

GENETIC SIMILARITY OF CITRUS FRESH FRUIT MARKET CULTIVARS¹

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ABSTRACT - The objective of this work was to assess the genetic similarity of the following citrus fresh fruit market seedless cultivars: Lane Late, Navelate, Navelina and Salustiana sweet oranges (*Citrus sinensis* (L.) Osbeck), Clemenules and Marisol mandarins (*C. reticulata* Blanco) and Okitsu satsuma mandarin (*C. unshiu* Marcovitch), and the hybrids Nova [*C. clementina* x (*C. paradisi* x *C. tangerina*)] and Ortanique (tangor probably derived from *C. sinensis* (L.) Osbeck x *C. reticulata* Blanco), utilizing isoenzymatic markers. Electrophoresis analysis of proteins extracted from leaf tissues was utilized to detect polymorphisms at ten isoenzymatic systems. Out of 30 alleles, 16 were polymorphic. The Jaccard coefficient was utilized to estimate the genetic similarity between the cultivars and the unweighed pair-group method using an arithmetic average (UPGMA) was used to obtain the phenogram (NTSYS 1.7). The cultivars showed high genetic similarity (>72.5%), and were classified in five main groups: sweet oranges, 'Clemenules' and 'Marisol' mandarins, 'Nova', 'Ortanique', and 'Okitsu' satsuma mandarin.

Index terms: biochemical markers, genetic similarity, isozymes.

SIMILARIDADE GENÉTICA DE CULTIVARES DE CITROS DE MESA

RESUMO - O objetivo deste trabalho foi estudar a similaridade genética das seguintes cultivares sem sementes de citros de mesa: laranjas Lane Late, Navelate, Navelina e Salustiana (*Citrus sinensis* (L.) Osbeck), tangerinas Clemenules e Marisol (*C. reticulata* Blanco), satsuma Okitsu (*C. unshiu* Marcovitch) e híbridos Nova [*C. clementina* x (*C. paradisi* x *C. tangerina*)] e Ortanique (tangor provavelmente entre *C. sinensis* (L.) Osbeck e *C. reticulata* Blanco), por intermédio de marcadores isoenzimáticos. O polimorfismo foi detectado por eletroforese de proteínas extraídas de tecido foliar em 10 sistemas isoenzimáticos. Foram obtidos 30 alelos, sendo 16 polimórficos. O coeficiente de Jaccard foi utilizado para estimar a similaridade genética entre as cultivares e o método UPGMA para gerar o fenograma por meio do NTSYS 1,7. As cultivares apresentaram elevada similaridade genética (>72,5%) e cinco grupos principais foram detectados: laranjas-doces, tangerinas 'Clemenules' e 'Marisol', 'Nova', 'Ortanique', e satsuma 'Okitsu'.

Termos para indexação: marcadores bioquímicos, similaridade genética, isoenzimas

The citrus production is one of the most important agribusiness, with high economic and social impacts for the Brazilian economy (Agriannual, 2004). Even though Brazil is the largest exporter of orange concentrated juice (IBGE, 2004), the citrus fresh fruit market should be better explored. Bud woods of 'Lane Late', 'Navelate', 'Navelina' and 'Salustiana' sweet oranges (*Citrus sinensis* (L.) Osbeck), 'Clemenules' and 'Marisol' (*C. reticulata* Blanco) mandarins and 'Okitsu' satsuma mandarin (*C. unshiu* Marcovitch), and the hybrids 'Nova' [*C. clementina* x (*C. paradisi* x *C. tangerina*)] and 'Ortanique' (natural hybrid, probably derived from the crossing between *C. sinensis* (L.) Osbeck x *C. reticulata* Blanco) were re-introduced into the country from Uruguay through Embrapa - Centro de Pesquisa Agropecuária de Clima Temperado. These cultivars are seedless, well accepted in international markets and commercially cultivated in several countries.

The taxonomy of the genus *Citrus* is very complex because of the occurrence of spontaneous mutations, structural chromosomal alterations, and/or interspecific hybridization (Cameron & Frost, 1968). These facts are important to understand and study genetic diversity and origin and for cultivar management.

Isoenzymatic markers have been extensively utilized for two decades in genetic studies of *Citrus*, since they are of low cost, easy to be conducted, show codominant inheritance, are related to genomic regions that have only a single copy, and allow the characterization of plants independent of age and season (Herrero et al., 1996).

The objective of this work was to assess the genetic similarity of 'Lane Late', 'Navelate', 'Navelina' and 'Salustiana' sweet oranges, 'Clemenules' and 'Marisol' mandarins, 'Okitsu' satsuma mandarin, and the hybrids 'Nova' and 'Ortanique', utilizing isoenzymatic markers.

