ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2011) **9**(2); 218–221
doi:10.1017/S1479262111000189

Analysis of genetic diversity in *Citrus*

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Abstract
Sugar and acidity levels are the main criteria of general fruit quality and for citrus juices pulp, in particular. The constituents of the acidity (organic acids) and the sweetness (glucose, fructose and sucrose) and the genes involved in their regulation have seldom been used to explore *Citrus* genetic diversity. We evaluated the juice composition of primary metabolic components for 87 varieties belonging to the eight major *Citrus* species grown under the same environmental and cultivation conditions by HPLC. We investigated the sequence polymorphism of nine candidate genes encoding for key enzymes of sugars and organic acids metabolic pathways by single strand conformation polymorphism (SSCP). Whatever the biochemical or molecular analyses, the observed structure of *Citrus* diversity was organized around three groups corresponding to the ancestral species (mandarin, pummelo and citron). As expected, the secondary species were closely related to their putative ancestors except for *Citrus aurantium*. Biochemical diversity was strongly correlated to molecular SSCP diversity at the genus level but not at the intraspecific level. Compared with other molecular marker types, higher diversity has been observed with SSCP technology, which makes it suitable for future quantitative trait loci mapping approach on gene polymorphism in citrus pulp acidity and sweetness regulation.

Keywords: acidity; *Citrus* spp; genetic diversity; single strand conformation polymorphism; sweetness

Introduction

Citron (*C. medica*), mandarin (*C. reticulata*) and pummelo (*C. maxima*) are considered to be modern cultivated types most similar to the ancestors (Barret and Rhodes, 1976; Nicolosi *et al.*, 2000; Luro *et al.*, 2001). Economically important types (orange, grapefruit, lemon and lime) are believed to have originated from one or more generations of hybridization between these ancestral genera. In terms of composition and commercial assessment of fruit maturity, sweetness and acidity are considered as major components of any citrus (Ting and Attaway, 1971; Tucker, 1993). Organic acids and sugars vary according to species, varieties, and also to environmental conditions (e.g. climate, irrigation, etc.) and fruit maturation (Bain, 1958; Sinclair, 1984; Marsh *et al.*, 2003). In mandarin, sweet orange and their hybrids, acidity decreases during fruit maturation and thus determines the most favourable time of harvest, suitable for commercial fruit quality (Sinclair, 1984). In terms of genetic evolution, acidity and sweetness had probably played a determinant role in the natural selection and dissemination of these species during their history. Unfortunately, their impact on natural selection is hard to verify and remains only a hypothesis suggested by the extension of pulp acid character on citrus varieties. Nevertheless, some 'acidless' varieties exist, due to spontaneous mutations, characterized by a very low acidity and a lack in citric acid (Bogin and Wallace, 1966; Canel *et al.*, 1995). More often, they are considered as useful biological models to display the cellular mechanisms and genes expression involved in acidity and sweetness regulation (Albertini *et al.*, 2006; Cercos *et al.*, 2006; Talon and Gmitter, 2008). The general behaviours in terms of primary metabolic contents and evolution for different cultivars are largely described in the literature. Nevertheless, these characteristics have never been investigated for a large panel of species representing

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the *Citrus* genus simultaneously in a standard time and environmental conditions. The objective of this work was to investigate the variation of pulp sweetness and acidity between and within major species for citrus history (ancestral species) and for citrus industry (cultivars) and then to explore its relationship within *Citrus* phylogeny. The polymorphism of gene sequences involved in the primary metabolic pathway was explored by single strand conformation polymorphism (SSCP) approach to find genetic markers related to this biochemical diversity.

Materials and methods

Materials

Fruit and leaves were sampled from 87 citrus varieties growing in "Institut National de la Recherche Agronomique-Centre International de Recherche Agronomique et de Developpement" citrus germplasm (San Giuliano, Corsica, France), in the first week of February. Each taxonomic group was represented by several genotypes to evaluate intra and interspecific diversity: 18 for mandarin (*Citrus reticulata* Blanco), 9 for pummelo (*C. maxima* (Burm) Merr.), 9 for citron (*C. medica* L.), 12 for orange (*C. sinensis* (L.) Osb.), 8 for grapefruit (*C. paradisi* Macf.), 7 for lemon (*C. limon* (L.) Burm.), 11 for limes (*C. aurantifolia* (Christm.) Swing), 9 for sour orange (*C. aurantium* L.), 1 for combava (*C. hystrix* D.C.) and 5 for various putative lemon hybrids. Lemon, orange, sour orange, lime and citron acidless mutants have also been included in the sample design.

