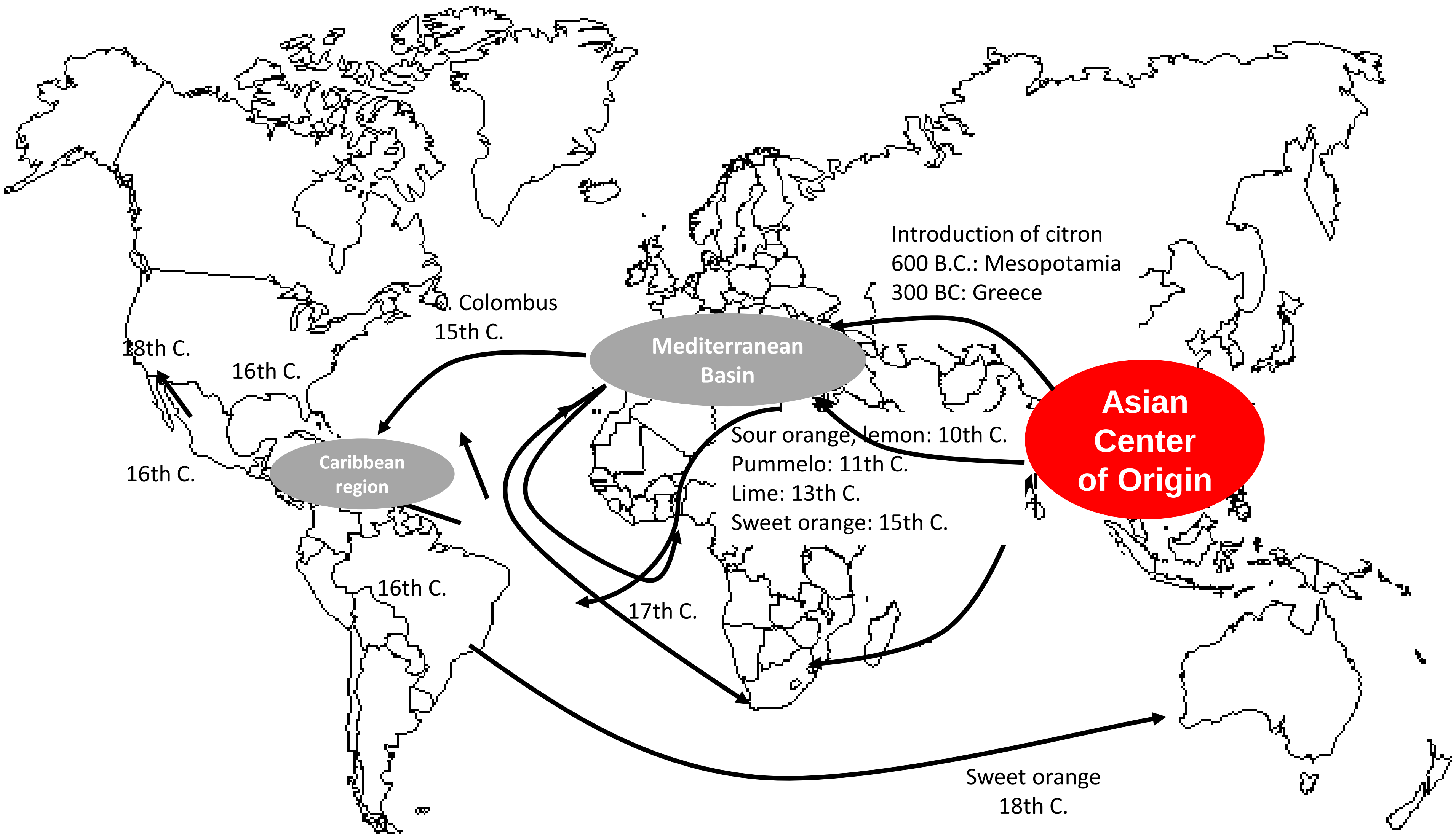


Figure 6. Maps of Structures of the *Ruby* Locus in the Different *Citrus* Species, Orange Accessions, and Hybrids.

Citrus origin and dispersion



Citrus diversity for genetic improvement

The objectives of breeding programs are:

- to diversify the panel of citrus cultivars,
- to extend the period of citrus production
- to produce new cultivars / rootstocks adapted to the environment and constraints

- How the citrus diversity can be mobilized ?
- Which main strategies for each kind of citrus ?
- How can breeding strategies be adapted to the characteristics of the species' biology ?

Constraints and advantages of conventional breeding

Reproduction

Self-incompatibility

backcross is hampered

Gametic sterility due to chromosomal aberrations embryo sac or seed abortion, or gene (CMS)

Partial **apomixis**

Polyembryonic seeds give somatic embryos from maternal tissue (nucellus).
Unfit for progeny development (competition)

Overcrowding of adult progenies

Long juvenile phase (~6 years)

The delays for fruiting are long and limit the number of generations in a genealogical selection

Genetic control of gametic self-incompatibility

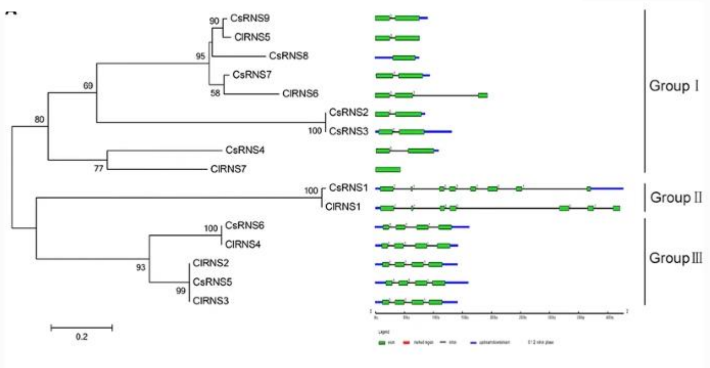
Liang et al. 2020

Mol Genet Genomics (2017) 292:325–341
DOI 10.1007/s00438-016-1279-8
CrossMark
ORIGINAL ARTICLE

Genome-wide identification and functional analysis of S-RNase involved in the self-incompatibility of citrus

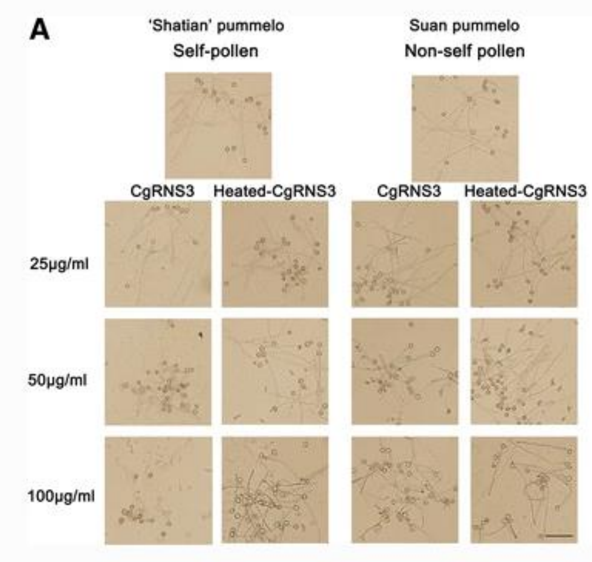
Mei Liang[1] · Wei Yang[1] · Shiying Su[1] · Lili Fu[1] · Hualin Yi[1] · Chuanwu Chen[2] · Xiuxin Deng[1] · Lijun Chai[1]

Homologous RNase genes from genome data base



CsRNS3 gene exprressed only in pistil

CgRNS3 protein significantly inhibited the growth of self-pollen tubes from 'Shatian' pummelo



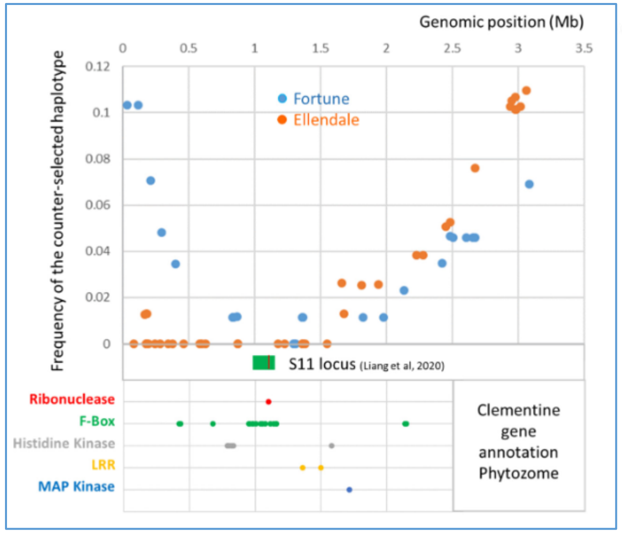
Ollitrault et al. 2021

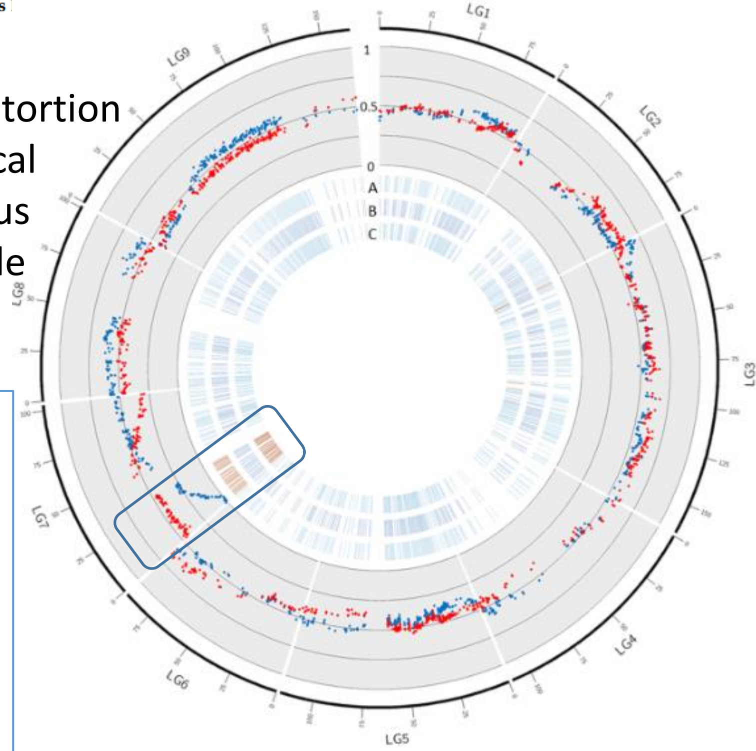
agriculture
MDPI
Article

Segregation Distortion for Male Parents in High Density Genetic Maps from Reciprocal Crosses between Two Self-Incompatible Cultivars Confirms a Gametophytic System for Self-Incompatibility in Citrus