As plant materials were investigated the following cultivars: Lane Late, Navelate, Navelina and Salustiana (*Citrus sinensis* (L.) Osbeck) sweet oranges, 'Clemenules' and 'Marisol' mandarins (*C. reticulata* Blanco), and Okitsu satsuma mandarin (*C. unshiu* Marcovitch), and hybrids Nova [*C. clementina* x (*C. paradisi* x *C. tangerina*)] and Ortanique (natural hybrid, probably derived from *C. sinensis* (L.) Osbeck x *C. reticulata* Blanco). Ten isoenzymatic systems

were used, i.e., esterase (EST), acid phosphatase (APS), phosphoglucomutase (PGM), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), leucine amino peptidase (LAP), malate dehydrogenase (MDH), peroxidase (PRX), superoxidase dismutase (SOD), and shikimate dehydrogenase (SKDH).

Mature leaves collected from three-year-old citrus mother trees cultivated in 20 L plastic pot under greenhouse conditions were used for protein extraction. Samples (10 mg of leaves) were transferred to porcelain plates with round grooves, where they were kept on ice. Each sample had four replications, each processed on different days. Samples were ground with an emery-glass pestle in the presence of specific buffers. Protein extraction was done in the presence of a buffer composed of histidine pH 6.5 with 0.15% β -mercaptoethanol (1:2) for the APS, GOT, IDH, LAP, MDH, PGM, PRX, SOD and SKDH. For EST, the buffer contained histidine pH 8.3 with 0.15% β -mercaptoethanol (1:1), according to Scandalios (1969). Subsequently, the extract was applied to 3MM Wattmann paper and loaded into 1.5 x 4.0 mm-well of polyacrylamide gels (PAGE) utilizing a stain steel comb.

The electrophoresis was performed in an horizontal system with 5% PAGE for IDH, MDH, PGM, SOD and SKDH in the presence of a running buffer according to Shields et al. (1983); with 6% PAGE for LAP, APS and GOT, and 5% PAGE for EST and PRX with a running buffer according to Scandalios (1969). Gel electrophoresis was done in a cold room at an average temperature of 4°C. The potential was kept approximately at 10 V cm⁻¹ and the electric current was paralyzed when the bromophenol blue dye reached 9 cm from the bottom.

The histochemical development of the gels followed the methods based upon Scandalios (1969) for the APS, EST, LAP and PRX; Vallejos (1983) for IDH, PGM and SKDH, and Ayala et al. (1972) for GOT, MDH and SOD. The gels were maintained at 37°C until bands were formed and, subsequently, gels were fixed in a solution composed of distilled water, methanol, and acetic acid (5:5:1).

The interpretation of the zymograms was done based upon the relative mobility of the bands, and only those stable and consistent were selected.

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TABLE 1 - Genetic similarity matrix among citrus fresh fruit market cultivars obtained with ten isoenzymatic systems by the Jaccard coefficient.

Genetic material	1	2	3	4	5	6	7	8	9
1. 'Lanelate'	1.0000								
2. 'Navelate'	1.0000	1.0000							
3. 'Navelina'	1.0000	1.0000	1.0000						
4. 'Salustiana'	1.0000	1.0000	1.0000	1.0000					
5. 'Clemenules'	0.8000	0.8000	0.8000	0.8000	1.0000				
6. 'Marisol'	0.7805	0.7805	0.7805	0.7805	0.9730	1.0000			
7. Satsuma 'Okitsu'	0.7436	0.7436	0.7436	0.7436	0.7436	0.7250	1.0000		
8. 'Nova'	0.7805	0.7805	0.7805	0.7805	0.8718	0.8974	0.8158	1.0000	
9. 'Ortanique'	0.7500	0.7500	0.7500	0.7500	0.8919	0.8684	0.7368	0.7750	1.0000

The Jaccard coefficient was used to estimate the genetic similarity between the parents and hybrids, and the phenogram was obtained based upon the unweighed pair group arithmetic average method (UPGMA), through the NTSYS 1.7 software (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1992). The bootstrap values for each cluster were determined by using the BOOD software (Coelho, 2000).

Thirty alleles were obtained from genetic materials of nine citrus fresh fruit market cultivars with ten isoenzymatic systems. Sixteen alleles showed polymorphic bands at least between two cultivars and the others were monomorphic (Figure 1). The number of polymorphic alleles produced through the isoenzymatic systems EST, APS, PGM, GOT, IDH, LAP, MDH, PRX, SOD and SKDH was 2, 0, 3, 0, 2, 2, 0, 0, 0 and 7, respectively.

