

The use of random amplified polymorphic DNA to evaluate the genetic variability of Ponkan mandarin (*Citrus reticulata* Blanco) accessions

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Abstract

RAPD analysis of 19 Ponkan mandarin accessions was performed using 25 random primers. Of 112 amplification products selected, only 32 were polymorphic across five accessions. The absence of genetic variability among the other 14 accessions suggested that they were either clonal propagations with different local names, or that they had undetectable genetic variability, such as point mutations which cannot be detected by RAPD.

INTRODUCTION

Ponkan mandarins probably originated in southern China and Taiwan or possibly India. Because of their excellent qualities these mandarins were widely cultivated throughout the Orient, reaching Europe in 1805 and North America in 1892 (Tanaka, 1954). This mandarin group has several varieties that are especially suited to the tropical climate of the Orient (from the Philippines to Iraq), Brazil, and, to a lesser extent, Florida (Young, 1986).

Several types of mandarins are known in India and probably represent the nucellar clonal budlines of 'Ponkan' originally known as 'Nagpur Suntara' or 'Santra'. The Ponkan varieties 'Oneco' and 'Warnurco' in Florida originated from seeds from north western India. 'Ponkan' mandarins are also known by other names such as 'Emperor' (Australia), 'Empress' and 'South Africa' (South Africa), 'Batangas' (Philippines), 'Muscia' (Italy) and 'Coorg', 'Assam' or 'Butwall' (India).

Climatically, 'Ponkan' is one of the most tropical mandarins, with the fruits reaching maximum size and quality under tropical conditions, there being little competition from other mandarins (Hodgson, 1967). Brazil is one of the major producers of mandarins, with about 41% of the produce being the 'Ponkan' variety (Figueiredo, 1991), most of which is destined for the fresh fruit market. The genetic uniformity of Brazilian mandarin orchards makes them vulnerable to pathogen or insect-caused catastrophes. Additionally, these mandarins have a limited harvesting period and, once ripe, they do not last long on the tree. The economic importance of the 'Ponkan' mandarin variety has led to the establishment of living tree germplasm collections. Data on the distribution of genetic variability and the relationships among Ponkan varieties are necessary in order to avoid duplications, and to establish core collections representative of the genetic variability of the group in which agricultur-

ally important genes may occur. Such information may help breeding programs and optimize the search for desirable characteristics.

Conventional breeding of citrus plants is traditionally slow and difficult, mainly because of the complex reproductive biology, involving polyploidy, high heterozygosity and single gene inheritance patterns in a few traits. Also, the prolonged juvenile period of seedlings in the field makes citrus breeding very lengthy. Recent technological advances have partially solved some of the limitations associated with classic citrus breeding. Molecular based markers have been used to construct linkage maps (Cai *et al.*, 1994; Kijas *et al.*, 1995), identify genotypes (Motohashi *et al.*, 1992; Albanese *et al.*, 1992), distinguish hybrids from nucellar seedlings (Bastianel *et al.*, 1998), explore phylogenetic relationships (Luro *et al.*, 1992; Herrero *et al.*, 1996) and evaluate citrus germplasm collections (Machado *et al.*, 1996; Coletta Filho *et al.*, 1998). In the latter two studies, random amplified polymorphic DNA (RAPD) analysis of several commercial and non-commercial mandarin accessions showed that this approach provided a quick and reliable method for evaluating diversity and genetic relationships. In both studies, closely related accessions were separated, with genotype-specific RAPD markers being obtained in some cases.

Until now, no attempts have been made to evaluate germplasm collections of Ponkan mandarins using RAPD molecular markers. We used this approach to study the genetic variability of a germplasm collection of Ponkan mandarins.

MATERIAL AND METHODS

Plant material

Nineteen Ponkan mandarin accessions belonging to the germplasm collections at the Centro de Citricultura

Table I - Regional names and origins of Ponkan mandarin accessions analyzed using RAPDs.

Accession	Name	Origin	Accession	Name	Origin
1	Muscia	Australia	11	Batangas	Phillippines
2	Ponkan	China	12	Sylhat	*
3	Ponkan Tuxpan	Mexico	13	Mandalina	Corsega
4	Loose Jacket	*	14	Emperor	Australia
5	Tangor	Brazil	15	Wildt	South Africa
6	Hung Kat	China	16	South Africa	South Africa
7	Warnurco	USA	17	Green Rind	South Africa
8	Empress	South Africa	18	Rosehaugh Nartjee	South Africa
9	Pan-American	Corsega	19	Oneco	USA
10	Swaton	*			

*Information not available.

Sylvio Moreira - IAC, Campinas, SP, Brazil, were investigated (Table I).

DNA isolation

Total DNA was isolated from 100 mg of lyophilized leaves using the method of Murray and Thompson (1980) as modified by Machado *et al.* (1996). The quality and quantity of DNA were determined as described by Sambrook *et al.* (1989).