Patrick Ollitrault [1,2,*], Dalel Ahmed [3], Gilles Costantino [3], Jean-Charles
Pierre Mourneт [1,2], Aude Perdereau [4] and Yann Froelicher [1,2]

S locus location by segregation distortion (rejection of one allele) in reciprocal crosses of 2 self-incompatible citrus sharing one self-incompatible allele



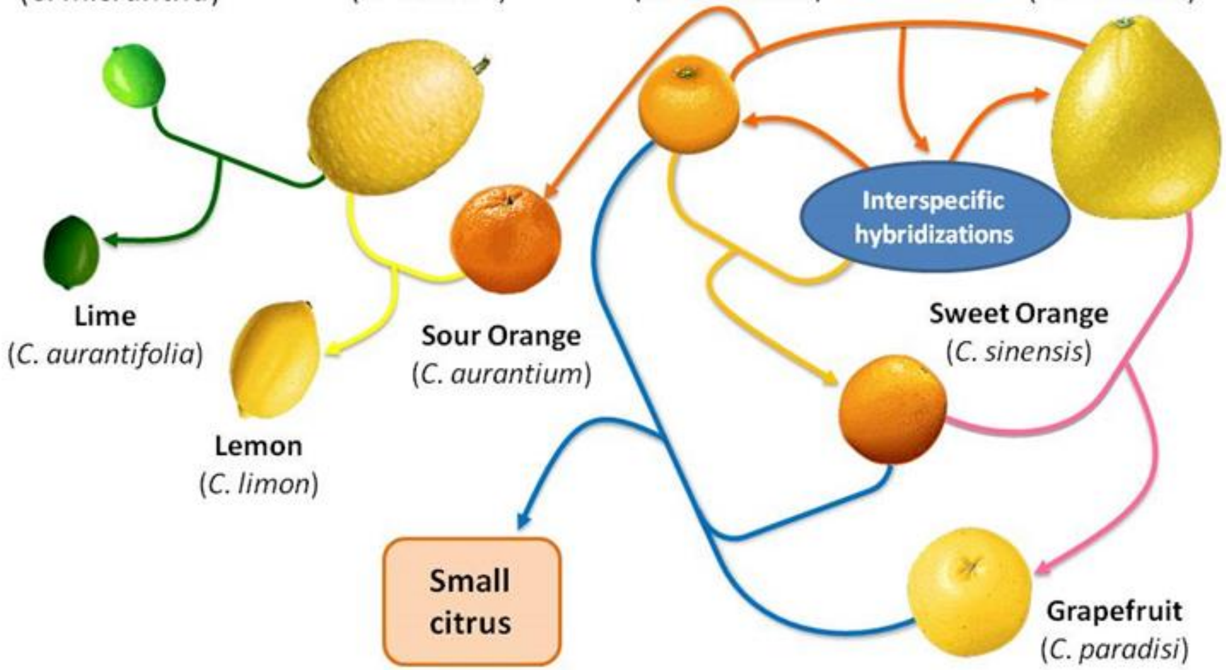


gametophytic S-RNase system with the S locus located at the beginning of chromosome 7

Constraints and advantages of conventional breeding

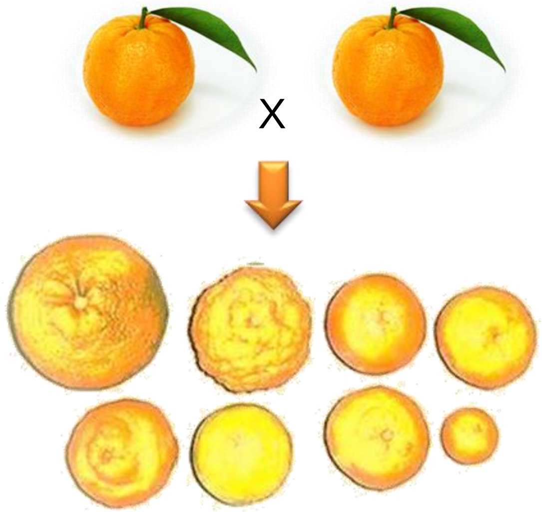
Complex genetic structures

Phylogeny of *Citrus* sp.



Citrus crops are interspecific hybrids fixed by vegetative propagation

High Heterozygosity



Conventional breeding of the citrus crops is hampered by the complex genetic structures that determine their specific phenotypes.

The **highly heterozygous** interspecific mosaic structure of their genome is broken by sexual recombination => high heterogeneous phenotype with expression of deleterious alleles

Solutions for secondary species breeding

The majotrity of citrus cultivars are spontaneous mutants

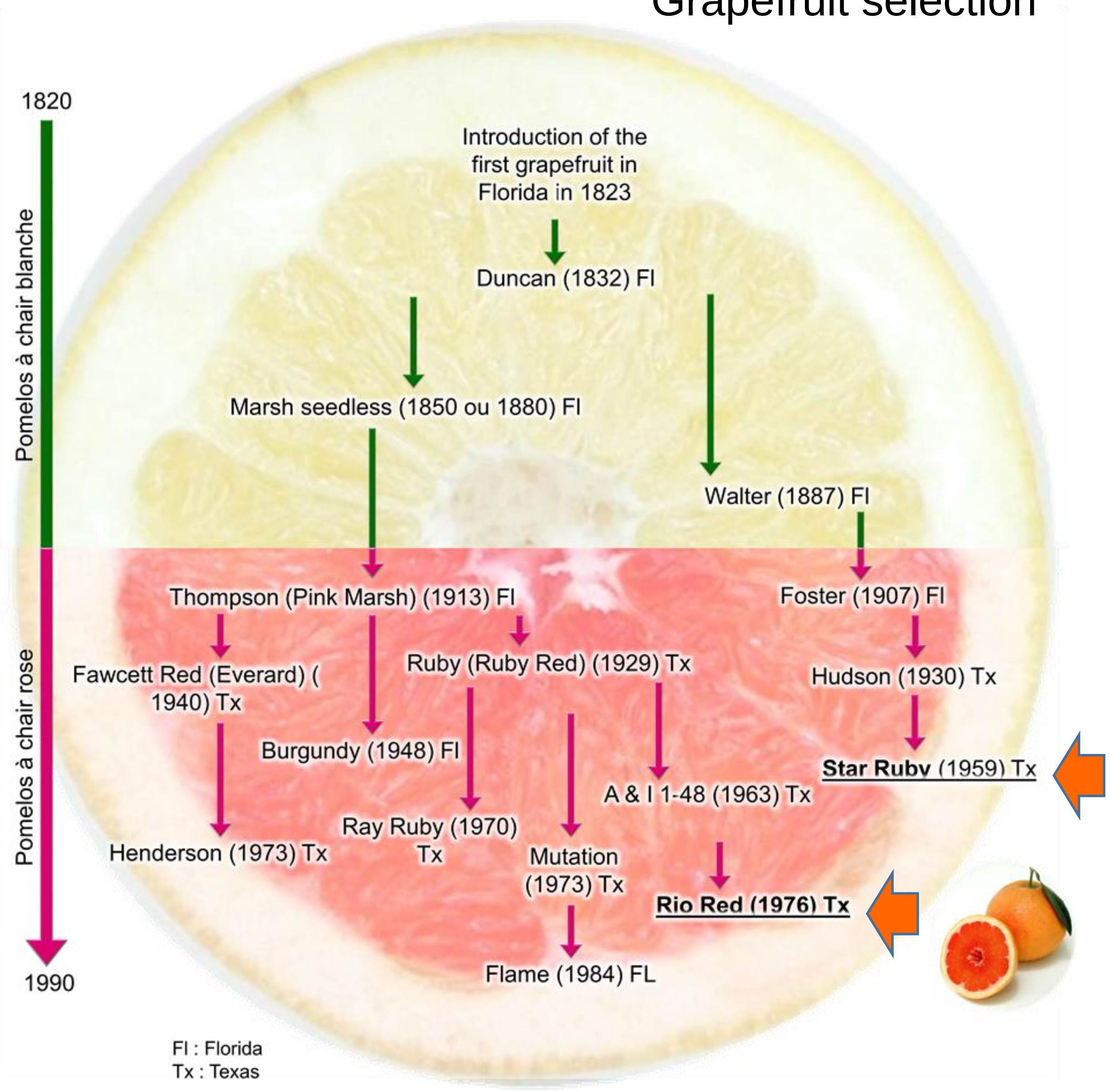
Mutagenesis

Mainly by gamma irradiation, very efficient for **sterility** / without modification on other traits

