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Phylogenetic relationships within the genus *Citrus* (*Rutaceae*) and related genera as revealed by RFLP and RAPD analysis

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Abstract Relationships among 88 accessions representing 45 *Citrus* species, three man-made hybrids, and six related genera were examined for restriction fragment length polymorphisms (RFLP). Thirty-two *Citrus* and three *Microcitrus* accessions were also examined by random amplified polymorphic DNA (RAPD) analysis. A measure of relative heterozygosity was estimated based on the mean of the number of fragments per individual per probe-enzyme combination (PEC) divided by total number of fragments per PEC for all non-hybrid *Citrus* individuals. The presence in a *Citrus* species of a rare band found also in a related genus was taken as an indication of possible introgression, while the presence of several fragments unique to 1 species was used to indicate non-involvement of that species in hybridization events. Most species that have been described in the literature as hybrids had high heterozygosity indices and no unique fragments. Distance matrices and dendrograms were generated using simple matching coefficient and neighbor-joining cluster analysis. RFLP and RAPD data gave approximately the same results. These data showed *C. maxima* was affiliated with the papedas *C. hongheensis* and *C. latipes*. *C. medica* clustered with *C. indica* when only non-hybrid taxa were examined, or among limes, lemons, and relatives when all species were considered. Mandarins did not show strongly supported groupings among themselves, nor with other species. These data showed that several accessions were probably assigned to the wrong species.

Key words *Citrus* · RFLP · RAPD · Phylogeny · Taxonomy

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Introduction

The genus *Citrus* has been variously described as consisting of from 1 to 162 species (Malik 1973; Tanaka 1977). The most widely accepted taxonomic systems today are those of Swingle (1946) and Tanaka (1977) who recognized 16 and 162 species, respectively. Relationships among taxa are complicated by several factors such as a high frequency of bud mutation, a long history of cultivation, and wide cross-compatibility. In species that are grown primarily for fruit, sports may be vegetatively propagated and maintained by budding, which can lead to small, mutation-based differences among varieties within cultivated species (Frost and Soost 1968). For example, little genetic variation was detected within the important cultivated species *C. sinensis* and *C. paradisi* when examined by microsatellite-based markers (Kijas et al. 1995; Luro et al. 1995; Fang and Roose, 1997). Moreover, many species have some degree of apomictic seed production, which tends to reduce variability within the species.

Hybridization can occur if taxa are in sufficiently close spatial proximity to one another, for there are few genetic barriers to interspecific hybridization within *Citrus*, and some related genera are also cross-compatible with *Citrus* (Iwamasa et al. 1988). Species which arose by hybridization between other taxa may have a high level of heterozygosity, especially if the species is highly apomictic.

Hybridization, natural or man-made, has probably played an important role in the evolution of many, or even most, *Citrus* species. Scora (1975) and Barrett and Rhodes (1976) contend that there are only 3 'basic' species of *Citrus* within the subgenus *Citrus* sensu Swingle: *C. medica*, *C. reticulata*, and *C. maxima*. They considered all other species within the subgenus *Citrus* might have derived from hybridization among these 3 species or between them and species of the subgenus Papeda or closely related genera. Blondel (1978) has suggested that even within *C. medica*, the cultivars 'Ethrog' and 'Buddha's Hand' may have arisen by hybridization with some other *Citrus* species.

Tanaka's and Swingle's systems of *Citrus* classification were based upon morphological and some biochemical criteria, such as the presence of acrid oils in papedas. More recently, biochemical data (Potvin et al. 1983), protein electrophoresis (Handa et al. 1986), isozymes (Torres et al. 1978; Fang et al. 1993; Herrero et al. 1996a, b), microsatellites (Kijas et al. 1995), and organellar genome analysis (Green et al. 1986; Yamamoto et al. 1993) have been used to examine relationships among *Citrus* taxa.

Restriction fragment length polymorphisms (RFLPs) are well-suited for taxonomic and evolutionary studies (Soller and Beckmann 1983; Gepts 1993; Whitkus et al. 1994). Organellar RFLPs have been used to study phylogenetic relationships of *Citrus* (Green et al. 1986) and *Citrus* and its relatives (Yamamoto et al. 1993), but there are no detailed reports of the use of RFLPs to study *Citrus* systematics.

The random amplified polymorphic DNA (RAPD) technique (Welsh and McClelland 1990; Williams et al. 1990) is less expensive per data point than RFLP but produces primarily dominant alleles. RAPD markers have been used to study phylogenetic relationships in *Rosa* (Millan et al. 1996) and *Hordeum* (Marillia and Scoles 1996). Dos Santos et al. (1994) showed that RAPD markers could be used as effectively as RFLPs to determine genetic relationships among *Brassica* genotypes. Although RAPDs have been used for mapping (Cai et al. 1994) and cultivar identification (Omura et al. 1993, Deng et al. 1995) in *Citrus*, they have not been used for phylogenetic analysis.

We used RFLP data from 17 probes to estimate the heterozygosity of 73 accessions of *Citrus* and 12 accessions from six related genera of the Citrinae subtribe in an attempt to clarify which species are possibly of hybrid origin. We examined relationships among non-hybrid accessions and among all accessions using these RFLP data and compared RFLP results to RAPD results for 35 of the accessions.

Materials and methods

Plant materials

Seventy-three accessions of *Citrus* from 45 species (*sensu* Tanaka), three man-made hybrids, and 12 accessions from six related genera (Table 1) in the Citrus Variety Collection at the University of California at Riverside were sampled for RFLP analysis. This collection includes both cultivated and wild species of *Citrus* and *Citrus* relatives collected over six decades and maintained as trees. PI lines 254727 and 254728 were from the University of California Lindcove field station. The data presented here are a combination of three independently prepared sets of data that included 55, 38, and 12 accessions, respectively. Ten accessions that had been used in the first set (Set A) were repeated in the second set (Set B), and seven from Set A were repeated in Set C to confirm that results were the same, even though methods of DNA extraction and Southern hybridization were not identical. One accession was included in all three sets. Only set B was analyzed for RAPD variation.

