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**E. Nicolosi · Z.N. Deng · A. Gentile · S. La Malfa**
**G. Continella · E. Tribulato**

# Citrus phylogeny and genetic origin of important species as investigated by molecular markers

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**Abstract** Citrus phylogeny was investigated using RAPD, SCAR and cpDNA markers. The genotypes analyzed included 36 accessions belonging to *Citrus* together with 1 accession from each of the related genera *Poncirus, Fortunella, Microcitrus* and *Eremocitrus*. Phylogenetic analysis with 262 RAPDs and 14 SCARs indicated that *Fortunella* is phylogenetically close to *Citrus* while the other three related genera are distant from *Citrus* and from each other. Within *Citrus*, the separation into two subgenera, *Citrus* and *Papeda*, designated by Swingle, was clearly observed except for *C. celebica* and *C. indica*. Almost all the accessions belonging to subgenus *Citrus* fell into three clusters, each including 1 genotype that was considered to be a true species. Different phylogenetic relationships were revealed with cpDNA data. *Citrus* genotypes were separated into subgenera *Archicitrus* and *Metacitrus*, as proposed by Tanaka, while the division of subgenera *Citrus* and *Papeda* disappeared. *C. medica* and *C. indica* were quite distant from other citrus as well from related genera. *C. ichangensis* appeared to be the ancestor of the mandarin cluster, including *C. tachibana*. Lemon and Palestine sweet lime were clustered into the Pummelo cluster led by *C. latipes*. *C. aurantifolia* was located in the Micrantha cluster. Furthermore, genetic origin was studied on 17 cultivated citrus genotypes by the same molecular markers, and a hybrid origin was hypothesized for all the tested genotypes. The assumptions are discussed with respect to previous studies; similar results were obtained for the origin of orange and grapefruit. Hybrids of citron and sour orange were assumed for lemon, Palestine sweet lime, bergamot and Volkamer lemon, while a citron × mandarin hybrid was assumed for Rangpur lime and Rough lemon. For Mexican lime our molecular data indicated *C. micrantha* to be the female parent and *C. medica* as the male one.

**Key words** *Citrus* · RAPD · SCAR · cpDNA · Phylogeny · Origin

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E. Nicolosi (✉) · Z.N. Deng · A. Gentile · S. La Malfa
G. Continella · E. Tribulato
Istituto di Coltivazioni arboree, University of Catania,
Via Valdisavoia, 5, 95123 Catania, Italy
Fax: +39-095-356322
e-mail: nicoeli@mbox.fagr.unict.it

## Introduction

Different hypotheses have been formulated on the origin of *Citrus*. In general, *Citrus* is believed to have originated in the tropical and subtropical regions of Southeast Asia and then spread to other continents (Webber 1967; Calabrese 1992). *Citrus* taxonomy and phylogeny, however, are very complicated, controversial and confusing, mainly due to sexual compatibility between *Citrus* and related genera, the high frequency of bud mutations and the long history of cultivation and wide dispersion.

In the past, studies on relationships between genera and species were carried out based mainly on morphological characteristics. Numerous classification systems have been formulated, among which those of Swingle (1943) and Tanaka (1977) have been the most widely accepted. Even these two researchers, however, have quite different concepts with respect to species classification, as Swingle included only 16 species in *Citrus* while Tanaka described 162. Later phylogenetic analysis by Scora (1975) and Barrett and Rhodes (1976) suggested that there were only 3 true species within the cultivated *Citrus*, i.e. citron (*Citrus medica* L.), mandarin (*C. reticulata* Blanco) and pummelo [*C. grandis* (L.) Osb.] (in 1988 Scora added another true species: *C. halimii* Stone). The other genotypes were derived from hybridization between these true species. More recently, this concept has gained further support from various studies using biochemical and molecular markers, including isozymes (Torres et al. 1978; Fang et al. 1993; Herrero et al. 1996), organeller genome analysis (Green et al. 1986; Yamamoto et al. 1993) and microsatellites (Fang and Roose 1997; Fang et al. 1998).