Grapefruits combined spontaneous and induced mutants: **Star Ruby** (irradiated bud woods to produce sterility), **Rio Red** (irradiated seeds to intensify the red flesh colour)

Used as the final step of selection process to produce sterility of improved hybrids
Mandarins: Afourer => **Tangold** , Murcott => **Mor**

Grapefruit selection



Citrus breeding: seedlessness

Seedless = major trait for citrus fresh fruit market

Breeding associates **parthenocarpy** and mechanisms that prevent ovule fertilization

Self-incompatibility was a very efficient way to select seedless cultivars :

Clementines and mandarins as ‘Fortune’, ‘Ellendale’, ‘Nadorcott’, ‘Nova’

Present in pummelos (*C. maxima*) : allogamic reproduction

Seedless fruit production is obtained if the trees are grown in monovarietal orchards to avoid cross fertilization

The possibilities of using hybridization for citrus improvement

Available for **creation of new varieties** in mandarins

The breeding strategies are generally based in one reproductive cycle

Sterility associated with **parthenocarpy** is the best way to obtain seedless fruits and to avoid cross-pollination in self-incompatible varieties such as clementine

The **selection of triploid hybrids** is the most widespread used strategy in the world to obtain new sterile seedless varieties (dysfunction of meiosis => abnormal gametes)

Natural citrus polyploids

- ✓ 3x hybrids = fertilization between unreduced and normal haploid gametes
- ✓ The frequency of 3x hybrids in 2x X 2x progenies varies to 0 at 25%
- ✓ 4x = doubled diploid from chromosome doubling in somatic embryos available for 3x production by 2x X 4x fertilization

Tahiti (Key) lime
C. latifolia

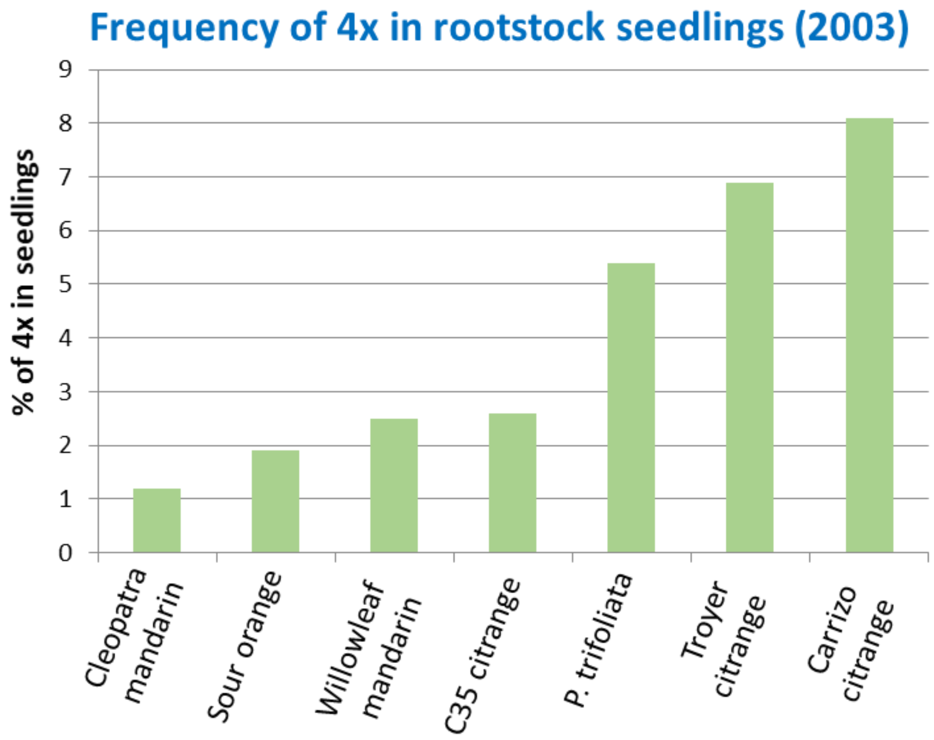
The frequency of polyploids in citrus fruits

Triploid hybrid frequency depends on variety and environment

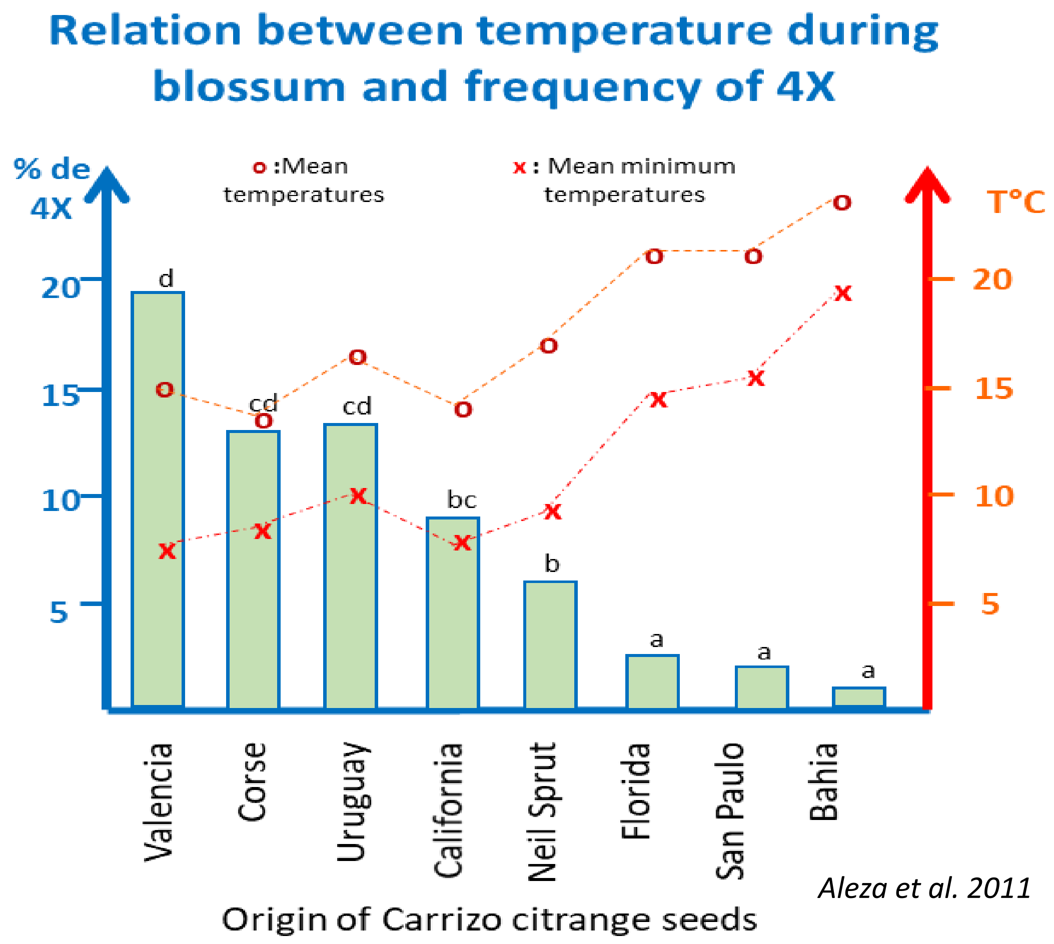
Clementine	1%	Wilking mandarin	14,6%
Citron	4%	Sweet Orange	8 to 33%
King mandarin	7%	Ortanique tangor	25%

Cold temperatures affect the meiosis process

The frequency of **doubled diploids** in seedlings depends on the variety… and the environment