DNA extraction

For Set A, total DNA preparation employed a CsCl gradient (Jarrell et al. 1992). For Sets B and C, DNA was extracted according to Fang et al. (1997).

RFLP analysis

All 17 probes (pRLc007, pRLc024, pRL031, pRLc032, pRLc038, pRLc039, pRLc040, pRLc041, pRLc049, pRLc053, pRLc056, pRLc060, pRLc089, pRLc091, pRLc103, pRLc107, and pRLc112) used in this experiment were inserts from a *C. jambhiri* cDNA library (Jarrell et al. 1992). Some fragments of 14 probes have been mapped to six of ten linkage groups of *Citrus* (Jarrell et al. 1992; Roose unpublished data). PRLc038, pRLc060, pRLc040, and pRLc091 mapped within a 70-centiMorgan (cM) interval on linkage group three. PRLc041 and pRLc112 were 4 cM apart on group two. Other probes were more than 50 cM apart or on separate linkage groups. Approximately 5 ug DNA from each sample was digested with 10 u restriction endonucleases *Eco*RI (Promega) and *Hin*dIII (Stratagene) in the manufacturers' buffer supplemented with 3 m*M* spermidine. Digestion was conducted at 37°C for 14 h. The procedures of DNA electrophoresis, Southern transfer, insert isolation, and probe labeling were according to Jarrell et al. (1992). Set A employed TM-NYX4 (Hoefer Scientific) positively charged membranes, and sets B and C employed Magnagraph membranes (MSI). Membranes were autoradiographed on X-OMAT AR film at −80°C for 24–96 h. Data from only one enzyme per probe were chosen for the analysis based on clarity of the results, smallest number of missing observations, and greatest variability among taxa.

RAPD analysis

A total of 23 decamer primers (OpI01, I03, I04, I07, I08, I11, I15, I16, L04, M04, M05, M06, M10, M11, M16, M19, M20, N02, N07, N08, N10, N13, and N14) (Operon Technologies) were used to amplify DNA. These primers were chosen because they produced repeatable, polymorphic and easily scored products after we screened about 100 primers. Reaction mixtures and temperature profiles followed Cheng and Roose (1995). Amplification products were separated on 1.8% agarose gel in 1 × TBE buffer and detected by ethidium-bromide staining.

Data analysis

For each accession an index of heterozygosity based on RFLP pattern was calculated as the mean number of fragments per probe-enzyme combination (PEC) divided by the mean number of fragments per PEC for non-hybrid *Citrus* accessions (based on the literature). For any accession that lacked data for some PECs, the denominator of the index was adjusted to include only those PECs studied for that accession. This index is necessary because RFLP phenotypes cannot be assigned to genotypes without segregation analysis, which we did not have for all the fragments we analyzed. Although, for a single RFLP probe, a multiple-banded pattern may reflect additional restriction sites rather than heterozygosity, when averaged over many probes only higher heterozygosity is likely to increase the index.

Table 1 Accessions used in sets A, B and C, identified by Tanaka species name, common name, and CRC identification number (Citrus Research Center, UC, Riverside)