Amplification reactions

The DNA amplification reactions were performed using 1.3 µl of 10x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 20 mM MgCl₂, 0.01% gelatin), 200 µM each of dATP, dCTP, dTTP, dGTP (Gibco BRL), 15 ng of 10-base pair primers (Operon) or 8-mer primer (Caetano-Anollés *et al.*, 1991), 10 ng of genomic DNA and 1.5 units of *Taq* polymerase (Promega). The final volume was completed to 12.3 µl with ultrapure water. The reaction mixtures were overlaid with mineral oil and the amplification was performed in a Robocycler (Stratagene) programmed for 36 cycles of 1 min at 92°C, 1 min at 36°C and 2 min at 72°C, using the fastest ramping time, with a final extension at 72°C for 10 min, followed by cooling at 20°C until recovery of the sample. Aliquots of the amplification products were analyzed by electrophoresis in 1.4% agarose gels containing 0.5 ng of ethidium bromide/ml. Molecular sizes of the amplifications products were estimated by a 1-kb DNA ladder (Gibco BRL). The gels were photographed using a UV-transilluminator with Polaroid film 667.

Data analysis

The amplifications obtained were compiled into a two-way matrix (accessions x markers) of the specific amplification products. Only reproducible and well-characterized RAPDs were used in this analysis (Grattapaglia and Sederoff, 1994). Cluster analysis was performed using the

unweighted pair-group method arithmetic average (UPGMA), with the Jaccard coefficient calculated by the software NTSYS-PC version 1.7 (Rohlf, 1992).

RESULTS AND DISCUSSION

All of the primers produced amplifications (Table II). However, 10 of them revealed no polymorphism among the 19 Ponkan mandarin accessions. A total of 112 bands were scored, of which only 32 were polymorphic. The

Table II - The sequences of the primers used and the number of fragments produced.

Primer	Sequence	Total number of fragments*	Number of polymorphic fragments**
P130	CGCGCCA	5	1
OPA01	CAGGCCCTTC	2	1
OPA16	AGCCAGCGAA	3	0
OPA18	AGGTGACCGT	3	0
OPG10	AGGGCCGTCT	6	3
OPG13	CTCTCCGCCA	2	0
OPG14	GGATGAGACC	6	4
OPG19	GTCAGGGCAA	6	3
OPH04	GGAAGTCGGC	2	1
OPH05	AGTCGTCCCC	2	0
OPH08	GAAACACCCC	4	1
OPH15	AATGGCGCAG	4	1
OPH16	TCTCAGCTGG	4	0
OPH17	CACTCTCCTC	6	4
OPH18	GAATCGGCCA	4	0
OPI11	ACATGCCGTG	5	0
OPK04	CCGCCAAAC	5	1
OPM04	GGCGGTGTC	6	1
OPM06	CTGGGCAACT	7	4
OPN07	CAGCCCAGAG	7	1
OPN20	GGTGCTCCGT	4	0
OPQ10	TGTGCCCGAA	7	3
OPW16	CAGCCTACCA	4	1
OPAT04	TTGCCTCGCC	5	0
OPAT14	GTGCCGCACT	2	2

*Includes selected polymorphic or monomorphic markers. **Includes only selected polymorphic markers.

number of selected products generated by each primer varied from two (OPA01, OPG13, OPH04, OPH05, OPAT14) to seven (OPQ10), with DNA bands of 0.2-4.0 kb. The greatest numbers of polymorphic bands (four) were generated by primers OPG14, OPH17 and OPM06 (Table II). Only two accessions (lanes 5 and 7) showed some difference in their DNA bands (Figure 1). Polymorphic amplifications were seen only with accessions 'Sylhat', 'Warnurco', 'Green Rind', 'Tangor' and 'Hung Kait'; the other 14 accessions had identical amplification profiles. Mediterranean mandarin accessions (*C. deliciosa* Ten.) also have low detectable genetic polymorphism, with most accessions appearing to be clonal (Machado *et al.*, 1996).

A similarity matrix based on the Jaccard coefficient was used to establish the level of relatedness between the 19 accessions studied. The highest estimated genetic similarity coefficient (GS) was 1.0, suggesting that these could

have originated from a single clone, or may reflect non-detectable genetic variability. On the other hand, the smallest GS among all accessions was 0.83. Similar results (GS = 0.88) have also been obtained with 39 Mediterranean mandarin (*C. deliciosa* Ten.) accessions (Machado *et al.*, 1996). An investigation of genetic variability in 35 mandarin accessions, including 10 species and seven hybrids, produced a minimum GS of 0.73 (Coletta Filho *et al.*, 1998). These high GS values in different mandarin genotypes emphasize the narrow genetic variability within cultivated mandarins.