**Table 1** Genotypes used for RAPD, SCAR and cpDNA analysis

| No. | Common name | Swingle system | Tanaka system | Source[a] |
|---|---|---|---|---|
| 1 | "Etrog" citron | *C. medica* L. | *C. medica* L. | A |
| 2 | "Avana" mandarin | *C. reticulata* Blanco | *C. deliciosa* Ten. | A |
| 3 | "Ponkan" mandarin | *C. reticulata* Blanco | *C. poonensis* Tan. | A |
| 4 | "Dancy" mandarin | *C. reticulata* Blanco | *C. tangerina* Hort. ex Tan. | A |
| 5 | "Huangpi Ju" mandarin | *C. reticulata* Blanco | *C. chuana* Hort. ex Tseng | C |
| 6 | "Zhu Ju" mandarin | *C. reticulata* Blanco | *C. succosa* Hort. ex Tan. | C |
| 7 | "Nan Ju" mandarin | *C. reticulata* Blanco | *C. tangerina* Hort. ex Tan. | C |
| 8 | "Zhu Hong Ju" mandarin | *C. reticulata* Blanco | *C. erythrosa* Hort. ex Tan. | C |
| 9 | "Sha Tian Yu" pummelo | *C. grandis* (L.) Osb. | *C. grandis* (L.) Osb. | C |
| 10 | "Duncan" grapefruit | *C. paradisi* Macf. | *C. paradisi* Macf. | A |
| 11 | Sour orange | *C. aurantium* L. | *C. aurantium* L. | A |
| 12 | "Biondo comune" sweet orange | *C. sinensis* (L.) Osb. | *C. sinensis* (L.) Osb. | A |
| 13 | "Femminello" lemon | *C. limon* (L.) Burm.f. | *C. limon* (L.) Burm.f. | A |
| 14 | Mexican lime | *C. aurantifolia* (Christm.) Swing. | *C. aurantifolia* (Christm.) Swing. | A |
| 15 | Palestine sweet lime | *C. aurantifolia* (Christm.) Swing. | *C. limettioides* Tan. | A |
| 16 | Tachibana orange | *C. tachibana* (Mak.) Tan. | *C. tachibana* (Mak.) Tan. | C |
| 17 | Indian wild orange | *C. indica* Tan. | *C. indica* Tan. | B |
| 18 | King mandarin[b] | *C. reticulata* Blanco | *C. nobilis* Lour. | A |
| 19 | "Okitzu" satsuma | *C. reticulata* Blanco | *C. unshiu* Marc. | A |
| 20 | Cleopatra mandarin[b] | *C. reticulata* Blanco | *C. reshni* Hort. ex Tan. | B |
| 21 | Alemow[b] | *C. aurantifolia* (Christm.) Swing. | *C. macrophylla* Wester | A |
| 22 | Rough lemon[b] | *C. limon* (L.) Burm.f. | *C. jambhiri* Lush. | B |
| 23 | Rangpur lime[b] | *C. limon* (L.) Burm.f. | *C. limonia* Osbeck | A |
| 24 | Murcott[b] | | | A |
| 25 | "Comune" clementine[b] | *C. reticulata* Blanco | *C. clementina* Hort. ex Tan. | A |
| 26 | Tankan[b] | | *C. tankan* Hay. | B |
| 27 | Volkamer lemon[b] | | *C. volkameriana* Ten. & Pasq. | A |
| 28 | Yuzu[b] | | *C. junos* Sieb. ex Tan. | B |
| 29 | Gou Tou Cheng[b] | *C. aurantium* L. ? | *C. aurantium* L. ? | B |
| 30 | Bergamot[b] | *C. aurantifolia* (Christm.) Swing. | *C. bergamia* Risso & Poit. | A |
| 31 | Celebes papeda | *C. celebica* Koord. | *C. celebica* Koord. | B |
| 32 | Mauritius papeda | *C. hystrix* D.C. | *C. hystrix* D.C. | B |
| 33 | Ichang papeda | *C. ichangensis* Swing. | *C. ichangensis* Swing. | B |
| 34 | Khasi papeda | *C. latipes* (Swing.) Tan. | *C. latipes* (Swing.) Tan. | B |
| 35 | Melanesian papeda | *C. macroptera* Montr. | *C. macroptera* Montr. | B |
| 36 | Small-flowered papeda | *C. micrantha* Wester | *C. micrantha* Wester | B |
| 37 | Trifoliate orange | *Poncirus trifoliata* (L.) Raf. | | B |
| 38 | Kumquat | *Fortunella margarita* (Lour.) Swing. | | A |
| 39 | Australian round lime | *Microcitrus australis* (Planch.) Swing. | | B |
| 40 | Australian desert lime | *Eremocitrus glauca* (Lindl.) Swing. | | B |

[a] A, Istituto di Coltivazioni arboree, University of Catania, Italy; B, Istituto Sperimentale per l'Agrumicoltura, Italy; C, Hunan Horticultural Research Institute, Changsha, China
[b] Not used for cpDNA analysis

DNA markers have been widely used for taxonomy studies in many plants (Tingey and Tufo 1993; Whitkus et al. 1994). Among them, random amplified polymorphic DNA (RAPDs) have been employed most widely, since this technique is more simple and less expensive than restriction fragment length polymorphism (RFLPs) (Dos Santos et al. 1994). In *Citrus*, RAPDs have been used for cultivar identification (Deng et al. 1995, 1996) and mapping (Cai et al. 1994). Federici et al. (1998) used RAPDs to study the phylogenetic relationship among *Citrus* and related genera in combination with RFLP polymorphisms.