Species name according to Tanaka system	Set	CRC number	Common name	Hybrid origin?
	C		PI254727	*C. maxima* × ?
	A	1462	Cuban Shaddock	*C. maxima* × *C. medica* or *C. limon* (Hodgson 1967)
	A	3769	New Zealand goldfruit	*C. maxima* × ? (Hodgson 1967)
C. amblycarpa Ochse	A B	2485	Nasnaran mandarin	*C. reticulata* × *C. aurantifolia*?
C. aurantifolia (Christm.) Swing.	A	1710	Mexican lime	*C. medica* × papeda (Scora 1975) or *C. medica* × *C. maxima* × *Microcitrus* (Barrett and Rhodes 1976)
C. aurantifolia (Christm.) Swing.	B	2188	Key lime	*C. medica* × papeda (Scora 1975) or *C. medica* × *C. maxima* × *Microcitrus* (Barrett and Rhodes 1976)
C. aurantifolia (Christm.) Swing.	B	2450	India lime	*C. medica* × papeda (Scora 1975) or *C. medica* × *C. maxima* × *Microcitrus* (Barrett and Rhodes 1976)
C. aurantium L.	A	0628	Standard sour orange	*C. maxima* × mandarin (Scora 1975)
C. bergamia Risso and Poit.	A B	2881	Bergamot orange	*C. aurantium* × *C. medica* (Scora 1988)
C. canaliculata Hort. ex Y. Tan.	A	3565	Kikudaidai	Like *C. aurantium* (Hodgson 1967)
C. clementina Hort. ex Tan.	A	0279	Clementine	*C. sinensis* × *C. reticulata* (Hodgson 1967)
C. deliciosa Ten	A	3843	Willowleaf, Mediterranean mandarin	
C. depressa Hay.	B	2448	Shekwasha mandarin	
C. erythrosa Hort. ex Tan.	B	3292	Fukushu mandarin	
C. halimii B. C. Stone	A	3900		?
C. hongheensis YLDL.	B	3797	Honghe papeda	
C. hystrix DC.	A B	3103	Mauritius Papeda	
C. ichangensis Swing.	A B C	2431	Ichang papeda	
C. ichangensis Swing.	C	2327	Ichang papeda	
C. ichangensis Swing.	C	3931	Ichang papeda	
C. indica Tan.	A	3163	Indian wild orange	*C. latipes* × ? (Swingle and Reece 1967)
C. jambhiri Lush.	A C	3879	Schaub rough lemon	*C. medica* × mandarin (Scora 1975)
C. keraji Hort. ex Tan.	A	3144		*C. reticulata* hybrid?
C. latifolia Tan.	B	0391	Tahiti lime	*C. aurantium* × *C. medica* or *C. limon* (Reece and Childs 1962)
C. latipes (Swingle) Tan.	B C	3052	Khasi papeda	
C. latipes (Swingle) Tan.	C		PI254728	
C. limetta Risso	A B	0569	Millsweet lemon	Like *C. limon*?
C. limetta Risso	B	2695	Faris sweet lemon	Like *C. limon*?
C. limetta Risso	B	3989	Limonette de Marrakech or Morrocan lemon	Like *C. limon*?
C. limetta Risso	B	3093	Sweet lemon	Like *C. limon*?
C. limettioides Tan.	A	0919	Sweet lime	Like *C. aurantifolia*?
C. limettioides Tan.	B	0363	Sweet lime	Like *C. aurantifolia*?
C. limettioides Tan.	B	0921	Sweet lime	Like *C. aurantifolia*?
C. limettioides Tan.	B	1482	Palestine sweet lime	*C. aurantifolia* × *C. sinensis* (Barrett and Rhodes 1976)
C. limon (L.) Bur. f.	A C	3176	Frost Lisbon lemon	*C. medica* × *C. aurantifolia* × ? (Malik et al. 1974)
C. limon (L.) Bur. f.	B	3043	Eureka lemon	*C. medica* × *C. aurantifolia* × ? (Malik et al. 1974)
C. limon (L.) Bur. f.	A B	2489	Rhobs-el-arsa lemon	*C. medica* × *C. aurantifolia* × ? (Malik et al. 1974)
C. limon (L.) Bur. f.	B	3492	Iraq sweet lemon	*C. medica* × *C. aurantifolia* × ? (Malik et al. 1974)
C. limonia Osbeck	A C	2424	Borneo rangpur	Mandarin × *C. jambhiri* (Singh and Schroeder 1962)
C. limonia Osbeck	B	2319	Australia red lime	Mandarin × *C. jambhiri* (Singh and Schroeder 1962)
C. limonia Osbeck	B	2318	Philippine red lime	Mandarin × *C. jambhiri* (Singh and Schroeder 1962)
C. lycopersicaeformis Hort. ex Tan.	B	3564	Monkey orange	
C. macrophylla Wester	A B	3842	Alemow	*C. celebica* × *C. maxima* (Swingle and Reece 1967)
C. maderaspatana Hort. ex Y. Tan.	A	3225	Kitchli	*C. aurantium* × ? (Hodgson 1967)
C. maxima (Burm.) Merrill	A C	2248	Kao panne pummelo	

Table 1 Continued

Species name according to Tanaka system	Set	CRC number	Common name	Hybrid origin?
C. maxima (Burm.) Merrill	A	2348	Pin Shan Kong Yau pummelo	
C. maxima (Burm.) Merrill	B	3926	Kao Phuang pummelo	
C. maxima (Burm.) Merrill	A B	2240	Siamese sweet pummelo	
C. medica L.	A	3523	Diamante citron	
C. medica L.	A B	3768	Buddha's Hand citron	*C. medica* × *C.* sp. (Blondel 1978)
C. medica L.	B	3891	Ethrog citron	*C. medica* × *C.* sp. (Blondel 1978)
C. miaray Wester	A	3574		*C. aurantium* × ?
C. micrantha Wester	B	3605	Aamuyao papeda	
C. natsudaidai Hay.	A	3235		*C. paradisi* × *C. reticulata* (Hodgson 1967)
C. nippokoreana Tan.	B	3228	Korai tachibana mandarin	
C. oleocarpa Hort. ex Tan.	A B	2692	Tim kat mandarin	
C. paradisi Macf.	A	3832	Duncan grapefruit	*C. maxima* × *C. sinensis* (Hodgson 1967)
C. paradisi Macf.	A	3770	Star ruby grapefruit	*C. maxima* × *C. sinensis* (Hodgson 1967)
C. paradisi Macf.	A	0343		*C. maxima* × *C. sinensis* (Hodgson 1967)
C. pennivesiculata Tan.	A	2434		*C. limon* × ?
C. reshni Hort. ex Tan.	A	3844	Cleopatra	
C. reticulata Blanco	A	3849	Ponkan	
C. shunkokan Hort. ex Tan.	A	3476		
C. sinensis (L.) Osbeck	A	2750	Olinda valencia	*C. maxima* × *C. reticulata* (Scora 1975)
C. sinensis (L.) Osbeck	A	3014	Newhall navel	*C. maxima* × *C. reticulata* (Scora 1975)
C. sinensis (L.) Osbeck	A	3827	Ruby blood orange	*C. maxima* × *C. reticulata* (Scora 1975)
C. succosa Hort. ex Tan.	B	3280	Jimikan mandarin	
C. sunki Hort. ex Tan.	A	3143		
C. tachibana (Mak.) Tan.	A	3150	Tachibana orange	
C. taiwanica Tan. and Shim.	A	2588	Nansho daidai	*C. aurantium* × ? (Swingle and Reece 1967)
C. tardiva Hort. ex Shirai	B	3297	Giri mikan mandarin	
C. unshiu Marc.	A	3820	Okitsu wase satsuma	?
C. yatsushiro Hort. ex Tan.	B	3466	Yatsushiro mikan mandarin	
Clymenia polyandra (Tan.) Swing.	A	3284		
Eremocitrus glauca (Lindl.) Swing.	A C	3463	Australian desert lime	
Fortunella margarita (Lour.) Swing.	A	3877	Nagami kumquat	
Fortunella polyandra (Ridl.) Tan.	A	3901	Malayan kumquat	*Fortunella* × *C. aurantifolia* (Swingle and Reece 1967)
Microcitrus australasica (F.Muell.) Swing.	B	3661	Australia finger lime	
Microcitrus australis (Planch.) Swing.	B	3666	Australian round lime	
Microcitrus papuana	B	3081		
Microcitrus warburgiana (F.M. Bail.) Tan.	A	3782	New Guinea wild lime	
Poncirus trifoliata (L.) Raf.	A	1717	Pomeroy trifoliate orange	
Poncirus trifoliata (L.) Raf.	A C	0838	Rubidoux trifoliate orange	
Poncirus trifoliata var. monstrosa (T. ito) Swing.	A	3330	Flying Dragon trifoliate orange	
Severinia buxifolia (Poir.)Tenore	A	1494	Chinese box orange	
Man made hybrids				
C. sinensis × *P. trifoliata*	A	1459	Troyer citrange	Yes
C. maxima (Siamese sweet) × *C. sinensis* (4N Ruby Blood orange)	A		2240 × 4N Ruby	Yes
C. limonia × *C. aurantium*	A		Rangpur × Sour	Yes

