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Isoenzymatic polymorphism in *Citrus* spp. and *Poncirus trifoliata* (L.) Raf. (Rutaceae)

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Abstract

Isoenzymatic polymorphism analysis was used to determine genetic variability among species and hybrids of *Citrus* spp. and one accession of *Poncirus trifoliata* (L.) Raf. Ten enzymatic systems aspartate aminotransferase (AAT), acid phosphatase (ACP), leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (6-PGD), isocitrate dehydrogenase (IDH), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), diaphorase (DIA), shikimate dehydrogenase (SKD) and peroxidase (PRX) were analyzed. Twenty loci and 48 alleles were identified. Sweet orange cultivars (*C. sinensis* (L). Osbeck) showed the highest polymorphism with the largest number of heterozygous loci, although the alleles of those loci were the same in all cultivars, with the exception of Westin and Lima graúda. Mandarins (*C. reticulata* Blanco) exhibited diverse patterns, whereas *Poncirus trifoliata* (L.) Raf. showed high variability with all *Citrus* species and hybrids. Exclusive phenotypes were observed in some enzymatic systems, and similar patterns were found among interspecific hybrids and their putative parents.

INTRODUCTION

The genus *Citrus* belongs to the subtribe Citrinae, tribe Citreae, subfamily Aurantioideae of the family Rutaceae (Swingle and Reece, 1967). Taxonomic relationships among members of this genus were established by Swingle and Reece (1967) and Tanaka (1954). However, these classifications differ considerably in the number of species, since Swingle and Reece recognized 16 species and Tanaka 163 species.

The *Citrus* genus includes the most widely producing fruit species in the world and is highly polymorphic. Several species are used as scion cultivars, such as sweet orange, mandarins, lemons and grapefruit. Many species and hybrids with related genera can also be used as rootstocks. *Poncirus trifoliata* (L.) Raf. has importance as a rootstock for several cultivars around the world. However, *Citrus* breeding programs have been hampered by factors associated with reproductive biology (sterility, incompatibility, nucellar embryony, juvenility) and scant information on the nature and mode of inheritance of economically important traits (Torres *et al.*, 1978; Jarrel *et al.*, 1992).

Researchers have recognized the need for genetical studies as well as identification of genetic markers as tools for clarifying taxonomic relationships and improving breeding programs in the genus (Esen and Scora, 1977; Torres *et al.*, 1978; Gogorcena and Ortiz, 1993). Furthermore, correct identification is important for certification and registration of new cultivars. The genetic variability of *Citrus* and associated genera has been evaluated by morphological descriptors, which have low discriminating capacity, as well as biochemical and molecular markers (Esen and Scora, 1977; Handa *et al*., 1986). Isoenzymes have been extensively used as genetic markers in *Citrus* spp. due to their low cost and feasibility as codominant markers (Torres *et al*., 1978; Gogorcena *et al*., 1990; Durham *et al*., 1992; Herrero *et al*., 1996).

We studied the genetic variability of isoenzymes in different species of *Citrus*, their hybrids and *Poncirus trifoliata* (L.) Raf., to provide basic information for breeding programs.

MATERIAL AND METHODS

All accessions analyzed (Table I) belong to the *Citrus* Germplasm Collection at the Centro de Citricultura "Sylvio Moreira", IAC, Cordeirópolis, SP, Brazil. Young leaves from fully expanded and mature plants of similar age were collected and maintained at low temperature in polyethylene bags. In the laboratory, the leaves were washed in distilled water and chopped into pieces. Leaf tissue (0.30 g) from each sample was ground with 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.8 mM DL-dithiothreitol (DL-DTT), 1.5 mM sodium metabisulfite, 1% polyethylene glycol (molecular mass, 6000), 10% sucrose and 0.2% Triton X-100 (Bechara, 1996, with modifications). The supernatants were stored at -20°C. The samples were assayed for the following enzymatic systems: phosphoglucoisomerase