Medecin 2003



Aleza et al. 2011

The INRAE-Cirad breeding program: exploitation of polyploidy

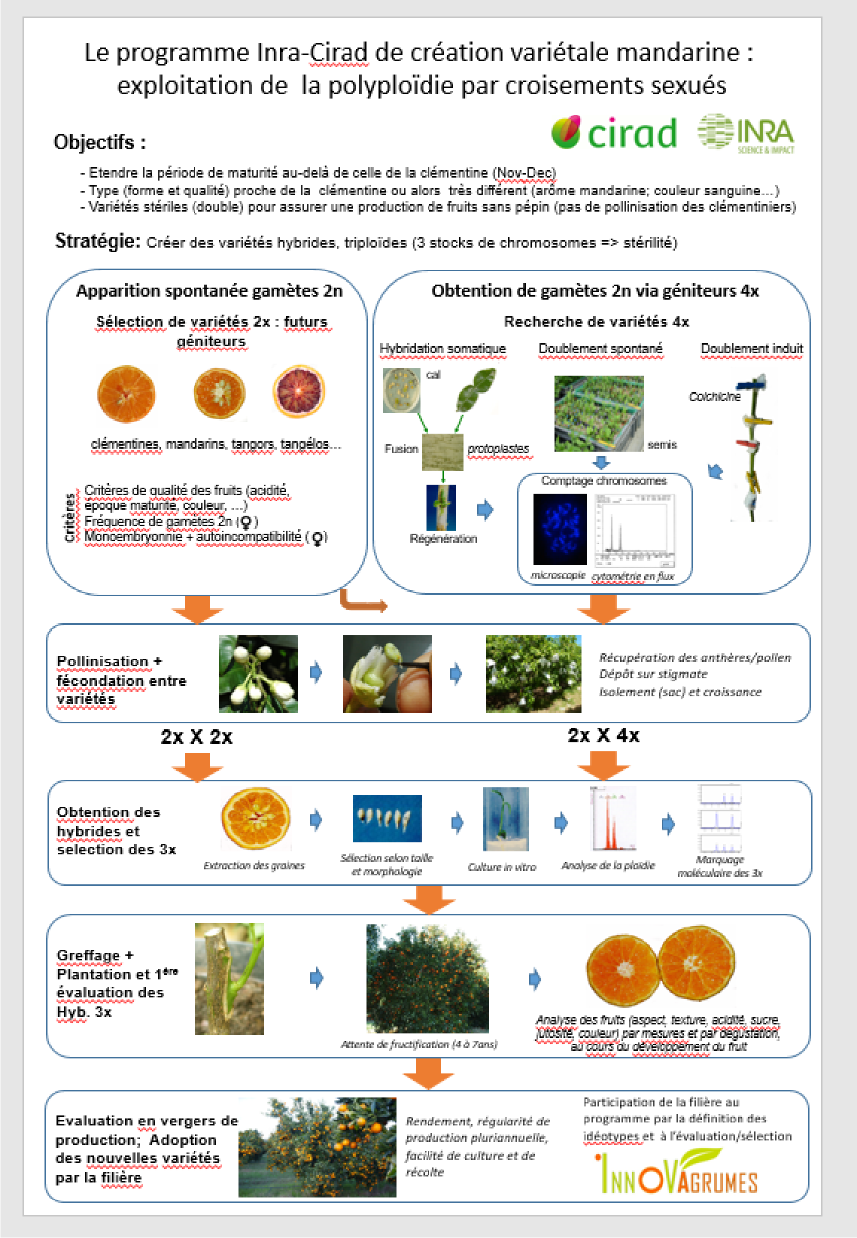
Mandarins : new cultivars

Lime (*C. latifolia*): reproduction of an elite genotype

Rootstocks : adaptation to environment constraints

Seapag

The INRAE-Cirad mandarin breeding program



Objectives

- Extend the ripening period beyond that of the clementine (after December)
- PhenoType (shape and quality) close to the clementine or very different (mandarin aroma; blood color...)
- Sterility of cultivars (double M&F) to ensure seedless fruit production (no pollination of clementine trees)

Strategy

Create 3x hybrid varieties (3 stocks of chromosomes => sterility)

Selection of triploid hybrids in 2x X 2x and 2x X 4x

Utilization and mobilization of the mandarin biodiversity

Selection of 2x genitors

Clementine

Mandarins

Tangors, tangelos

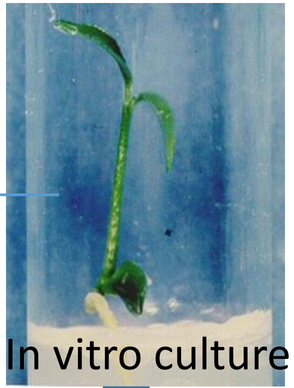
Selected Criteria

Fertilized parent :

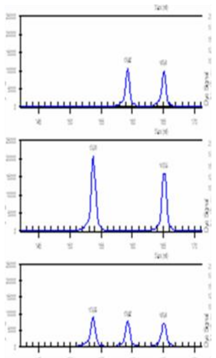
- ✓ Parthenocarpic + self-incompatible + monoembryonic
- ✓ 2n gamete frequency

Pollinators:

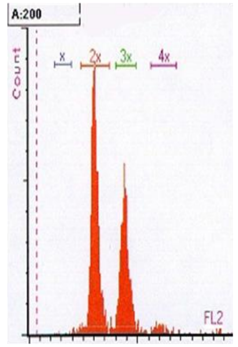
Quality traits (acidity, color, aroma, maturity period…)



In vitro culture



SSR genotyping



Flow cytometry

breeding program =
15 – 20 years

Safor : Spanish triploid mandarin is exploited commercially (*Aleza et al., 2010*)

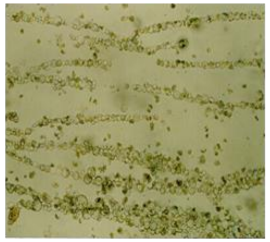
Selection of triploid hybrids in 2x X 2x and 2x X 4x

How to select 4x varieties?

Somatic hybridization

Embryogenic callus

Protoplasts fusion



Embryogenesis

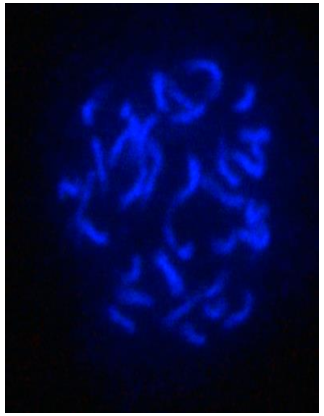
Graft rescue

Spontaneous 4x (doubled diploids)

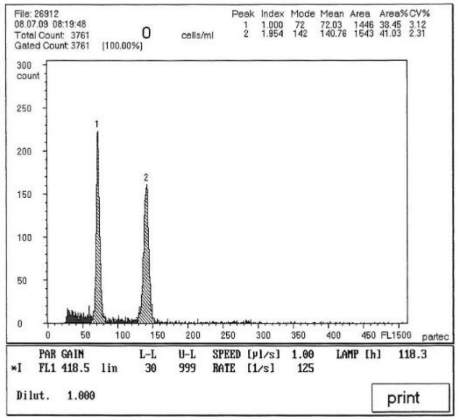
Seedlings of polyembryonic varieties

Chromosome doubling induction

Ploidy evaluation



chromosome counting



Flow cytometric evaluation

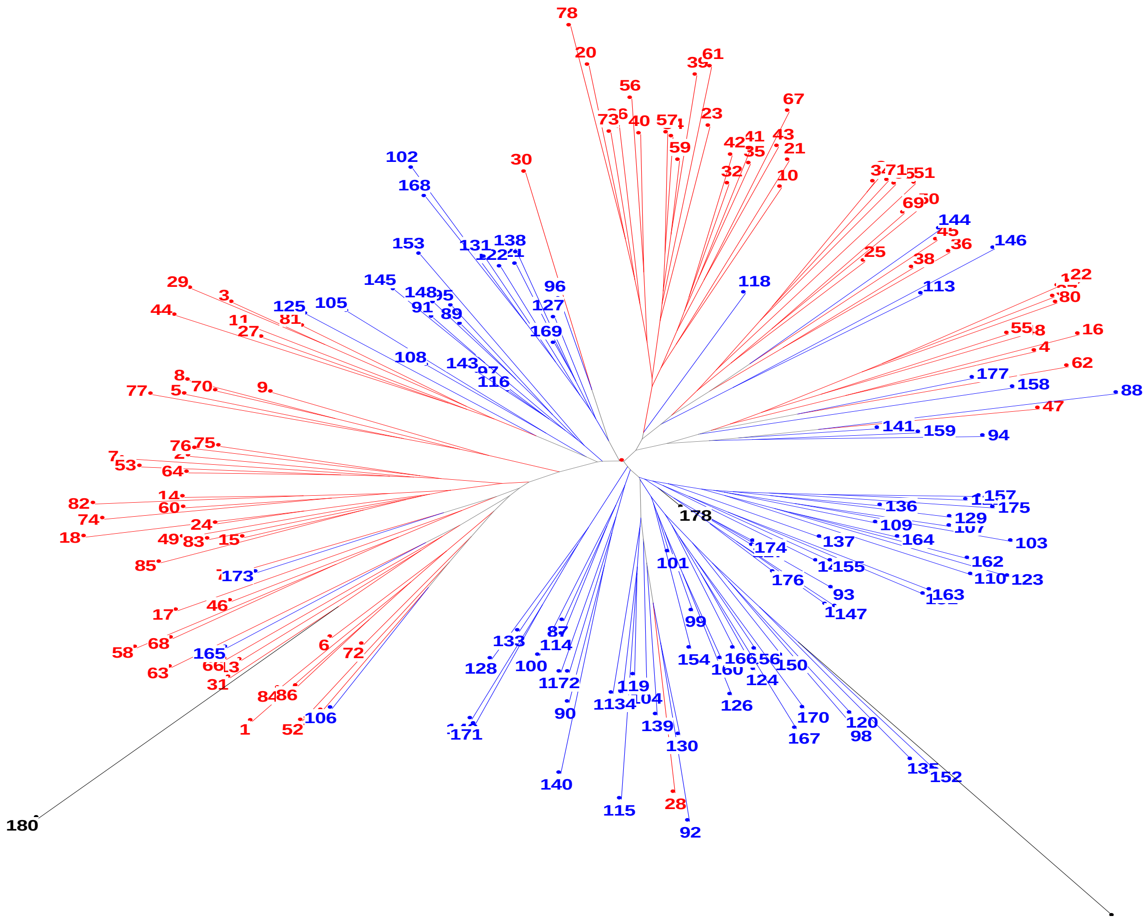
Recombination and segregation in 3x progenies

SDR is the main mechanism of 2n megagametophyte formation in mandarins
Luro et al. 2004; Cuenca et al. 2011

In somatic tetraploid, the meiosis is intermediate beetween disomic and tetrasomic depending of the genetic divergence of the two genomes
Kamiri et al. 2012

Complementarity of **SDR 2n gametes** and **2n gametes of tetraploid** clementines on genetic diversity (evaluated with SSR and SNP markers)

Aleza et al. 2012



Better prediction of traits inheritance in popyploid progenies

The INRAE-Cirad lime breeding program

How phylogenetic origin and genomic structures of modern cultivars can help the reconstruction of the complex genetic structure ?