HPLC analysis

Each fruit juice was diluted tenfold for sugar analysis and twofold for organic acids analysis and centrifuged at 2250 *g* for 10 min. The supernatant was filtered through 0.45 μm acetate cellulose membrane filter. The separation of organic acids and sugars was achieved by method described in Albertini *et al.* (2006), using an analytical HPLC unit (Series 200; Perkin-Elmer, France).

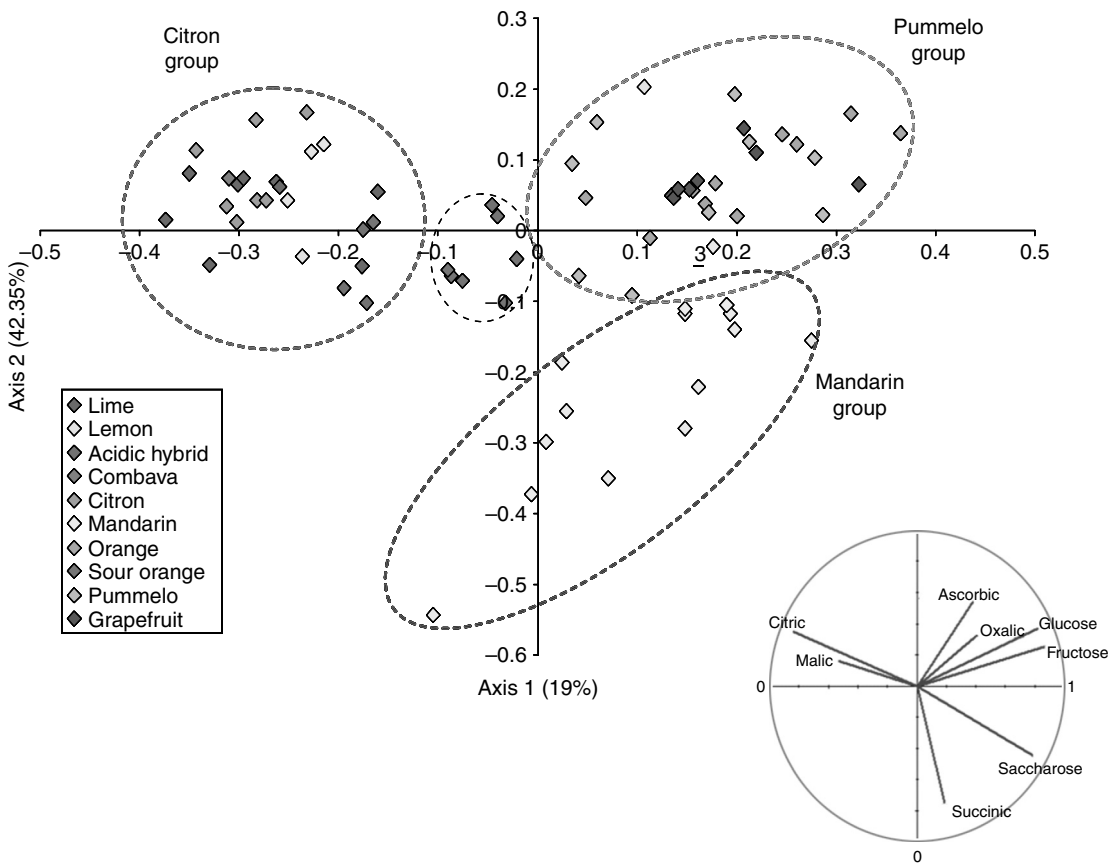

Fig. 1. PCA plot of citrus species based on the pulp concentrations of soluble sugars and organic acids and the contribution of each component to the diversity represented on the two first axes of PCA (at right and lower part of the plot); 'acidless' mutants have been removed from the citrus samples.

https://doi.org/10.1017/S1479262111000189 Published online by Cambridge University Press

SSCP analysis

Primers were designed using Primer3 software for nine genes involved in primary metabolic pathway: vacuolar acid invertase (AB074885), mitochondrial malic enzyme (CB417399), aconitase (AF073507), phosphoenolpyruvate carboxylase (EF058158), isocitrate dehydrogenase (AF176669), vacoluar citric acid transporter (EF028327), phosphofructokinase (AF095520), phosphoenolpyruvate carboxykinase (Csi5808) and malate dehydrogenase (DQ901430). DNA extraction from citrus leaves was carried out according to Doyle and Doyle (1987). PCR reactions (mixture and amplification conditions) were performed in a 'Mastercycler gradient' thermocycler (Eppendorf) according to Luro *et al.* (2008). SSCP analyses of amplified fragments were displayed according to the method of Markoff *et al.* (1997).

Statistical analysis

DARwin5 v. 4.0 (Perrier *et al.*, 2003) was used to examine the molecular genetic diversity. From SSCP data, a dissimilarity matrix was established using Dice's distance (Dice, 1945) and a dendrogram was elaborated by Hierarchical and Ascendant classification method. A principal component analysis (PCA) was performed with R software for HPLC data.