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(PGI), phosphoglucomutase (PGM), leucine aminopeptidase (LAP), isocitric acid dehydrogenase (IDH), catodic and anodic peroxidase (PRX), 6-phosphogluconate dehydrogenase (6-PGD), shikimate dehydrogenase (SKD), aspartate aminotransferase (AAT), diaforase (DIA) and acid phosphatase (ACP). Electrophoresis was performed in horizontal starch gels according to Conkle *et al.* (1982), Cheliak and Pitel (1984) and Ballvé *et al*. (1995). The gels were stained for specific systems (Conkle *et al.*, 1982; Tanksley and Orton, 1983; Cheliak and Pitel, 1984; Soltis and Soltis, 1989). The stained gels were rinsed in distilled water and fixed using acetic acid:glycerol:water (1:1:8). Specific genotypes were inferred from the banding patterns. Gene loci and alleles were named and interpreted according to Torres *et al.* (1978, 1982).

A similarity matrix was generated using the Nei unbiased genetic identity (GI) coefficient (1978, in Swofford and Selander, 1989) with the software BIOSYS 1.7 based on allelic frequencies (Swofford and Selander, 1989), and a cluster analysis using the unweighted pair-group method using arithmetic averages (UPGMA) was performed from the similarity matrix (Rohlf, 1992).

RESULTS

The number of loci and alleles in each accession varied according to the isoenzymatic system tested. Twenty loci and 48 alleles were detected from the 10 enzymatic systems analyzed (Table II). The degree of polymorphism detected (from most to least) was PRX, PGM, SKD, DIA, IDH, 6-PGD, AAT, ACP, PGI and LAP. Several accessions presented exclusive phenotypes in different enzymatic systems, and similar patterns were found among hybrids and their putative parents (Table II).

Table I - *Citrus* species, cultivars and hybrids analyzed (identified according to Tanaka, 1954).

Species	Cultivars	Acessions
Rootstock cultivars		
Sour orange (*Citrus aurantium* L.)	Sour orange Tunis	CV 237
Rangpur lime (C*itrus limonia* Osbeck)	Rangpur lime Limeira	Limeira
Cleopatra mandarin (*Citrus reshni* Hort. ex. Tan.)	Cleopatra mandarin	Mother plant
Sunki mandarin (*Citrus sunki* Hort. ex. Tan.)	Sunki mandarin	Mother plant
Trifoliata orange (*Poncirus trifoliata* (L.) Raf. Sylva Tellur)	Trifoliata orange	Mother plant
Scion cultivars		
Sweet orange (*Citrus sinensis* (L.) Osbeck)	Hamlin	Multiplication block
	Lima graúda EEL	CV 1587
	Mortera	CN 131
	Natal	Multiplication block
	Pera	Multiplication block
	Valência	Multiplication block
	Valência folha murcha	Multiplication block
	Westin	Multiplication block
Mandarins (*Citrus reticulata* Blanco)	Carvalhaes Portugal	CN 546
	Clementina	CV 174
	Cravo	Multiplication block
	Dancy	CN 206
	Fremont EUA	CN 543
	Hansen Austrália	CN 596
	Kara	CN 207
	Mel	CN 205
	Paraguaia EEP - RS	CN 492
	Poncan	Multiplication block
	Vermelha 17 - RS	CN 511
Mandarin *Citrus nobilis* Loureiro	King	CV 179
Mandarin *Citrus unshiu* Marcovitch	Satsuma Japão	CN 527
Hybrids		
C. paradisi- 'Duncan' x *C. reticulata* 'Dancy'	Orlando Tangelo	Mother plant
C. reticulata 'Clementina' x Tangelo Orlando (*C. paradisi* -'Duncan' x *C. reticulata* 'Dancy')	Lee IPEACS - RJ	CV 441
C. sinensis x *C. reticulata*	Murcott Tangor	Mother plant
C. reticulata 'Clementina' x Tangelo Orlando (*C. paradisi*- 'Duncan' x *C. reticulata* 'Dancy')	Nova EEL Tangelo	CV 1583
C. reticulata 'Clementina' x Tangelo Orlando (*C. paradisi*- 'Duncan' x *C. reticulata* 'Dancy')	Osceola IPEACS - RJ	CV 443

Table II - Genotypes of cultivars of *Citrus* spp. and *Poncirus trifoliata* (L.) Raf. obtained with different enzymatic systems.