Tahiti lime
C. latifolia

The case of Tahiti lime (*C. latifolia*)

Context:

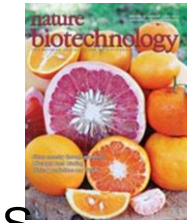
- ✓ Limes (*C. latifolia*) not well adapted to the Mediterranean climate (early de-greening),
- ✓ Fruit skin contains toxic phenolic compounds (furocoumarins),
- ✓ Sensitive to Huanglongbing (very severe bacterial disease),
- ✓ Flowering is mostly grouped on one period while the needs are all of the year.

Objectives :

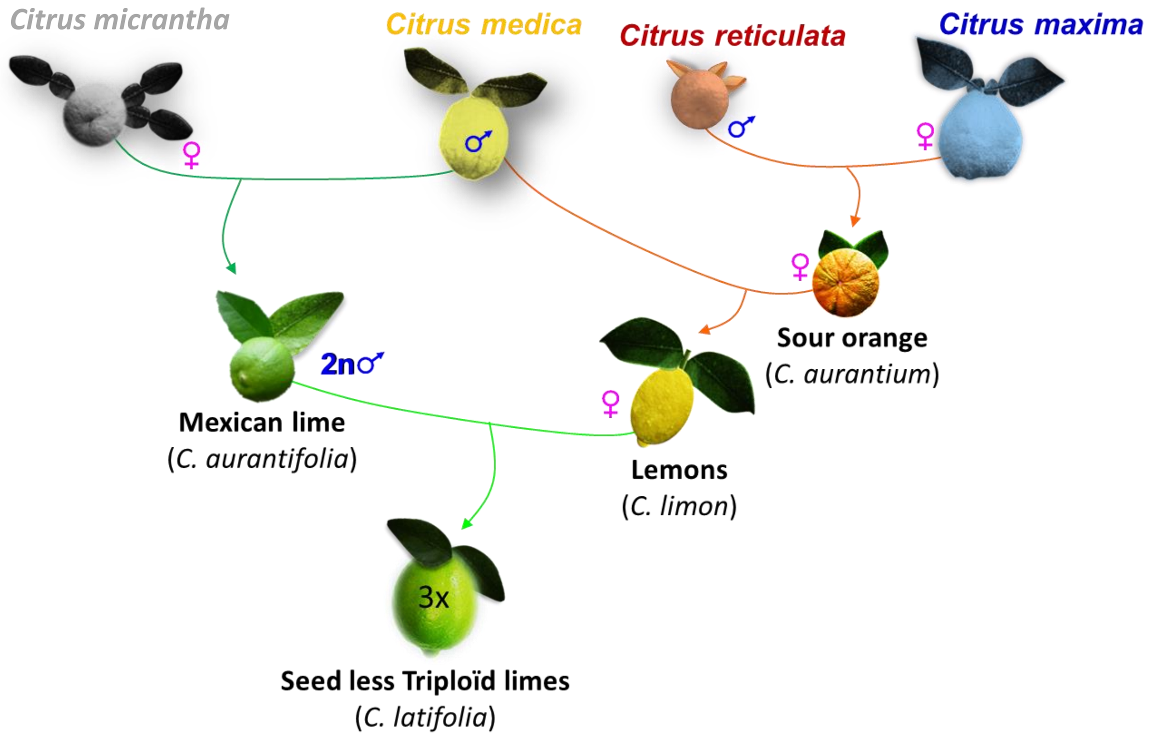
- ➢ Discover the genetic origin (parents & meiosis mechanisms),
- ➢ Reproduce the original parental cross,
- ➢ Select by DSNP the hybrid genotypes close to the Tahitian lime,
- ➢ Evaluate the phenotype

How genomic investigations help the reconstruction of a complex genotype

Tahiti lime
C. latifolia



C. latifolia phylogeny deciphered by diagnostic SNPs

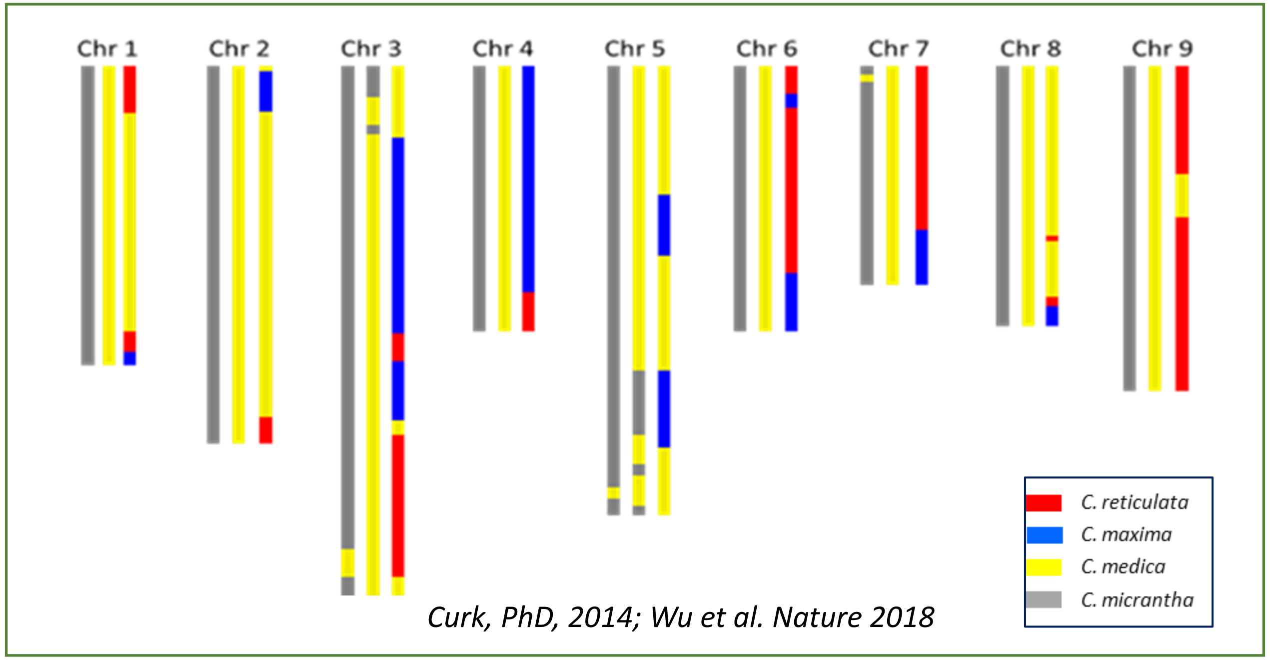


C. latifolia (Tahiti lime) genome is an admixture of 4 ancestral species and is derived from fertilization of a lemon ovule (1n) by unreduced pollen (2n) of *C. aurantifolia* (Key lime)