The dendrogram generated by the UPGMA analysis confirmed the above pattern of diversity (Figure 2). Fourteen accessions (from 'Mandalina' to 'Rosehaugh Nartjee') are probably clones and are known by different local names. Using RAPD markers generated by 23 random primers, Coletta Filho *et al.* (1998) found no genetic difference among the mandarin accessions 'Ponkan',

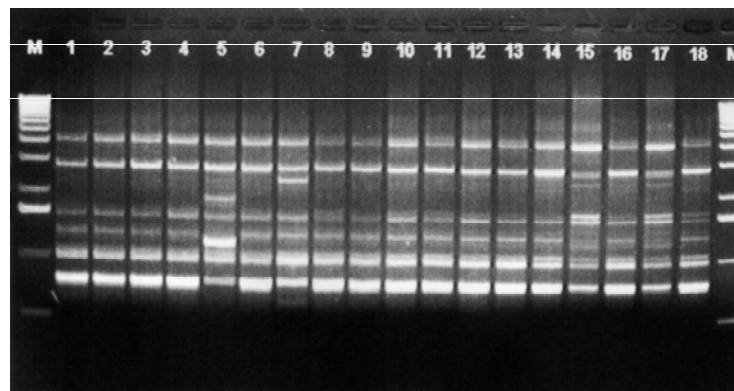


Figure 1 - RAPD amplification products of primer OPQ10 in 18 mandarin accessions. M: Molecular weight markers (1-kb DNA ladder). Lanes 1-18: DNA accessions as listed in Table I.

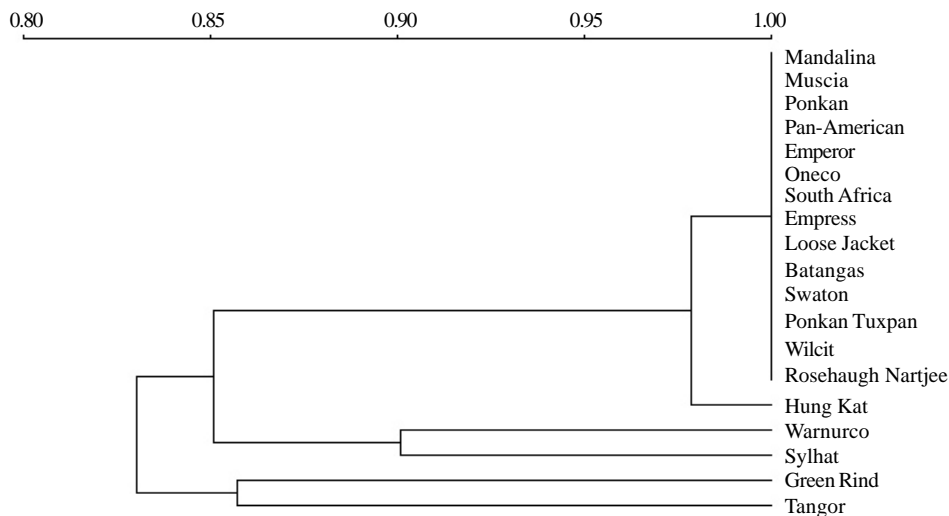


Figure 2 - Dendrogram of 19 accessions of mandarins based on RAPD markers using UPGMA cluster analysis of the Jaccard genetic similarity coefficient.

'Muscia' and 'Batangas'. These results agree with the observation that Ponkan mandarins spread from Asia to the rest of the world and received different local names. Consequently, 'Emperor' (Australia), 'Empress' (South Africa), 'Oneco' (United States), 'Batangas' (Philippines) and others are probably nucellar clonal budlines of 'Ponkan' mandarin. Similar observations were reported by Hodgson (1967) who noted that 'Ponkan' fruits were sent to the United States in 1893, and seedlings were grown from these and later propagated under the names 'Warnurco' and 'Oneco'. 'Emperor' mandarin originated from seedlings derived from fruits imported from the Orient and is very similar, if not identical, to 'Oneco' and 'Ponkan'. 'Empress' originated either as a mutation or a hybrid derived from South African seedlings believed to be 'Emperor'.

The accession 'Hung Kat' is closely related to the Ponkan clones (Figure 2, GS 0.98). 'Hung Kat', of Chinese origin, probably derived from a mutation or a hybrid with characteristics very similar to 'Ponkan'. The distance between 'Warnurco' and Ponkan clones (GS 0.86) showed that the former was not a 'Ponkan' clone. In addition, the 'Warnurco' used in this research was probably not the same variety described as 'Warnurco' by Hodgson (1967). The most distant accessions were 'Green Rind' and 'Tangor' (GS 0.83), which are probably hybrids derived from the Ponkan mandarin.

Although Ponkan mandarins spread widely from the Orient a long time ago, our results indicate a close genetic relationship among them, perhaps as a result of low mutation and natural hybridization rates. Since five accessions phenotypically similar to Ponkan mandarins were differentiated by RAPD, this procedure represents a powerful tool which can be used to evaluate the genetic diversity of mandarin germplasm collections.

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RESUMO

Amplificações RAPD foram desenvolvidas utilizando-se 25 "primers" de sequência aleatória e 19 acessos de tangerinas pertencentes ao grupo das Ponkan. Dos 112 produtos de amplificação selecionados somente 32 foram polimórficos, sendo este polimorfismo restrito somente a 5 acessos. Nos 14 acessos restantes não pôde ser detectada qualquer variabilidade genética, sugerindo que estes podem ser propagação clonal da tangerina

'Ponkan', tendo os mesmos adquirido diferentes nomes regionais ao longo do tempo. Ou, por outro lado, a técnica RAPD utilizada pode não ter detectado qualquer mutação de ponto que tenha ocorrido nestes materiais ao longo do tempo.

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