Cvs/loci	Pgi-1	Pgi-2	Pgm-1	Pgm-2	Lap-1	Lap-2	Idh-1	Prx-an	Prx-ca	Aat-1	Aat-2	Aat-3	6-Pgd1	6-Pgd2	Skd-1	Dia-1	Dia-2	Dia-3	Acp-1	Acp-2
1. Hamlin	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
2. Lima graúda	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	SS
3. Mortera	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
4. Natal	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
5. Pêra	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
6. Valência	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
7. Valência folha murcha	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FS
8. Westin	FF	FS	FI	PM	FI	MS	IM	FF	MM	FS	FF	SS	FI	MS	IS	F_1S_1	F_2S_2	F_3S_3	FF	FS
9. Rangpur lime	FF	FS	FF	MM	FI	MM	IS	MM	FS	FF	FF	FS	II	MS	FM	F_1S_1	F_2S_2	F_3S_3	FF	FF
10. Cleopatra	FF	FF	FF	MM	FI	MM	II	FF	MM	FF	FF	SS	II	MM	FF	F_1F_1	S_2S_2	S_3S_3	FF	FF
11. Sour orange	FF	WS	II	PS	FI	MM	II	FS	II	FF	FF	SS	FI	MS	II	S_1S_1	F_2F_2	F_3S_3	FF	SS
12. Trifoliata orange	FF	FS	FF	-	FI	MM	FF	FF	IM	FF	FF	MP	FI	MM	FF	F_1F_1	S_2S_2	-	FF	-
13. Sunki mandarin	FF	FF	FF	MM	FI	MM	II	MS	MM	FF	FF	SS	II	MM	II	F_1F_1	S_2S_2	-	FF	SS
14. King	FF	FF	II	PP	FI	MM	II	FF	FM	FF	FF	SS	II	MM	II	F_1F_1	S_2S_2	-	FF	FF
15. Satsuma Japão	FF	FS	FF	PM	FI	MM	II	FF	MM	FF	FF	SS	FI	MM	FF	F_1F_1	S_2S_2	F_3S_3	FF	FF
16. Carvalhaes	FF	FS	FF	PP	FI	MS	II	FF	IM	FF	FF	SS	II	MS	SS	F_1S_1	F_2S_2	F_3S_3	FF	FS
17. Clementina	FF	WS	FF	PP	FI	MM	II	FF	IM	FS	FF	SS	II	MS	FM	F_1S_1	F_2S_2	-	FF	FF
18. Cravo	FF	FS	FF	PM	FI	MS	II	FM	MM	FS	FF	SS	II	MM	II	F_1F_1	S_2S_2	-	FF	FF
19. Dancy	FF	FF	FF	PP	FI	MM	II	FF	MM	FF	FF	SS	II	MM	IM	F_1F_1	S_2S_2	-	FF	FF
20. Fremont	FF	FS	FF	PP	FI	MS	II	FF	MM	FF	FF	SS	II	MS	IM	F_1F_1	S_2S_2	-	FF	FF
21. Hansen	FF	WS	FF	MM	FI	MS	II	FF	MM	FF	FF	SS	II	MM	FM	F_1F_1	S_2S_2	-	FF	FF
22. Kara	FF	FF	FI	PP	FI	MM	II	FF	FM	FF	FF	SS	II	MM	FF	F_1F_1	S_2S_2	-	FF	FF
23. Mel	FF	FF	FF	PM	FI	MM	II	FF	MM	FF	FF	SS	II	MM	FF	F_1F_1	S_2S_2	-	FF	FS
24. Paraguaia	FF	FF	FF	PP	FI	MM	II	FF	MM	FF	FF	SS	II	MM	FM	F_1F_1	S_2S_2	-	FF	FS
25. Poncan	FF	FF	FF	PP	FI	MM	II	MM	MM	FF	FF	SS	II	MM	II	F_1F_1	S_2S_2	-	FF	SS
26. Vermelha	FF	FF	FF	PM	FI	MM	II	FF	IM	FF	FF	SS	II	MM	FM	F_1F_1	S_2S_2	-	FF	FS
27. Lee	FF	FS	FF	PP	FI	MS	II	FF	MM	FF	FF	SS	II	SS	MM	F_1F_1	S_2S_2	-	FF	FS
28. Murcott Tangor	FF	FF	FF	PP	FI	MM	II	FF	MM	FF	FF	SS	II	SS	MM	F_1F_1	S_2S_2	-	FF	FF
29. Nova EEL Tangelo	FF	FS	FF	PP	FI	MM	SS	FF	MM	FF	FF	SS	II	MM	FF	F_1F_1	S_2S_2	F_3S_3	FF	FS
30. Osceola	FF	FS	FI	PP	FI	MS	II	FF	MM	FS	FF	SS	II	MS	IM	F_1F_1	S_2S_2	-	FF	FS
31. Orlando Tangelo	FF	FS	FI	PP	FI	MS	II	FF	MM	FS	FF	SS	II	SS	SS	F_1F_1	S_2S_2	-	FF	FF