Results and discussion

The general organization of *Citrus* diversity based on the amount of primary metabolic compounds is presented in the PCA (Fig. 1) It is organized around three groups where the main ancestor species are citrons (associating lemons, lemon hybrids, limes and combava), pummelos (associating oranges and grapefruits) and mandarins (without any other species). This representation totally agreed with the *Citrus* diversity and supposed phylogenic relationships established with molecular markers (Nicolosi *et al.*, 2000; Luro *et al.*; 2001; Barkley *et al.*, 2006). Lemon may have originated from a cross between citron and sour orange, orange from a cross between pummelo and mandarin, and grapefruit from a cross between orange and pummelo. These hypotheses were confirmed with our analysis of sugars and organic acids composition of citrus pulp. Sample 3, representing Clementine, is linked to pummelo group in total accord with its mandarin × orange origin. One major deviation from the putative phylogeny was observed for sour orange origin supposed to be a mandarin × pummelo cross, which is not supported by our analysis since sour orange varieties have an intermediate position between pummelo and citron groups. Mandarin is the most diversified group characterized by a high variation of succinic and citric acid amounts. Moreover, sucrose content is a valuable criterion to distinguish mandarins from all other taxi.

SSCP technique of DNA fragments from genes involved in the metabolic pathway of sugars and organic acids allowed the detection of diversity between varieties undetectable at the amplified fragment-size level. Then, these polymorphisms were supposed to be related to single nucleotide variations and not insertions or deletions. The general organization of *Citrus* diversity obtained with the SSCP data is quite similar to that observed with biochemical criteria (Fig. 2). The three major groups are maintained. However, some cultivated species such as sour oranges are associated with mandarins, whereas, phylogenetically, they have been suggested to derive from a pummelo and mandarin crossing. This is also the case for sweet oranges, associated with pummelo group while they are supposed to derive from a mandarin backcross following an initial pummelo × mandarin cross. The second major information resulting from this analysis was the high level of diversity between varieties of each species. This is a common situation for ancestral species that have evolved by intersexual crosses. But molecular diversity between varieties resulting from somatic

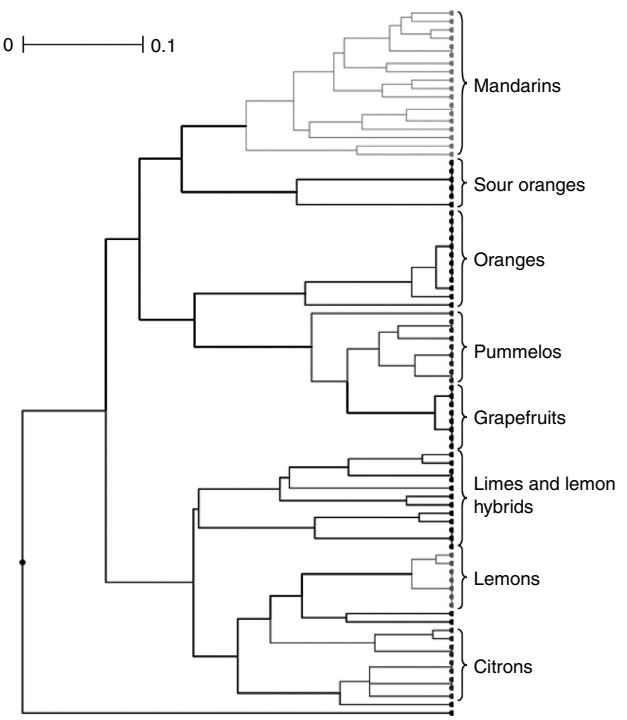

Fig. 2. Hierarchical and Ascendant classification tree showing the relationships of *Citrus* species accession as determined by Darwin using a distance matrix calculated from the proportion of shared alleles scored on SSCP gels.

https://doi.org/10.1017/S1479262111000189 Published online by Cambridge University Press

mutations, such as for oranges, lemons or grapefruits, is very rare or not detected previously by neutral markers (Luro *et al*., 2001; Barkley *et al*., 2006). Four genotypes are detected for oranges and two for both lemons and grapefruits. 'Acidless' mutant profiles were not distinguishable from the other, suggesting independency between molecular polymorphism and biochemical variations.

The overall biochemical diversity of *Citrus* reinforced the idea that *Citrus* diversity is distributed among three ancestral species suggesting a speciation of fruit pulp sweetness and acidity prior to secondary species genesis. SSCP approach revealed a polymorphism apparently neutral against acidity and sweetness regulation but suitable for further genetic studies such as quantitative trait loci mapping and gene expression profiling.

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