The efficiency of the isozyme technique to produce polymorphic bands among mandarins and hybrids of citrus species and its inefficiency when compared to sweet orange cultivars, and the genetic similarity matrix are demonstrated in this work (Figure 1; Table 1). These results can be explained based upon evolutionary facts, i.e., the differentiation of mandarins is due to natural hybridization processes, while that from sweet oranges is due to spontaneous mutation in only a few genes (Scora, 1975). Therefore, it has been very difficult to differentiate sweet orange cultivars even using molecular markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and micro satellites (Novelli et al., 2000), although there are phenotypic differences between these plants.

The genetic analyses showed that the cultivars had high genetic similarity (> 72.5%) (Table 1). Similar results were previously documented by Oliveira et al. (2002), who evaluated different cultivars of *C. sinensis* and *C. reticulata*. In spite of the high similarity observed, the cultivars were clustered into five groups according to the phenogram obtained via the UPGMA method. Group I comprises 'Lanelate', 'Navelate', 'Navelina' and 'Salustiana' sweet oranges; group II was formed by the 'Clemenules' and 'Marisol' mandarins; group III clustered the hybrid 'Nova', while group IV had the hybrid 'Ortanique', and group V was formed by the 'Okitsu' satsuma mandarin (Figure 2). Clusters I and II showed the greatest consistency in the bootstrap analyses, with 100% and 87%, respectively.

There are several discrepancies related to the phylogeny and taxonomy of *Citrus* due to its high variability, spontaneous mutations, apomixes, and cultivation in a wide geographic area (Cameron & Frost, 1968). Various classification systems were proposed for *Citrus*, and they differ mainly in the number of known species: 159 (Tanaka, 1977), 16 (Swingle & Reece, 1967) and 3 (Barret & Rhodes, 1976). Likewise, there are disagreements for mandarin's taxonomy. Swingle & Reece (1967) and Coletta Filho et al. (1998) suggest that mandarins should be clustered into one species, even though they agree with the split of the taxonomic groups. On the other hand, Tanaka (1977) classified mandarins in 36 species.

In the present work, the cultivars 'Clemenules' and 'Marisol' showed high genetic similarity (G_j 0.9730) (Table 1), which was expected because both are considered the same species, *C. reticulata*, based upon all classification systems in the literature. The hybrids 'Nova' and 'Ortanique' also showed strong genetic similarity with the cultivars