The GI obtained from the allelic frequencies (Nei, 1978 in Swofford and Selander, 1989) and the UPGMA cluster analysis ranged from 1, the most related accessions, to 0.65 for the less related (Figure 1). The highest GI was observed among the different cultivars of *C. sinensis* (0.94 to 1.0). This group was very distinct from the other accessions.

The *C. reticulata* accessions did not cluster into one group. Some were more similar to accessions of other species. Carvalhaes, Clementina and Fremont accessions (*C. reticulata*) were grouped together with Osceola, Lee, Murcote and Orlando hybrids, that have *C. reticulata* as one of the progenitors. Another subgroup included some *C. reticulata* accessions and *C. reshni*, *C. nobilis* species and Nova tangelo.

DISCUSSION

According to Iglesias *et al.* (1974), isoenzymatic variability in *Citrus* is expected since many species and cultivars probably originated through natural hybridization, which is the route to heterozygosity in plants.

Sweet orange cultivars (*C. sinensis*) showed the highest level of polymorphism, with 13 or 14 heterozygous loci out of 20 (Table II), although the alleles at these loci were almost always the same. Researchers suggest that this species originated from hybridization between *C. grandis* (L.) Osbeck and *C. reticulata* Blanco (Scora, 1975, 1988; Esen and Scora, 1977). Although heterozygosity within these cultivars is high, the need for uniform cultivation must have resulted in the selection of a few variants, that had the de-

