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**New Insights on Lime and Lemon Origins from Targeted Nuclear Gene Sequencing, SSRs, InDels and Cytoplasmic Markers Genotyping**

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**Introduction**

It is believed that four basic taxa, *citron* (*C. medica*), *pummelo* (*C. maxima*), *mandarin* (*C. reticulata*) and *C. microtha* have generated all cultivated Citrus species. Depending on the botanical classifications, lemons and limes are classified either into two species, *C. limon* and *C. aurantifolia* (Swingle and Reece) or into more than 30 (Tanaka). In order to clarify the phylogeny of this Citrus group, we used nuclear SNPs from 15 targeted DNA fragments mined by longer sequencing, 10 nuclear Indels (Garcia-Lor et al., 2012b, 23 nuclear SSRs (Froelicher et al. 2008, Luro et al., 2008), 3 mitochondrial Indels (Froelicher et al., 2011) and 5 chloroplast SSR markers (Lotfy et al., 2003) for 15 lemons and limes compared with 19 representatives of the basic taxa and 8 secondary species.

**Maternal phylogeny**

*Fig. 1: Neighbor Joining Tree from Mitochondrial Marker data*

*Fig. 2: Neighbor Joining Tree from Chloroplast Marker data*

Four groups of lemons and limes were differentiated (Fig 2): Group 1 *Palestinian sweet lime*, *C. sinensis* var. *limonoides* Tan., *Meyer lemon* (*C. meyerii* Tan.), and *Ponderosa* (*C. pynum* H. Z.), and Group 2 *Limonette de Marrakech* (*C. irretita* Russo), *Kama lime* (*C. aurantifolia* Russo), *Lobron* (*C. aurantifolia* Russo), and *Citronberg* (*C. aurantifolia* Russo) have a pummelo type cytoplasm (Fig 1). Group 2 share its cytoplasm with *Sour Orange* (*C. aurantum* L.) while Group 1 share it with Sweet Orange and *Grapefruit* (Fig 2). Group 3 *Rough lemon* (*C. limonii* L.), *Vollamer lemon* *C. aurantium* (Obl.), and *Rangpur lime* (*C. limon* var. *C. microtha* (Christm. Swing). *Alcover* (*C. aurantifolia* Russo) and *Kaki lime* (*C. webberi* West.) has a Citrus microtha type cytoplasm. None of the 4 groups shows any citrus, *Microcitrus*, *Zizyphor*, *Fortunella* or *Poncirus* matricul contributions.

**Nuclear Phylogeny**

*Fig. 3: Population Structure Representation (10 Indels and 23 SSRs)*

All analyzed lemons and limes are related to *citron* (Fig 3). Sour oranges appear as a parental group of some lemons and limes like *Karma lime*, *Limonette de Marrakech*, *Lobron*, *Eureka* and *Sweet limes*, and *Citronberg*. An another group constituted by *Palestinian sweet lime*, *Rough lemon*, *Vollamer lemon*, *Rangpur lime* and *Meyer lime* seem to results mainly from *citron* and mandarin contributions. *Ponderosa* seems to be directly related to *pummelo* and citron, *Mexican lime*, *Alemow* and *Kaki lime* share equilibrated contributions of *Citrus microtha* and citron. They are very clearly differentiated from the others limes and lemons in the Cluster analysis (Fig 6). The sub-clustering of the others limes and lemons (Fig 6) is globally in agreement with the structure analysis.

**Multilocus SNPs Analysis**

*Fig. 4: 2D PCA representation for genotype assignment of the 456 pb sequenced fragments of the SO3S gene*

*Fig. 5: Phylogenetic nuclear genotypic structure assumed from multilocus analysis of 15 gene fragments*

575 SNPs were identified in 1608 pb of the 15 targeted sequenced fragments. For each gene a Principal Component Analysis (PCA) was performed from SNP data to analyze the phylogenetic genotypic structure (e.g. gene SO3S, Figure 4). It was suggested that intermediary position between two basic taxa corresponded to interspecific heterozygosis; this hypothesis was confirmed by checking the heterozygosity for phylogenetic diagnostic SNPs. For most of the amplified fragment, the polymorphism was enough for unambiguous multiple discrimination of the species and assignment of a phylogenetic origin for the analyzed limes and lemons (Fig 5). This analysis display the preponderance of *citron* in complete heterozigous status in many limes and lemons confirming that *citron* was a direct parent of the other species. The parents appear to be (*i*) interspecific hybrids *M x P* for *Karma*, *Marrakech*, *Lobron*, *Eureka*, *Sweet*, *Meyer*, and *Palestinian* (*ii*) mandarin parents for *Rough lemon*, *Vollamer lemon*, and *Rangpur lime* (*iii*) *C. microtha* for *Kaki* and *Mexican lime*. *Mandarin/Pummelo* heterozygosity was observed for *C. bergamia*, *homocygosity citrus/citron in Alemow and Ponderosa* (as well as *pummelo/pummelo for Ponderosa*) suggesting more complex origins such as (*Pumel+Cit*) x (*Pumel*) for *C. bergamia*, (*Micro+Cit*) x (*Alemow*) and (*Pumel+Cit*) for *Ponderosa*.

**Conclusion**

Nuclear and cytoplasmic data confirm previous hypothesis (Nicolosi et al., 2000) for some limes and lemons and allow proposing new origin hypothesis for others:

- *Eureka*, *Lobron* and sweet limes as well as *Marrakech lime* built from *sour orange* x *citron* hybridization
- *Citrus bergamia* is probably an hybrid between *sour orange* and *yellow lemon* (*Eureka type*) and *Ponderosa* a cross between *pummelo* x *citron* hybrids,
- *Vollamer lemon*, *Rangpur lime* and *Rough lemon* should be direct hybrids between a mandarin from the acid group and *citron*, *Mexican and Kaki lime* should result from *C. microtha* x *citron* hybridizations,
- *Alemow* should be a (*C. microtha* x *citron*) x *citron* backcross,
- The origin of *Karma* and *Palestinian sweet lime* and *Meyer lemon* appears more complex,
- Future multilocus SNPs analysis of a larger number of sites for phylogenetic assignment of DNA fragments will allow to decipher the interspecific mosaic genome structure of this particular Citrus group and will give more answers about his origins.

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