Bands for each PEC or primer were scored as present or absent (coded A or T, respectively). Distances between taxa were calculated using a simple matching coefficient, the proportion of shared A's and T's subtracted from 1. All cluster analysis was performed with *MEGA* (Kumar et al. 1993) using neighbor-joining. Bootstrap estimates were calculated for 500 re-samplings. A dendrogram was constructed using data of these 17 PECs for taxa that could be considered to be species not likely of hybrid origin based on the heterozygosity index and previous workers' assessments (Fig. 1). Another dendrogram was constructed that also included probable hybrid species (Fig. 2). Separate RFLP and RAPD dendrograms were constructed for Set B data (not shown), as well as a combined RFLP plus RAPD dendrogram (Fig. 3).

Results

The following PECs were used in the analysis: *Eco*RI digests of pRLc007, pRLc031, pRLc041, pRLc053, pRLc056, pRLc060, pRLc103, pRLc112, and *Hin*dIII digests of pRLc024, pRLc032, pRLc038, pRLc039, pRLc040, pRLc049, pRLc089, pRLc091, pRLc107. These generated 143 fragments, ranging from 3 to 24 fragments per PEC.

For RAPD data from Set B, 23 primers generated 197 bands. The number of polymorphic fragments per primer ranged from 3 (OpI15) to 15 (OpM06).

The heterozygosity index ranged from 0.766 to 1.472, with the man-made hybrids at the middle to high end of the range (1.155, 1.261 and 1.436) (Table 2).

Unique fragments

A search was made of the RFLP data for fragments that were present only in 1 species. The species that possessed 3 or more unique fragments in this data set are *C. halimii* with 4, *C. ichangensis* with 4, *C. latipes* with 5, *Cl. polyandra* with 4, and *E. glauca* with 4. *F. margarita* and *F. polyandra* each possessed 1 unique band plus 4 more found only in both *Fortunella* species but in no other genus.

Clustering of non-hybrid species

A dendrogram (Fig. 1) was constructed from the RFLP data that excluded most of the species that were cited in the literature as possible hybrids (Table 1). The very low heterozygosity indices (Table 2) of 'Ethrog' and 'Buddha's Hand' were taken as evidence that these *C. medica* cultivars are no more likely to be hybrids than *C. medica* cv 'Diamante'; therefore they were not excluded. *F. polyandra* had a lower heterozygosity index than *F. margarita*, so it was also included. *C. indica* was also included based on its low heterozygosity index and 4 unique fragments.

The species which were represented by more than 1 accession, *C. ichangensis*, *C. latipes*, *C. maxima*, and *C. medica*, all had high bootstrap values for the branch bearing the species; however, associations among species were generally poorly supported. The 15 mandarin accessions clustered together, but the bootstrapping values among them were all very low except for the branch containing *C. lycopersicaeformis* and *C. oleocarpa*. *C. indica* was strongly linked to *C. medica* (bootstrap value 96%). *C. latipes* and *C. hongheensis* were associated, as were *C. hystrix* and *C. micrantha*, but these two papeda branches did not cluster together. *C. latipes* and *C. hongheensis* were grouped with *C. maxima*, whereas *C. hystrix* and *C. micrantha* clustered with *C. halimii*, within a group containing *C. ichangensis*, *Fortunella*, *Poncirus*, *Microcitrus*, *Eremocitrus*, and *Clymenia*.

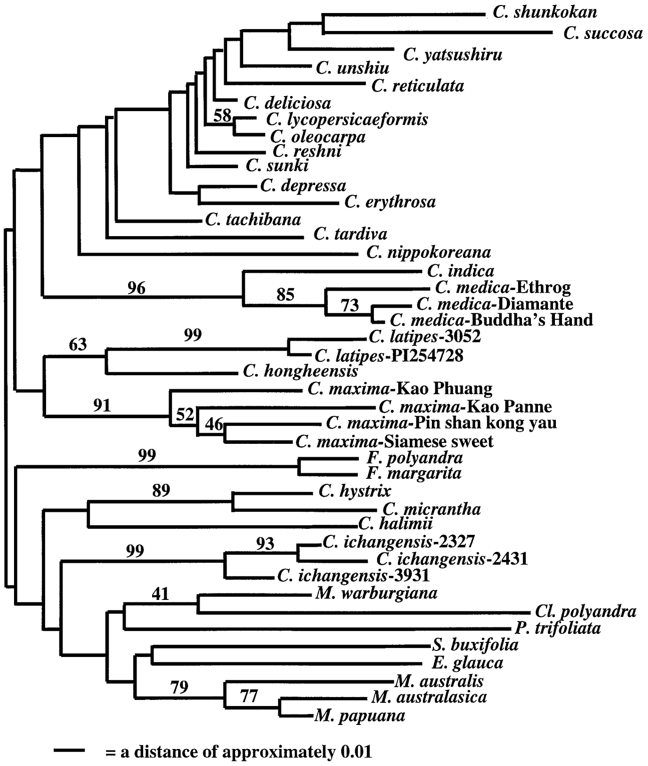

Fig. 1 Dendrogram of putative non-hybrid *Citrus* accessions and related genera from RFLP data. Numbers are bootstrap values higher than 40%, based on 500 re-samplings. The three *P. trifoliata* cultivars had identical RFLP profiles