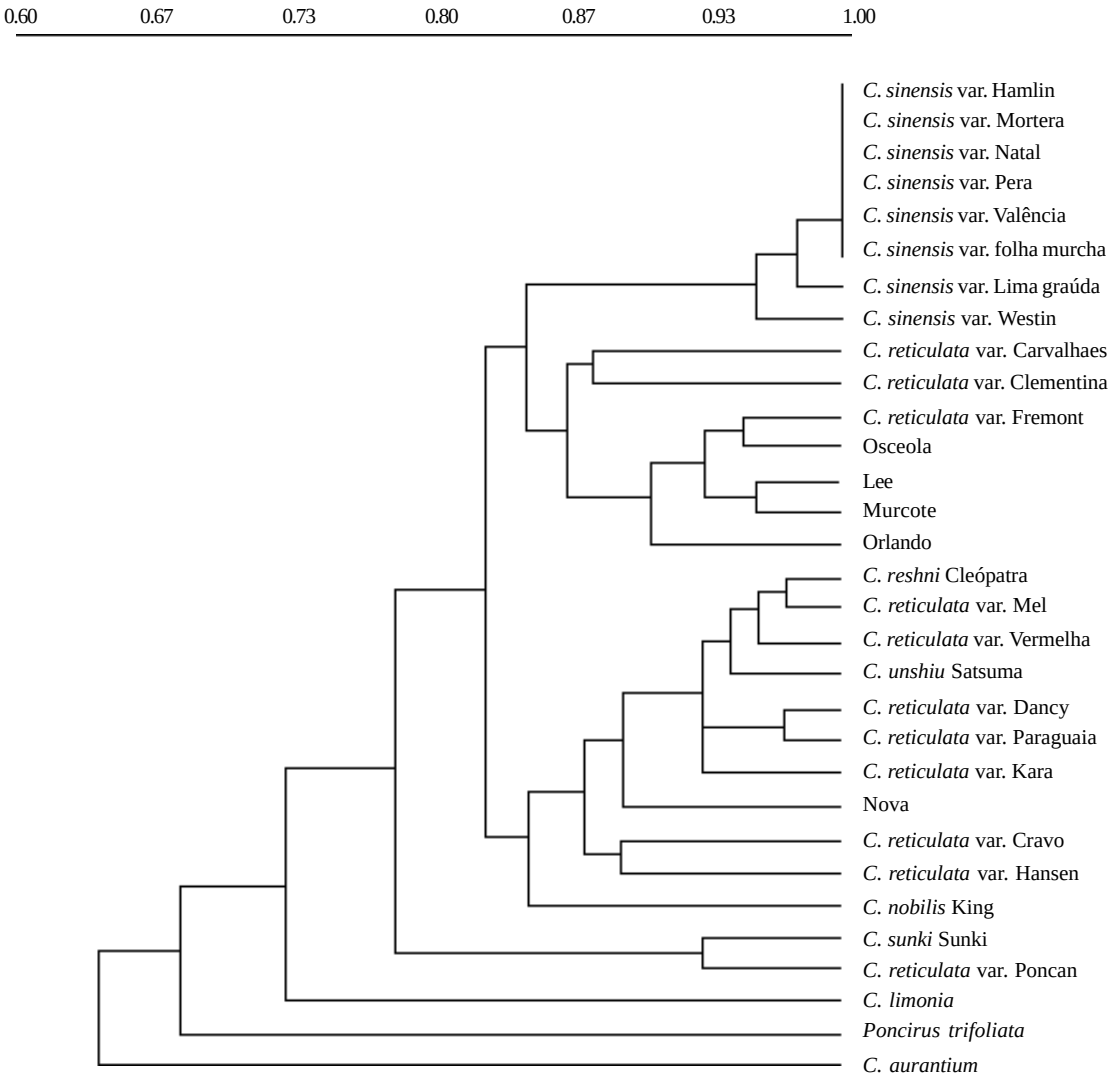

Figure 1 - UPGMA cluster (Nei, 1978, in Swofford and Selander, 1989) identity genetic coefficient matrix of isoenzymatic polymorphism for different species of *Citrus*, their hybrids and *Poncirus trifoliata* (L.) Raf.

sirable genotypic constitution, and these were multiplied through vegetative propagation (Barret and Rhodes, 1976).

However, cultivars Westin and Lima graúda showed specific isoenzymatic patterns for SKD (IS) and ACP (FF/SS), respectively, suggesting that less hybridization has occurred among these cultivars. These enzyme systems could be useful for identification. This could be important, since few studies have focused on polymorphism within cultivars of *C. sinensis* (Esen and Scora, 1977; Vardi, 1988; Sawasaki *et al.*, 1992; Herrero *et al.*, 1996). However, several cultivars of sweet orange originated from somatic mutations of seedlings or limbsport and such mutations have been difficult to detect by codominant markers, like isoenzymes.

In general, mandarins were found to be less polymorphic than sweet orange cultivars. Fourteen different heterozygous loci were observed, and the number of heterozygous loci per individual plant ranged from one to nine (Table II). The lower degree of polymorphism in this group may be due to the fact that this species originated from a cross either between two unknown *C. reticulata* cultivars or one *C. reticulata* cultivar and a different species. In all accessions, six homozygous *loci* and one heterozygous *locus* presented the same phenotype. Therefore, *C. reticulata* cultivars showed less polymorphism within groups than among them, suggesting some intraspecific variability from a narrow genetic base.

Esen and Scora (1977) observed complete homology in amylase in Clementina and Dancy (*C. reticulata*), Cleópatra (*C. reshni*), and King (*C. nobilis*). In the present study seven enzymatic systems (PGI, PGM, PRX, AAT, 6-PGD, SKD and DIA) were useful in detecting differences between some of the accessions analyzed from the cultivars cited above. In fact, some phenotypes were specific, such as King (PGM = II/PP), Cleópatra and Clementina mandarins (DIA = $F_1F_1/S_2S_2/S_3S_3$, and F_1S_1/F_2S_2, respectively) (Table II).