Curk et al., PlosOne, 2015; Annals of Botany 2016

Deciphering interspecific mosaic structures from WGS

Structure of C. latifolia (3x) genome

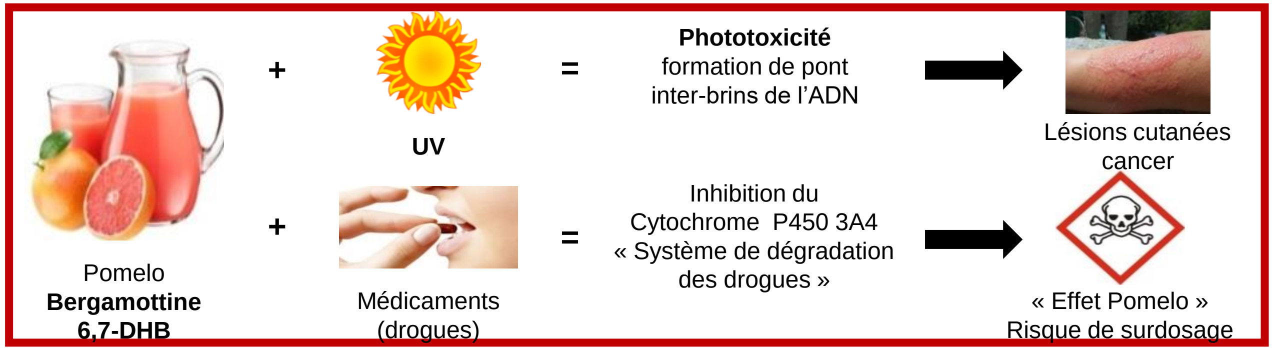


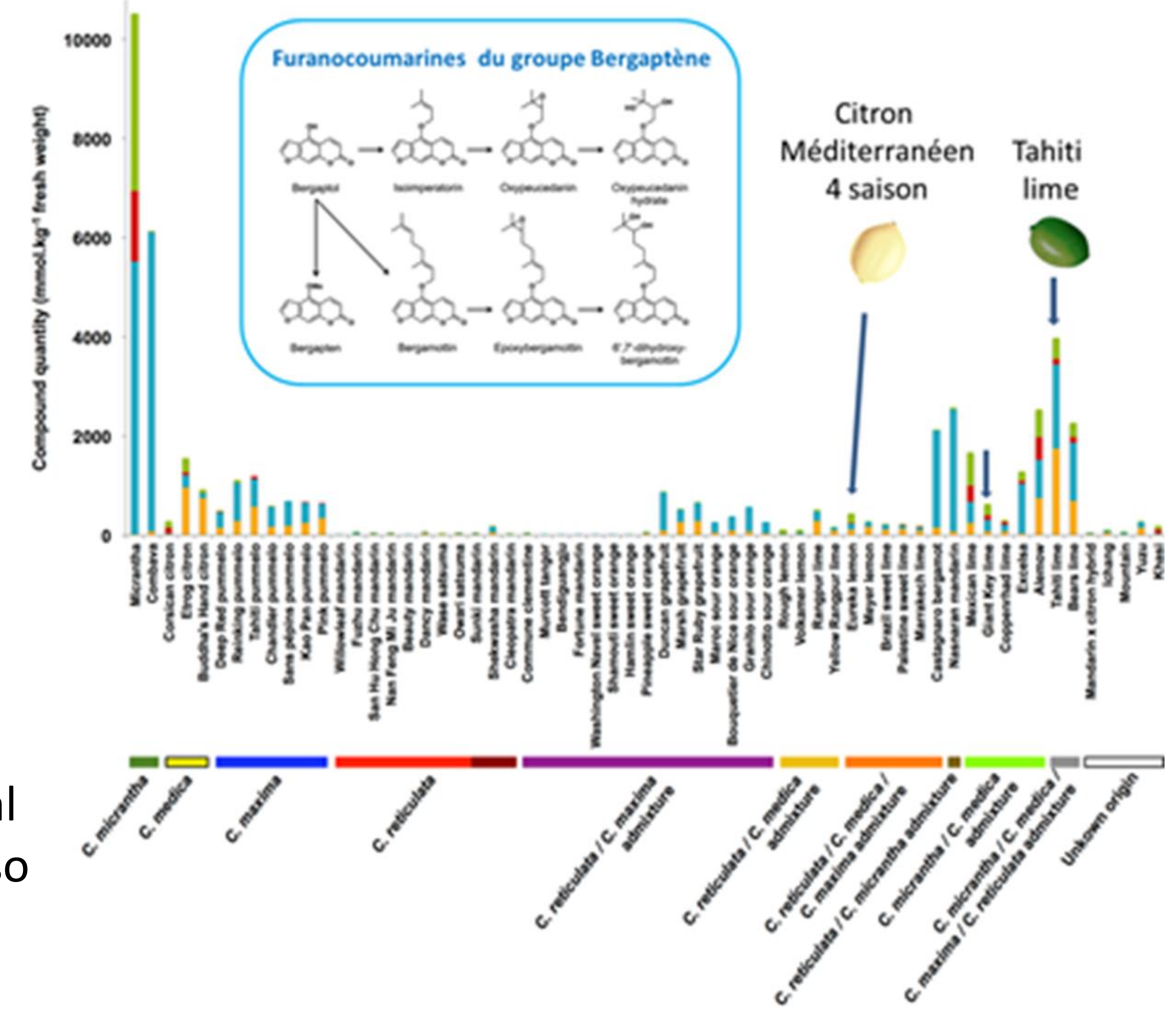
Giant Key lime (*C. aurantifolia*, 4X) meiosis is predominantly disomic; this inheritance is consistent with the *C. limon* (2x) X *C. aurantifolia* (4x) cross that produced *C. latifolia* (3x)

Riouss et al., Annals Bot. 2018

Some inappropriate traits of lime *C. latifolia*

Furanocoumarins (Flavanoids) are toxic





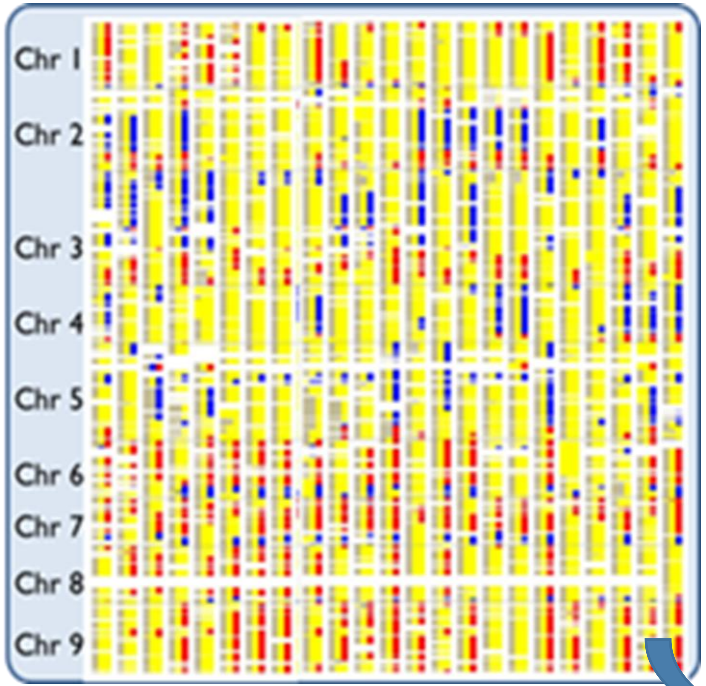
C. latifolia, like its ancestor *C. micrantha*, has very high levels of furanocoumarins; the lemon tree, its maternal parent, is almost devoid of them and Giant Key (4x) also

Dugrand-Judek et al. PlosOne 2015

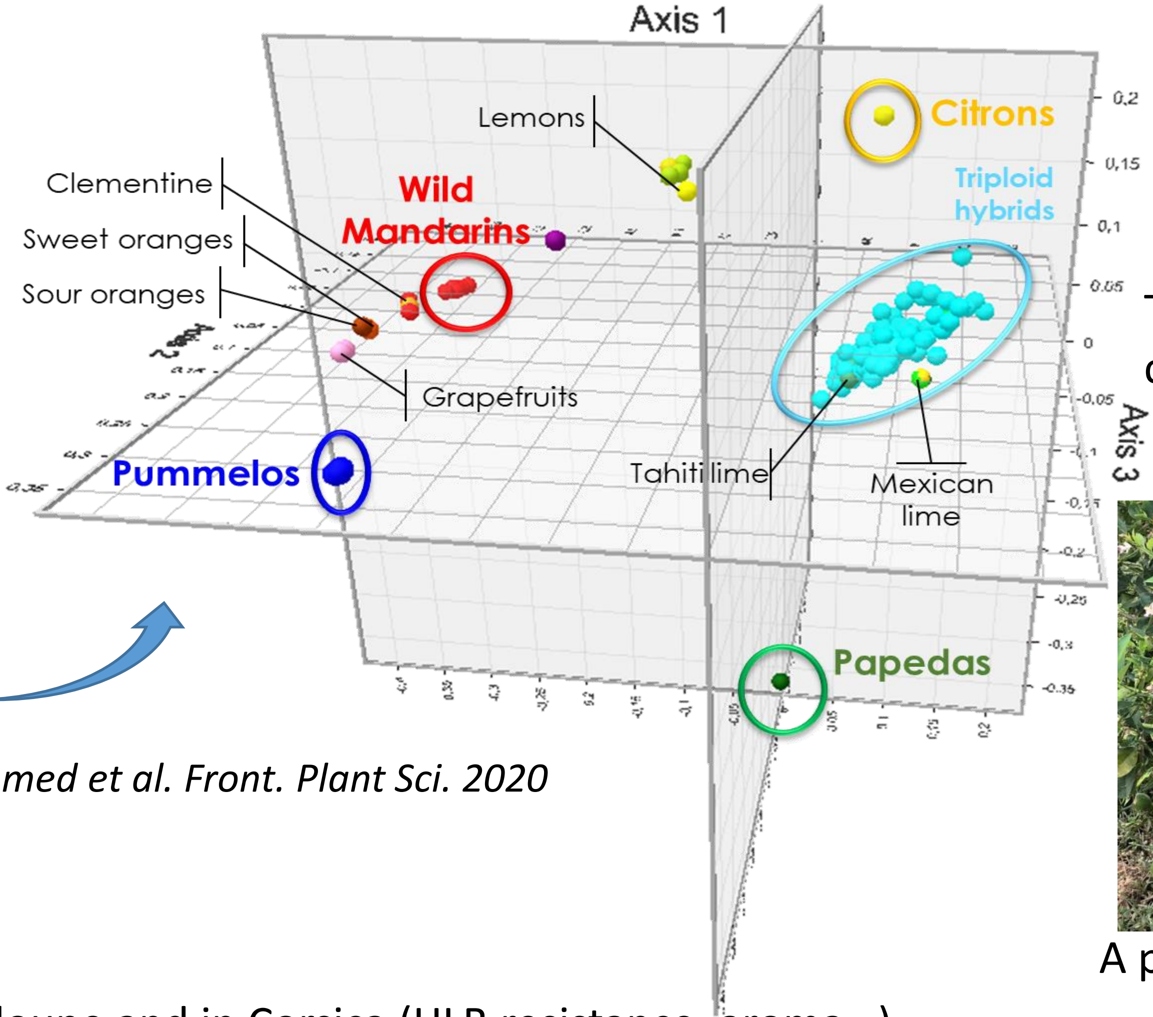
Reproduction of the cross at the origin of the Tahiti lime

C. limon (2x) x *C. aurantifolia* 4X (Giant Key) => 500 hybrids (3x)

Analyze of hybrid genomes by DSNP (GBS)