Clustering of non-hybrid and hybrid species

As with the non-hybrid species clustering, the bootstrap values here are usually high within species but not between species, with a few exceptions (Fig. 2). *Microcitrus australis*, *M. australasica*, and *M. papuana* have bootstrap values greater than 60%. *C. hystrix* and *C. micrantha* are well linked as are *C. latipes* and *C. hongheensis*, although these two papeda branches again do not cluster together. *C. latipes* and *C. hongheensis* cluster with *C. maxima*, as they did above. *C. canaliculata* and PI254727 fall within the same group. *C. hystrix* and *C. micrantha* are loosely associated with *C. aurantifolia*, *C. macrophylla*, and *C. latifolia*. These limes and papedas are part of a larger cluster including *C. ichangensis* and all the non-*Citrus* genera. Mandarins did not form a unified cluster as they had in the non-hybrid dendrogram, but were divided into three groups. *C. clementina*, *C. reticulata*, *C. shunkokan*, *C. yatsushiro*, and *C. succosa* were in a cluster with *C. sinensis*, *C. paradisi*, *C. natsudaidai*, *C. taiwanica*, and 'New Zealand goldfruit'. *C. tachibana*, *C. nippokoreana*, *C. tardiva*, and *C. keraji* grouped together, with the remaining 9 mandarins in the third group. *C. aurantium* and *C. bergamia* branched together next to a cluster containing *C. limon*, *C. limetta*, *C. limettioides*, *C. limonia*, *C. jambhiri*, *C. medica*, *C. pennivesiculata*, and *C. indica*.

Table 2 Heterozygosity index

Accession	Heterozygosity index	Accession	Heterozygosity index
C. medica-Ethrog[a]	0.766	Pummelo 2240 X 4N Ruby[b,c]	1.155
M. warburgiana	0.780	*C. tardiva*	1.159
C. medica-Diamante	0.783	*C. miaray*[a]	1.168
C. medica-Buddha's Hand[a]	0.783	*C. sinensis*-Olinda valencia[a]	1.185
E. glauca	0.816	*C. sinensis*-Newhall navel[a]	1.185
C. indica[a]	0.829	*C. sinensis*-Ruby Blood[a]	1.185
Cl. polyandra	0.842	*C. nippokoreana*	1.188
C. ichangensis-3931	0.865	*C. taiwanica*[a]	1.205
C. tachibana	0.878	New Zealand goldfruit[a]	1.210
M. australasica	0.879	*C. pennivesiculata*[a]	1.222
M. australis	0.879	*C. clementina*[a]	1.240
C. ichangensis-2327	0.897	*C. limetta*-3093[a]	1.241
C. oleocarpa	0.905	*C. limettioides*-1482[a]	1.241
C. lycopersicaeformis	0.905	*C. aurantium*[a]	1.246
C. maderaspatana[a]	0.918	*C. succosa*	1.253
S. buxifolia	0.921	*C. limonia*-Borneo rangpur[a]	1.257
C. sunki	0.944	*C. limonia*-Philippine red lime[a]	1.257
C. deliciosa	0.950	*C. limonia*-Australia red lime[a]	1.257
C. unshiu	0.969	Rangpur X Sour Orange[b]	1.261
F. polyandra[a]	0.972	*C. paradisi*-Duncan[a]	1.279
C. hongheensis	0.989	Cuban Shaddock[a]	1.283
C. reshni	0.989	*C. limetta*-Limonette de Marrakech[a]	1.285
F. margarita	0.999	*C. bergamia*[a]	1.289
C. ichangensis-2431	1.001	*C. amblycarpa*	1.309
P. trifoliata-Rubidoux	1.002	*C. aurantifolia*-India lime[a]	1.316
P. trifoliata-Flying Dragon	1.002	*C. latifolia*[a,c]	1.317
P. trifoliata-Pomeroy	1.002	*C. limettioides*-0919[a]	1.319
C. halimii	1.010	*C. limon*-Frost Lisbon lemon[a]	1.330
M. papuana	1.020	*C. aurantifolia*-Mexican lime[a]	1.337
C. reticulata	1.032	*C. natsudaidai*[a]	1.340
C. micrantha	1.038	*C. limettioides*-0921[a]	1.341
C. latipes-PI254728	1.039	*C. aurantifolia*-Key lime[a]	1.343
C. hystrix	1.044	*C. limettioides*-0363[a]	1.345
C. erythrosa	1.050	*C. limon*-Iraq sweet lemon[a]	1.363
C. maxima-Pin shan kong yau	1.059	*C. limon*-Eureka lemon[a]	1.365
C. maxima-Siamese sweet	1.065	*C. jambhiri*[a]	1.365
C. latipes-3052	1.068	PI254727[a]	1.375
C. canaliculata[a]	1.079	*C. macrophylla*[a]	1.387
C. depressa	1.092	*C. paradisi*-343[a]	1.434
C. maxima-Kao panne	1.093	Troyer[b]	1.436
C. yatsushiro	1.108	*C. limon*-Rhobs-el-arsa[a]	1.448
C. shunkokan	1.118	*C. limetta*-Millsweet[a]	1.453
C. keraji	1.121	*C. limetta*-Faris sweet lemon[a]	1.472
C. maxima-Kao phuang	1.127		

[a] Species that are reputed to be of hybrid origin
[b] Man-made hybrids
[c] Triploids