According to Scora (1975) and Barret and Rhodes (1976), the sour orange, *C. aurantium*, probably originated from a cross between *C. grandis* (L.) Osbeck (pummelo) and *C. reticulata* Blanco (mandarin). In this work some alleles were common to *C. aurantium* and one of its probable progenitors, *C. reticulata*. In sour orange, specific phenotypes were detected in three enzymatic systems (PGM (II/PS), PRX (FS/II), DIA ($S_1S_1/F_2F_2/F_3S_3$)), making these three systems useful in cultivar identification. Similarly, *C. limonia* Osbeck showed specific phenotypes in three enzymatic systems: PRX (MM/FS), AAT (FF/FF/FS) and IDH (IS); *C. sunki* (Sunki) and *C. reticulata* cvs Cravo and Poncan could be identified by the following PRX specific patterns, MS/MM, FM/MM and MM/MM, respectively.

Some enzymatic phenotypes were specific to hybrids, such as Nova tangelo, which showed the IDH specific phenotype SS. The hybrids Lee, Murcote and Orlando shared the same phenotype II/SS of 6-PGD, while Lee and Murcote had the phenotype MM of SKD. As expected, several alleles occurred in some hybrids as well as their probable progenitors.

The accession of *Poncirus trifoliata* showed specific phenotypes for four enzymatic systems: PGM (FF/--), IDH (FF), AAT (FF/FF/MP) and ACP (FF/--). In general, the phenotypes were very similar to those described by Torres *et al.* (1978, 1982), Ballvé *et al.* (1991), Sawazaki *et al.* (1992) and Jarrel *et al.* (1992). Some alleles shared by *P. trifoliata* and other *Citrus* species suggest that the two genera, *Citrus* and *Poncirus*, are structurally and functionally related at the genomic level, although taxonomically distinct on a morphological level, which would explain the results of crossing these species (Torres *et al.*, 1985; Jarrel *et al.*, 1992).

According to Scora (1975), Esen and Scora (1977), Handa *et al.* (1986), Scora (1988), Vardi (1988) and Roose (1988), *C. reticulata*, a possible progenitor of several species, is related to many of the accessions. Thus, the similarity of *C. reticulata* to the other species is understandable, considering the polyphyletic origin of *Citrus* cultivated species.

Our data show that *C. limonia*, *Poncirus trifoliata* and *C. aurantium* are related species, but with a minor degree of intragroup similarity and *C. aurantium* is the most differentiated among them (Figure 1). It has been suggested that *C. reticulata* is involved in the origin of *C. aurantium* and *C. limonia* (Hodgson, 1967). The similarity observed here between the genera *Poncirus* and *Citrus* had been confirmed at the molecular level by Torres *et al.* (1985) and Jarrel *et al.* (1992), who detected high structural and functional homology between the genomes of the two genera.

In conclusion, the isoenzyme phenotype results showed a high level of heterozygosity and allows one to infer the genotype of the 31 accessions studied and the genetic similarity among them.

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RESUMO

A análise do polimorfismo isoenzimático foi usada para determinar a variabilidade genética entre espécies e híbridos de *Citrus* spp. e um acesso da espécie *Poncirus trifoliata* (L.) Raf. Dez diferentes sistemas enzimáticos foram analisados, incluindo aspartato aminotransferase (AAT), fosfatase ácida (ACP), leucina aminopeptidase (LAP), 6-fosfogluconato desidrogenase (6-PGD), isocitrato desidrogenase (IDH), fosfoglucose isomerase (PGI), fosfoglucomutase (PGM), diaforase (DIA), shiquimato desidrogenase (SKD) e peroxidase (PRX). Um total de 20 locos e 48 alelos foram identificados. Os cultivares de laranja doce (*C. sinensis* (L.) Osbeck) apresentaram um grande número de locos heterozigotos, mas similares entre eles, com exceção dos cultivares Westin e Lima graúda. Os cultivares de mandarim (*C. reticulata* Blanco) apresentaram diferentes padrões entre eles, enquanto que *Poncirus trifoliata* (L.) Raf. apresentou elevada diferenciação em relação a

todas as espécies de *Citrus* e híbridos. Fenótipos exclusivos foram observados em alguns sistemas enzimáticos, sendo encontrados padrões similares entre os híbridos interespecíficos e seus possíveis parentais.

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