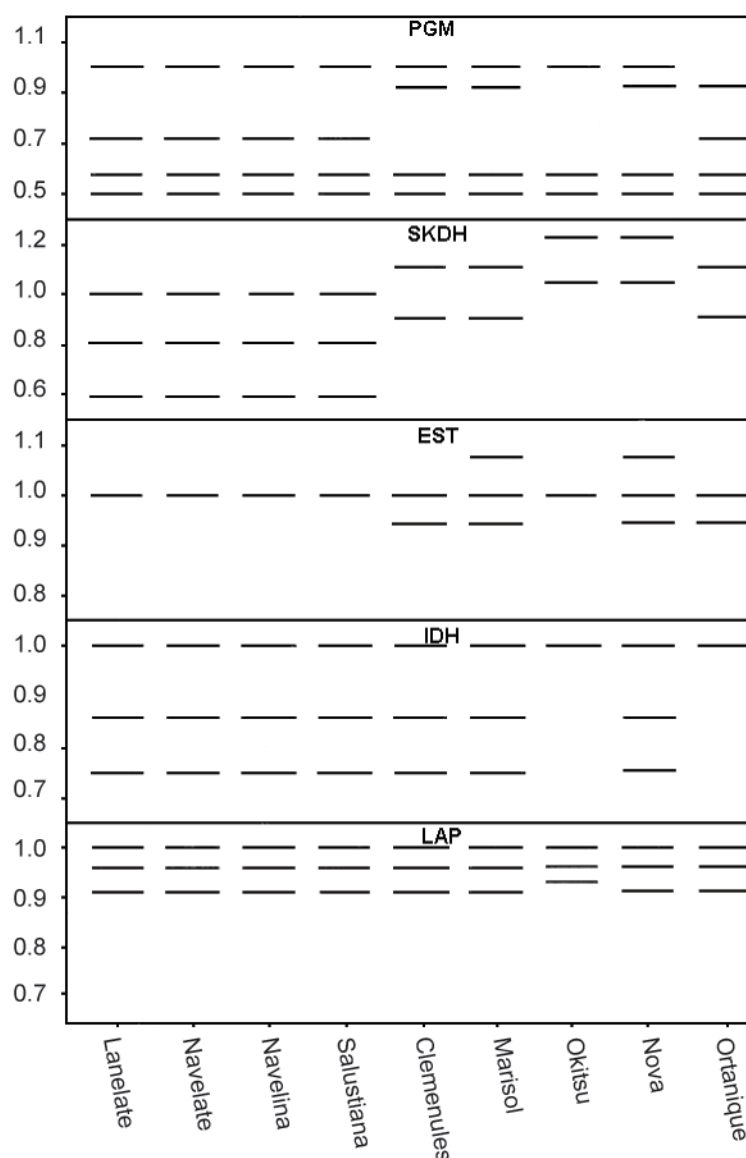


FIGURE 1 - Representation of the isoenzymatic polymorphic banding patterns obtained with the PGM (phosphoglucomutase), SKDH (shikimate dehydrogenase), EST (esterase), IDH (isocitrate dehydrogenase) and LAP (leucine aminopeptidase) in citrus fresh fruit market cultivars.

from *C. reticulata*. 'Ortanique' tangor is considered a natural hybrid between *C. sinensis* and *C. reticulata*, while 'Nova' is a man-made hybrid produced by double crossings [*C. clementina* x (*C. paradisi* x *C. tangerina*)] (Swingle & Reece, 1967). Therefore, a higher genetic similarity was expected among 'Ortanique' and the evaluated cultivars from *C. reticulata* ('Clemenules' and 'Marisol') than for 'Nova'. Coletta Filho et al. (1998) obtained similar results comparing different cultivars from the same species through RAPD markers. They found that hybrids from *C. clementina* x (*C. paradisi* x *C. tangerina*) had higher genetic

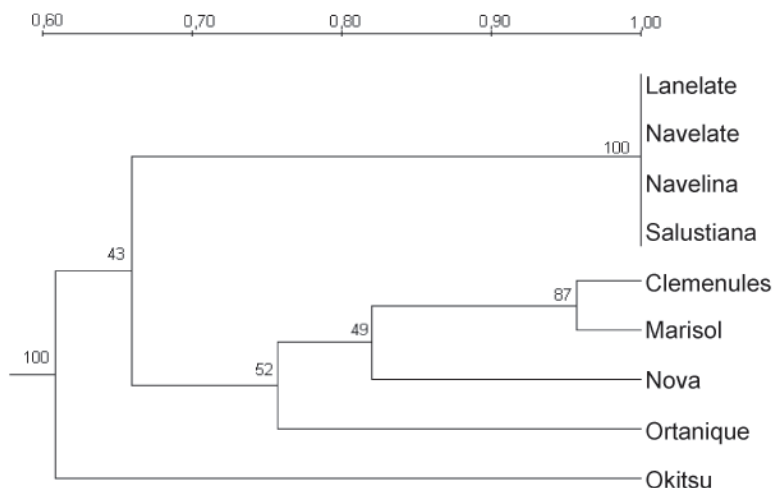


FIGURE 2 - Genetic diversity among citrus fresh fruit market cultivars obtained by the Jaccard coefficient and clustered by UPGMA (unweighted pair group arithmetic average method). The consistency of each cluster was determined by bootstrap analysis.

similarity in relation to *C. reticulata* than those from *C. reticulata* x *C. sinensis*.

Cultivar Okitsu satsuma had the highest genetic distance when compared to the other samples assessed (Table 1 and Figure 2), and this result was highly consistent through the bootstrap analysis (100%). The genetic similarity of this cultivar varied from G_j 0.7250 ('Marisol') to G_j 0.8158 ('Nova'). The polymorphism obtained in this work is not in accordance to the data reported by Swingle & Reece (1967) and Coletta Filho et al. (1998), who considered all mandarins in a single species. This work also showed that the sweet orange cultivars (*C. sinensis*) had greater genetic similarity when compared to mandarins 'Clemenules' and 'Marisol' (*C. reticulata*) than in relation to satsuma 'Okitsu' (*C. unshiu*). This was an unexpected result even considering the hypothesis proposed by several authors that sweet orange cultivar *C. sinensis* could be natural hybrids and had *C. reticulata* as one of the parents (Scora, 1975; Barret & Rhodes, 1976). Coletta Filho et al. (1998) also documented a lower genetic similarity between *C. reticulata* and *C. unshiu* when they assessed several mandarin cultivars; however, since they did not evaluate other plant materials, the differences were not relevant to distinguish the mandarin cultivars in more than one species. Certainly, the use of a higher number of polymorphic fragments and other species of *Citrus* will help to clarify this discussion.

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