Set B RFLP and RAPD results

The dendrogram based on RFLP data generally had lower bootstrap values and shorter branch lengths than the dendrogram based on RAPD data, which had about one-third more observations than the RFLP data set. The topologies were very similar with a few exceptions. The RAPDs placed *C. bergamia* with *C. limon* cv 'Eureka' 52% of the time, whereas the RFLPs placed *C. bergamia* with *C. limon* cv 'Rhobs-el-arsa' 35% of the time. The clustering between *C. medica*, *C. latifolia*, *C. aurantifolia*, and *C. macrophylla* was stronger with RFLPs than with RAPDs. *C. nippokoreana* was not as clearly clustered with other mandarins by RFLPs as by RAPDs. *C. maxima* was nested among papedas by RAPDs but branched next to them by RFLPs. *Microcitrus* was part of a cluster containing papedas and *C. maxima* with the RFLP data, but on a separate branch outside all *Citrus* species with the RAPD data. When the two types of data were combined, giving a total of 340 observations, most bootstrap values went up compared to either individual set for the branches that remained the same (Fig. 3). Four major clusters could be seen: (1) *Microcitrus* species,

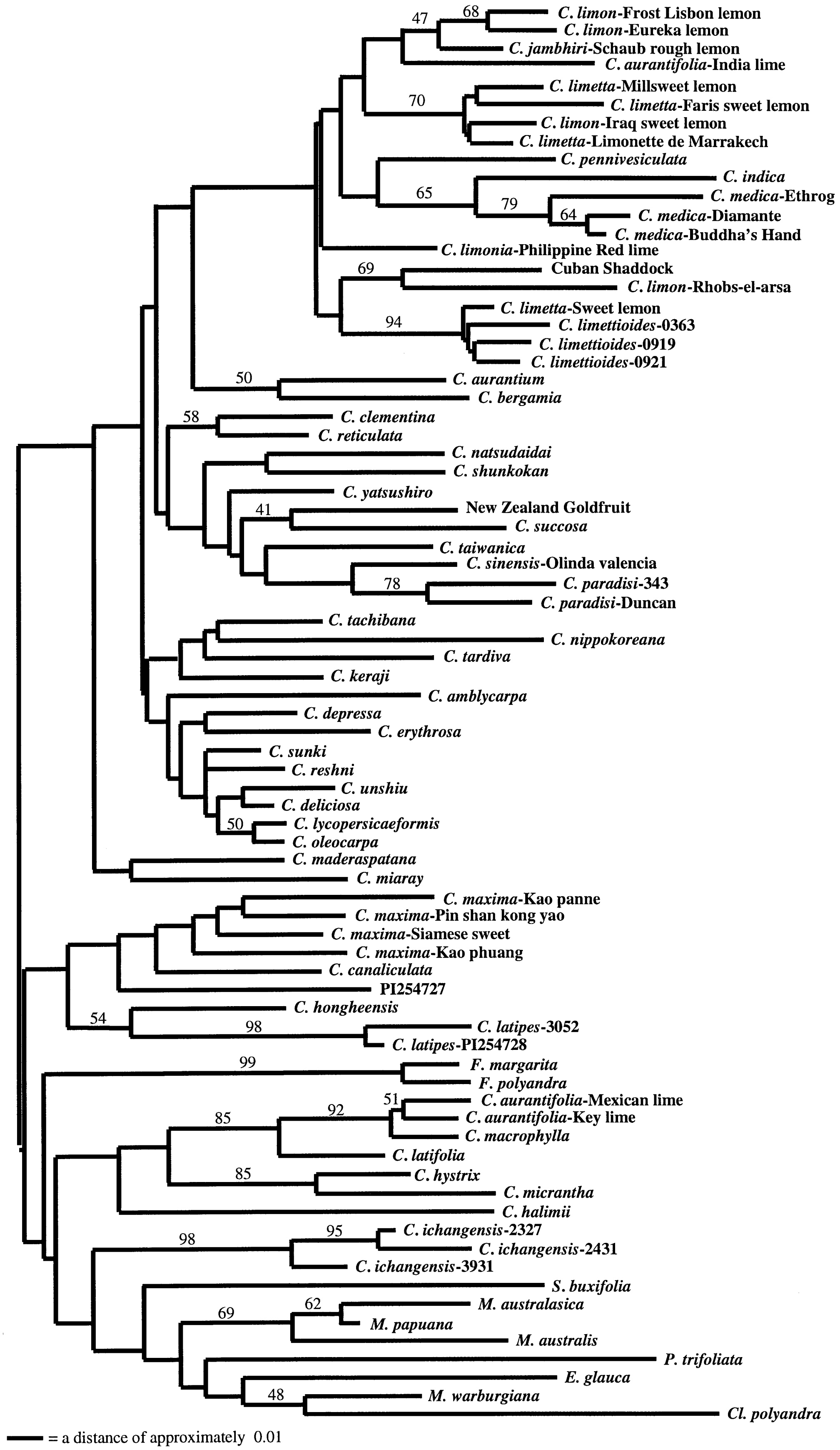

Fig. 2 Dendrogram of 85 accessions of *Citrus* and related genera from RFLP data. Numbers are bootstrap values higher than 40%, based on 500 re-samplings. Each of the following five groups were represented by only one individual on the dendrogram to save space. Their RFLP profiles were not different from one another within each group: *C. sinensis*, cvs 'Newhall' navel, 'Olinda' valencia, and 'Ruby' blood orange; *C. limetta* CRC accession 3093 and *C. limettioides* 'Palestine sweet lime'; *C. limonia* accessions 'Australian red lime', 'Borneo rangpur' and 'Philippine red lime'; *P. trifoliata* cvs 'Flying Dragon', 'Pomeroy' and 'Rubidoux'; and *C. paradisi* cvs 'Duncan' and 'Star Ruby'