Diversity of hybrid population in relation with parental genotypes and ancestral species (DSNP)



Triploid hybrids are close to *C. latifolia*

Ahmed et al. Front. Plant Sci. 2020

Evaluation of hybrids in progress in Guadeloupe and in Corsica (HLB resistance, aroma…)

A promising 3x hybrid with permanent blooming

Rootstock ploidy manipulation for a better adaptation; new breeding strategies

Adapting to climate change through rootstock selection

Constraints related to climate change

Abiotic stress:

- ★ Water deficit / drought
- ★ Salinity
- ★ Temperature (low)
- ★ Ferric chlorosis (Alkalinity)

Biotic stress:

- ★ Root rot Gommosis (*Phytophthora)*
- Brown spot (*Alternaria alternata*)
- ★ Mal secco *(Deuterophoma tracheiphila)*
- ★ Tristeza (*CTV)*
- Variegated Chlorosis (*Xylella fastidiosa*)
- Citrus canker (*Xanthmonas campestris*)
- ★ HLB (*Canditatus liberobacter*)

Fruit quality traits:

- ★ Acidity / early collapse
- ★ Peel coloration
- ★ Fruit size
- ★ Juiceness

★ Solution par sélection P-G
C Non présent en Méditerr.
C Non présent en Corse

Most constraints can be managed or modulated via the choice of rootstock

Adapting to climate change through rootstock selection

There are sources of tolerance to the various stresses in the different gene pools, which are useful for selecting tolerant rootstocks.

	Alk.	Chl.	Bore	Dr.	Trist.	Nem.	Phyto.
Citrus relatives							
Eremocitrus glauca	T	TT	TT	TT	T	S	T
Severinia buxifolia	pT	TT	TT	pT	T	T	T
Cleopatra mandarin	T	T	pT	pT	T	S	S
Sour orange	T	T	pT	T	S	S	T
Sweet Orange	pT	S	pT	T	T	S	S
C. junos	S	pT	?	T	T	S	T
C. taiwanica	T	T	pT	pT	pT	S	T
Lemon group							
Rangpur lime	T	TT	T	TT	T	S	S
Rough lemon	pT	S	pT	T	T	S	pT
C. macrophylla	T	pT	pT	T	S	S	T
C. volkameriana	T	pT	pT	T	T	S	T
P. trifoliata	S	S	S	pT	TT	TT	TT

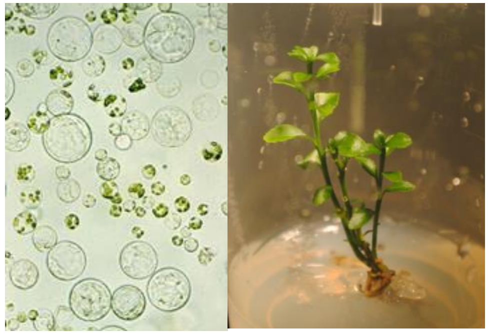
Rootstock ploidy manipulation for a better adaptation; physiological insights

Doubled diploid AB → AABB

Obtention: spontaneous chromosome stock doubling in nucellar cells (Aleza et al., 2011)
Most traditional citrus rootstock are actually available as doubled diploid
Advantages:

- global conservation of tolerance to biotic stress,
- enhancement of tolerance to some abiotic stresses,
- frequent vigor reduction in doubled diploid

Somatic hybrids AB + CD → ABCD



Obtention: protoplast fusion (Grosser et al., 210; Dambier et al., 2011)
Advantages:

- addition of dominant traits of complementary parents (Poncirus/Citrus)
- frequent vigor reduction

Tetrazyg AABB x CCDD; ABCD x EFGH

Obtention: sexual hybridization at tetraploid level (Grosser et al. 2010)
Advantages:

- very large possibility of allelic combinations
- teraploid behavior facing abiotic stresses

Limits: totally new genotypes and numerous selection objectives
Needs: efficient early selection ; marker trait association

Sweet orange grafted on FLHORAG1 somatic hybrid (CIRAD)
Calcareous soils Morocco

Tolerance to water deficit: better tolerance and resilience of 4x rootstocks

Allario et al., 2013

Valencia delta orange grafted on tetraploid (DD) Rangpur lime

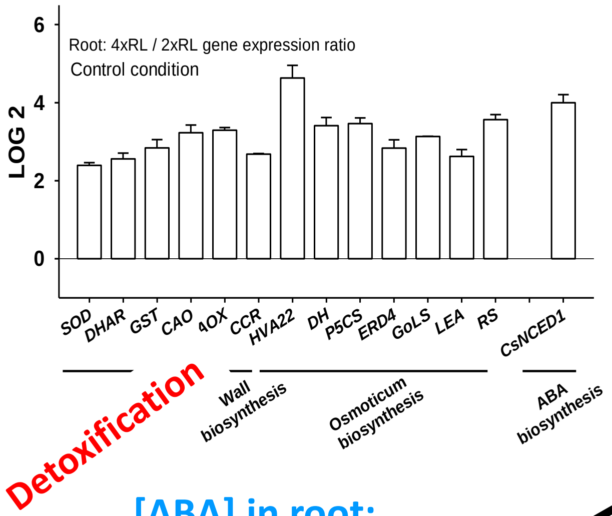
11 days without watering

Rangpur L. 2x/V

Rangpur L. 4x/V

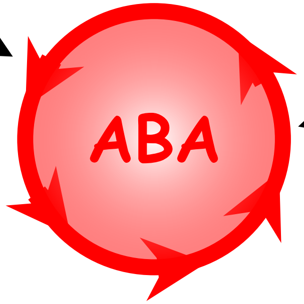
Rootstock ploidy manipulation for a better adaptation; physiological insights

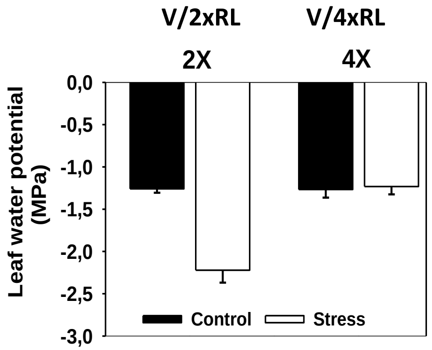
Candidate gene for adaptation expression at the root level: 4x > 2x



Plant growth: 2x > 4x

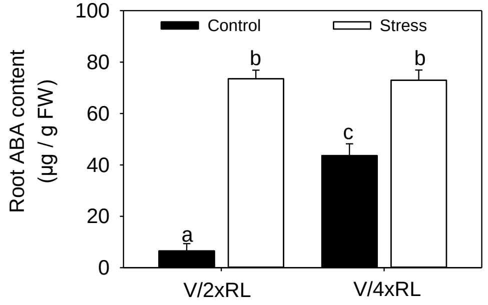
Tree water consummation: V /2xRL > V/4xRL

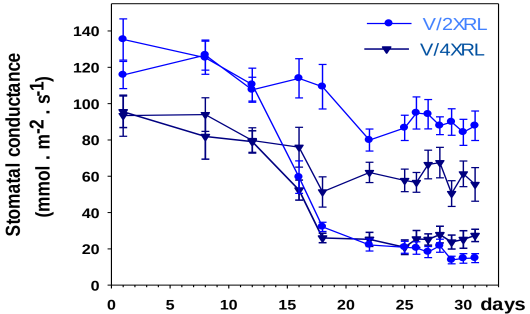




Leaf water potential in stress: V/2xRL < V/ 4xRL

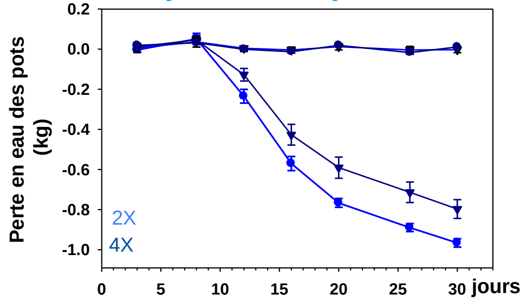
[ABA] in root: 2x < 4x





Leaf transpiration in control condition: V/2xRL > V/4xRL

Pot water loss along water deficit: V/2xRL > V/4xRL



Allario et al., Plant Cell Environ. (2013)
CIRAD; IVIA; Embrapa; USC

Huanglongbing: does exist varietal solution for HLB ?

- No strict resistance to *Liberobacter asiaticus* in the genetic resources
- However, the impact is different depending of the variety

- Comportment that is different in infected orchards: grapefruit (Jackson et Triumph)
- Minor sensitivity in some small citrus Temple, KanSan mandarins)
- Better comportment of 3x (Tahiti) compared to 2x (Mexican lime, etc)
- Appetence of psyllids for the variety is importance regarding the sensibility

Orange trees infected using sensitive and « tolerant » rootstocks

- Some 4x rootstocks present a reduced sensitivity (or a partial tolerance?)

Huanglongbing: what do we know ?