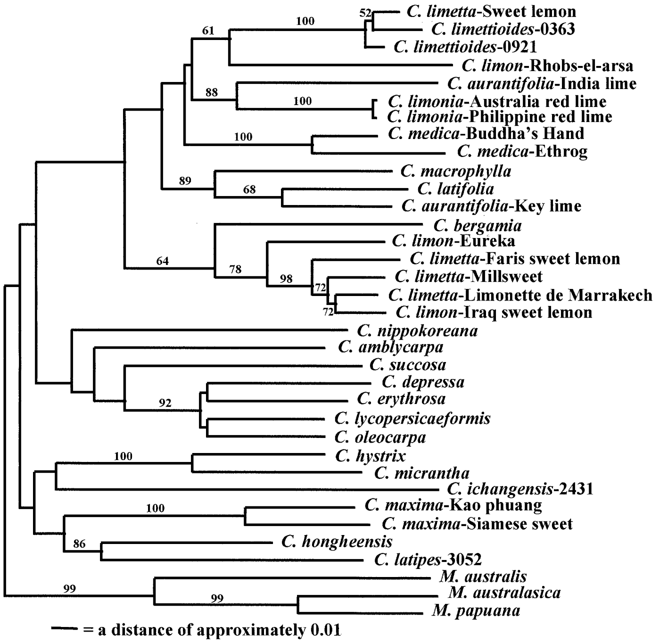

Fig. 3 Dendrogram of 32 accessions of *Citrus* and three of *Microcitrus* from combined RFLP and RAPD data. Numbers are bootstrap values higher than 40%, based on 500 re-samplings

(2) Papeda species and *C. maxima*, (3) Mandarin species, (4) *C. medica*, *C. bergamia*, *C. macrophylla*, *C. limon*, *C. limetta*, *C. aurantifolia*, *C. limettioides*, and *C. limonia*.

Discussion

Because of the widely accepted belief that some or even most *Citrus* species have been derived from hybridization, we attempted to extract from these data some information that could help decide which species are hybrids. We calculated a heterozygosity index and examined unique RFLP fragments. A higher index of heterozygosity meant that the accession had a larger number of different fragments than other accessions, as might be expected for an accession that originated by hybridization between divergent taxa. When so many species are examined, any taxon possessing several fragments not found in any other taxon is likely to have existed for a significant time in reproductive isolation from the rest. It is unlikely to be derived from hybridization between other taxa represented in the set.

The species that have been previously inferred to be of hybrid origin almost all have a higher mean fragment number than the species that are not suspected to have arisen by a hybridization event and do not possess a large number of unique fragments (Table 2). Exceptions are *C. indica* and *C. maderaspatana*, which have a low mean fragment number but have been suggested as possible *C. latipes* (Swingle and Reece 1967), or sour orange hybrids (Hodgson 1967), respectively. That *C. indica* had 4 unique RFLP fragments coupled with its low heterozygosity index argues against a hybrid origin for this species. *C. maderaspatana* did not have any unique fragments, so in spite of its low heterozygosity index we treated it as a hybrid species. The RFLP profiles of *C. medica* cvs 'Buddha's Hand' and 'Ethrog' were nearly identical to that of *C. medica* cv 'Diamante', and all 3 had very low heterozygosity indices so they probably did not derive their characteristic phenotypes from hybridization with other species as had been suggested by Blondel (1978). *C. latipes* had an intermediate heterozygosity index but 5 unique RFLP fragments, so it is probably not of hybrid origin.

Although *Severinia* is placed in subtribal group A, Primitive Citrus fruit trees (Swingle and Reece 1967), whereas the other genera examined here are all placed with *Citrus* in subtribal group C, True Citrus fruit trees, *Severinia* clusters within the related genera, rather than outside (Fig. 1). On the hybrid and non-hybrid dendrogram (Fig. 2), *Severinia* is closer to *Citrus* than other genera except *Fortunella*. The isozyme data of Herrero et al. (1996b) clustered *Poncirus* farther from *Citrus* than *Severinia* or *Microcitrus*. *Severinia* may be more closely related to *Citrus* than published phylogenies suggest.

Fortunella is nested within *Citrus* in both RFLP dendrograms, as Herrero et al. (1996b) found as well. Although *Fortunella* is well-differentiated from *Citrus* on the basis of detailed morphological studies, apparently there has not been the same level of divergence at the molecular level.

M. warburgiana clusters more closely with *Cl. polyandra* and *E. glauca* than with other *Microcitrus*. It is native to New Guinea, whereas the other three *Microcitrus* are native to Australia.

C. maxima clustered with papedas, particularly with *C. hongheensis* and *C. latipes*. Herrero et al. (1996b) found *C. maxima* clustered with the papeda *C. hystrix* within a lime/lemon/citron/pummelo group.

On the non-hybrid species dendrogram (Fig. 1), *C. medica* is on a branch with *C. indica*, between mandarins and other *Citrus*. However, *C. medica* is contained within the lemon/lime/citron cluster on the combined RFLP dendrogram (Fig. 2) as well as on the RFLP plus RAPD dendrogram (Fig. 3). This supports previous suggestions that *C. medica* is probably a parent of limes and lemons (Malik 1974; Scora 1975; Barrett and Rhodes 1976), so these species cluster together.