- **High concentration of starch and sugars in leaves**
- **Callosis synthesis in the cells of phloem**
- Low presence of assimilates at root level
- Deficiency in leaf lead to accumulation of starch and sugars

Symptoms seem to be associated to physiological and metabolomic perturbations:

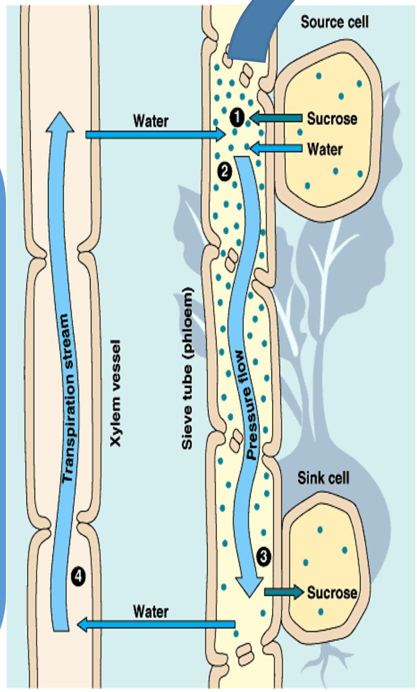
- Leaf symptoms are similar to leaf removing => limitation of the transport of phloem

- **Limitation of the conversion of starch to sugars**

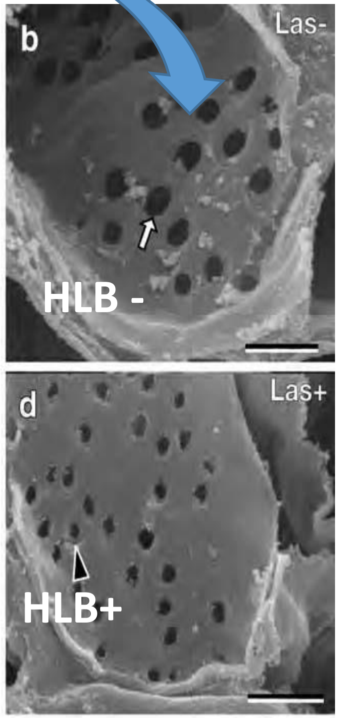
⇒ perturbation of the photosynthesis

- **Callosis in the cells of the phloem: obstruction of connections between cells**

⇒ Limitation of the symplast transport



Xylem Phloem



Conclusion

Citrus diversity is the result of a speciation phase during the allopatric evolution of ancestral populations and subsequent interspecific crosses.

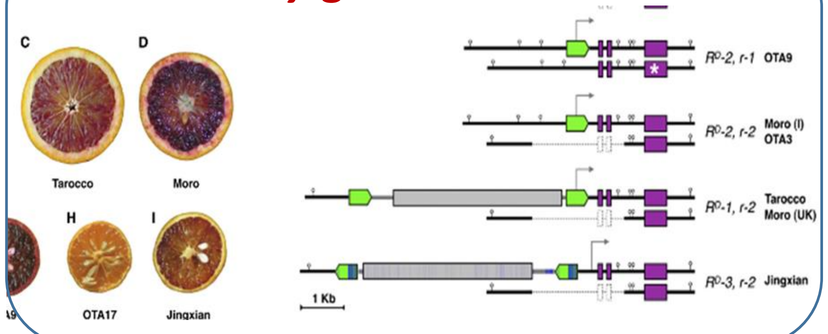
Citrus reproduction is complex and diverse depending on the species, and apomixis is responsible for the clonal multiplication of interspecific hybrids elevated to the rank of secondary species.

Breeding strategies are adapted to biological constraints: mutagenesis for secondary species, hybridization for primary species mixing triploidy (sterility) and tetraploidy (increased tolerance to biotic and abiotic stress)...

Genome investigations (DSNP, GBS and NGS) are useful for reproduction of a complex interspecific hybrids and for MAS

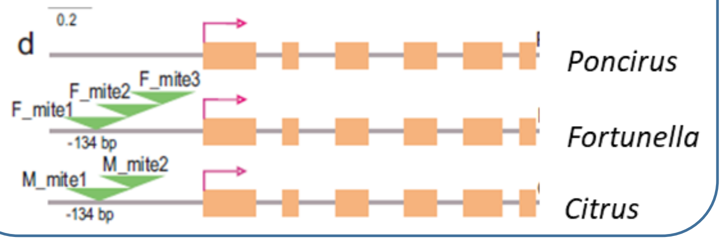
Toward marker assisted selection (MAS) or genome editing

Ruby gene



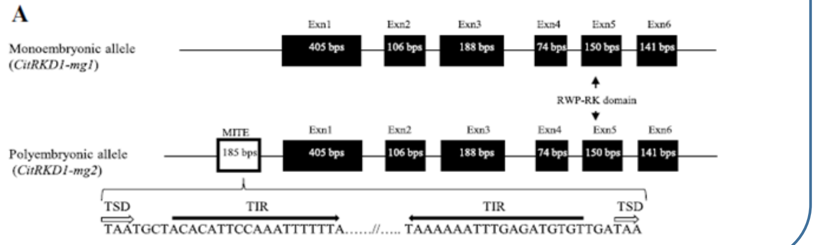
pulp acidity

(Wang et al. 2022)



Apomixis (Polyembryony)

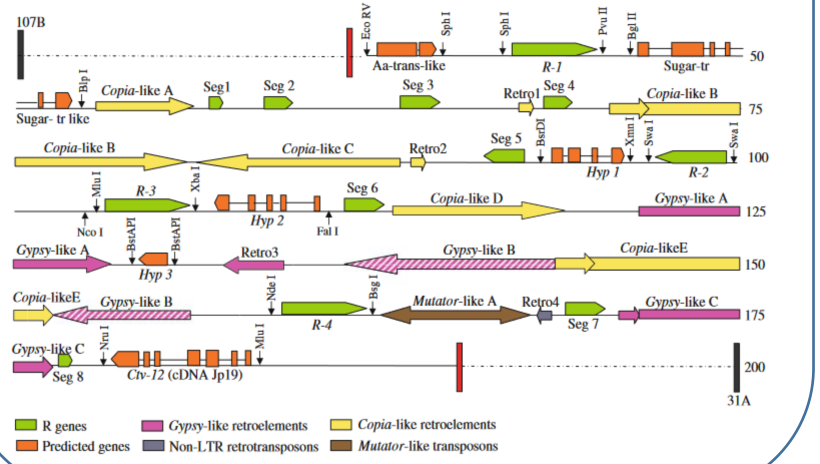
(Shimada et al. 2018)



CTV resitance gene

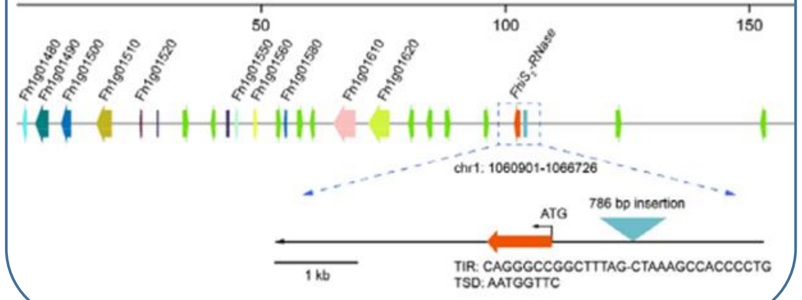
Plant Molecular Biology (2006) 61:399–414
DOI 10.1007/s11103-006-0018-7

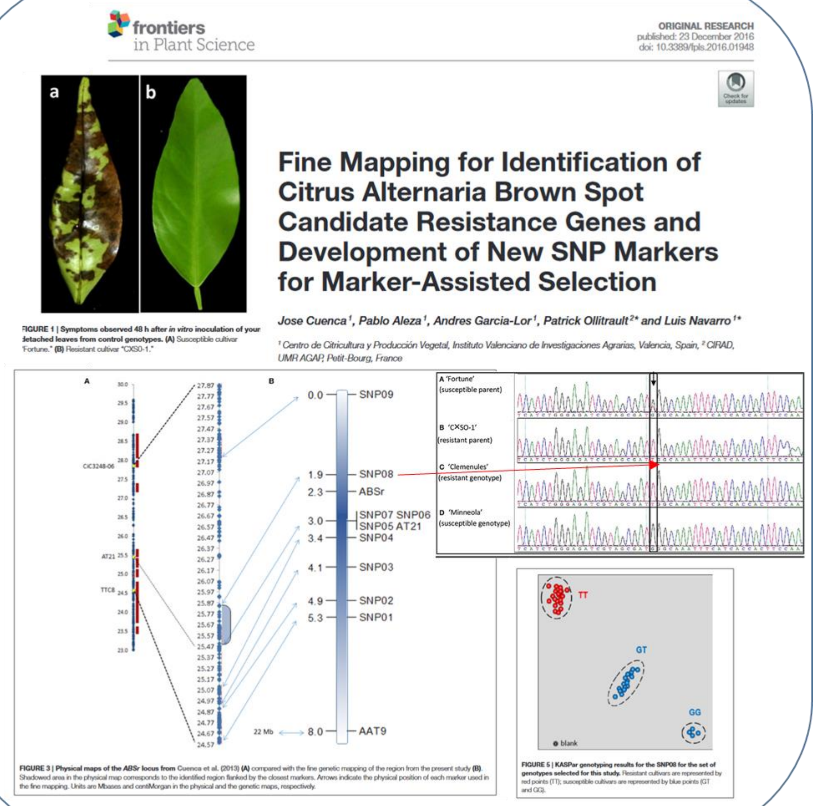
Refinement of the Citrus tristeza virus resistance gene (*Ctv*) positional map in *Poncirus trifoliata* and generation of transgenic grapefruit (*Citrus paradisi*) plant lines with candidate resistance genes in this region



self-compatibility

(Hu et al. 2023)





SNP and InDel markers can be used for selection of different traits in breeding programs

Thank You...

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