Swingle's (1946) and Tanaka's (1977) systems differed over the systematic treatment of mandarins. Except for *C. indica* and *C. tachibana*, wild species of India and Japan, respectively, Swingle placed all mandarins in 1 species, *C. reticulata*. However, Tanaka placed them in section Acrumen and further separated them into 36 species. In this study, the 9 mandarin species in Set B clustered in one subgroup with *C. nippokoreana* being the most distinct (Fig. 3). (Although *C. tardiva* and *C. yatsushiro* are not shown in Fig. 3 because about 20% of the RAPD data was missing, when a dendrogram was constructed with them they were in the mandarin cluster, but bootstrap values were lower.) The 15 mandarins included in the non-hybrid species dendrogram (Fig. 1) formed one cluster, but associations within the cluster were all weak. Only *C. lycopersicaeformis* and *C. oleocarpa* had bootstrap values higher than 50%. The hybrid and non-hybrid RFLP data (Fig. 2) separated mandarins into three groups but, except for the 58% bootstrap value linking *C. clementina* and *C. reticulata*, all bootstrap values were very low. This indicates that the genetic relationships among these mandarin species are fairly close. From this point of view, it might be appropriate to accept Swingle's system regarding the taxonomy of mandarins, except that our data do not support separating *C. tachibana* from the rest of the mandarins.

Tanaka places *C. indica* in the Microgroup Angustifolia with *C. tachibana*, *C. erythrosa*, *C. oleocarpa*, *C. sunki*, *C. reshni*, and *C. tardiva.* As shown in Fig. 2, *C. indica* clusters with none of these but instead clusters with *C. medica* within the lemon/lime/citron cluster. It possesses 1 unique RFLP fragment. If the plant in the UC Riverside collection is typical of the species, *C. indica* cannot be classified as a mandarin.

Major differences exist between Swingle's (1946) and Tanaka's (1977) systems regarding the taxonomy of *C. ichangensis.* Swingle placed it in the subgenus Papeda, while Tanaka classified it into the subgenus Metacitrus, which contained all mandarin species and some hybrids of *C. ichangensis* but no other papeda species at all. Zhu (1988) showed that *C. ichangensis* was a primitive *Citrus* species. Herrero et al. (1996) found that isozyme data clustered *C. ichangensis* with *C. karna* and *C. meyeri*, which are lemon types. The analysis of Fraction I protein conducted by Handa et al. (1986) showed that *C. ichangensis* was divergent from other papedas. Also, *C. ichangensis* obviously differs from the other papeda species which originated in tropical or subtropical regions by its cold hardiness and having single flowers. The present RFLP and RAPD results show that *C. ichangensis* is a distinct species very different from most other *Citrus* species, loosely aligned with *C. hystrix* and *C. micrantha*, but not easily placed by these data in relation to other species in the set. It shows more affinity than other *Citrus* with the other genera in the analysis (except *Fortunella*). It has 4 unique RFLP and 4 unique RAPD bands. It is not appropriate to place it into the subgenus Metacitrus with mandarins. Based on isozymic investigation of 4 accessions, Fang et al. (1993) suggested raising *C. ichangensis* to the third subgenus of *Citrus*, i.e. subgenus Ichang Papeda. These data do not support such a reclassification.

C. halimii clustered with *C. micrantha* and *C. hystrix*. It did not show the strong affiliation with *Fortunella* that had been seen with isozymes (Scora et al. 1988; Herrero et al. 1996b), although they are not very distant and the current placement is not well supported. Stone et al. (1973), in describing the species, noted that it could not be classed as a papeda because it lacked broadly winged petioles and droplets of acrid oil in its pulp vesicles. It possesses 4 unique RFLP fragments, a relatively high number, which emphasizes its difference from other *Citrus* species.

Swingle (1946) considered *C. macrophylla* to be a hybrid of *C. celebica*, or some other species of the subgenus Papeda, with a species of the subgenus *Citrus*, probably *C. maxima.* Tanaka placed it in the section Limonellus along with *C. aurantifolia.* Herrero et al. (1996a, b) showed that *C. macrophylla* clustered with papedas, but not near *C. aurantifolia.* Our results indicate that *C. macrophylla* clusters with *C. aurantifolia*, and the papedas *C. hystrix* and *C. micrantha. C. macrophylla*, and *C. aurantifolia* possess 2 fragments that are also found only in *C. micrantha* and *C. hystrix*, 1 that is only in *C. micrantha* and *C. ichangensis* and 1 that is found in all four papeda species and *M. australis.* This supports Scora's (1988) assertion that *Microcitrus* is a possible ancestor of *C. aurantifolia.*

The papedas are a very diverse group. The RFLP data do not cluster them all together. The RFLP plus RAPD data do, although *C. maxima* is also within the same cluster and the branch lengths are very long.

C. limetta includes a group of cultivars which are called sweet lemon, while *C. limettioides* composes a group of so-called sweet lime cultivars. Unfortunately, some so-called sweet limes, such as Mediterranean sweet lime, should be placed in *C. limetta* (Hodgson 1967). Likewise, some sweet lemon cultivars may really be *C. limettioides.* The current results show that except for *C. limetta* CRC accession 3093, all other *C. limetta* cultivars ('Millsweet' lemon, 'Faris' sweet lemon and 'Limonette de Marrakech') are in the same small group along with *C. limon* accession Iraq sweet lemon. The four *C. limettioides* cultivars branch closely together along with *C. limetta* CRC accession 3093. They might be nucellar or sport mutations from one source. *C. limetta* CRC accession 3093 might be a *C. limettioides* cultivar or its hybrid with *C. limon* or *C. limetta.*

One accession in this set, PI254727, was originally classified as *C. latipes.* When it clustered with *C. canaliculata* and *C. maxima* rather than with other *C. latipes*, the tree in the field was examined again and found to differ from other *C. latipes* in leaf morphology. This example, as well as *C. limon*-Iraq sweet lemon and *C. limetta* CRC accession 3093, reveal the power of molecular markers to clarify the status of incorrectly classified plants. They can also show relationships among individuals of the same or closely related species, but the markers studied here seem to be less powerful at defining relationships between more distantly related taxa.

There is support in these data for a hybrid origin of many species of *Citrus*. Relationships between major groups revealed by the data indicate *C. maxima* has some affiliation with some papedas. *Fortunella* and *Citrus* are not separated. *M. warburgiana* is very different from other *Microcitrus*. Mandarin species do not cluster in the groups Tanaka used.

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