In 1993, Paran and Michelmore, in order to characterize RAPD markers, developed a technique which they named sequence-characterized amplified regions (SCARs), which generated polymerase chain reaction (PCR) products of higher specificity and fidelity than RAPDs. SCAR analysis has led to the detection of some special markers strictly linked to genes encoding the resistance to anthracnose in bean (Adam-Blondom et al. 1994) and to mosaic virus in tomato (Ohmori et al. 1996), as well as to tristeza virus in citrus (Deng et al. 1997).

Chloroplast DNA (cpDNA) analysis is especially useful in phylogenetic analyses due to its evolutionary conservatism, relative abundance in plant tissue, small size and predominant uniparental inheritance (Olmstead and Palmer 1994). Organeller RFLPs have been used for determining the phylogenetic inheritance (Olmstead and Palmer 1994). Organeller RFLPs have been used for determining the phylogenetic relationships among *Citrus* (Green et al. 1986) and among species and cultivars in the genera *Citrus*, *Poncirus* and *Fortunella* (Yamamoto et al. 1993).

PCR was employed to amplify non-coding regions of mitochondrial (mt) and chloroplast DNA in plants with "universal" primers, and polymorphisms were subsequently generated by digestion of the PCR products with endonucleases (Demesure et al. 1995). While this type of analy-

**Table 2** SCAR primers identified by designated names with original RAPDs, sequences and annealing temperatures

| SCAR primer | RAPD fragments | Number of bases | Sequences[a] | Annealing temperature (°C) |
|---|---|---|---|---|
| SC1 | Pummelo K10-900 | 24 | gTgCAACgTgAATTgAgAggATTT | 60 |
| | | 24 | gTgCAACgTgTgCCCATgAACAAT | |
| SC2 | Pummelo H14-450 | 20 | ACCAggTTgggAgTATTATA | 60 |
| | | 20 | ACCAggTTggCgTCTTgCCg | |
| SC3 | Citron S07-600 | 24 | TCCgATgCTgTTgAgTTggCTAAT | 65 |
| | | 24 | TCCgATgCTgCAgTTgTAgCTTCC | |
| SC4 | Citron H07-500 | 21 | CTgCATCgTgTATTTgATAgg | 60 |
| | | 21 | CTgCATCgTgACTCATgTAgT | |
| SC5 | Mandarin G11-800 | 24 | TgCCCgTCgTATgTTTATAACAgT | 68 |
| | | 24 | TgCCCgTCgTCCAAgTTAACAggg | |
| SC6 | Mandarin W01-550 | 20 | ggACCggCgAgACgCACTAC | 60 |
| | | 20 | TCCAgCAgTTggCgATAgCC | |
| SC7 | Palestine sweet lime U11-500 | 20 | AgACCCAgAgTgAAgATgAg | 62 |
| | | 20 | AgACCCAgAgAgCACACAAA | |
| SC8 | Sour orange G04-800 | 20 | AgCgTgTCTgggTTggAgTg | 65 |
| | | 20 | AgCgTgTCTgAAACAAAAgT | |
| SC9 | Mexican lime V05-500 | 20 | TCCgAgAGGGCAATAAAACC | 60 |
| | | 20 | ATCTggAgCTACgTTggCAA | |

[a] The underlined nucleotides are sequences of the original Operon random primers

**Table 3** cpDNA universal primer sequences, size of amplified fragments and percentage of bases searched by endonucleases

| Primer sequences | | | | Amplified fragments (bp) | Analyzed bases (%) |
|---|---|---|---|---|---|
| Forward | | Reverse | | | |
| *trn*H | 5′-ACGGGAATTGAACCCGCGCA-3′ | *trn*K | 5′-CCGACTAGTTCCGGGTTCGA-3′ | 1750 | 9.54 |
| *trn*D | 5′-ACCAATTGAACTACAATCCC-3′ | *trn*T | 5′-CTACCACTGAGTTAAAAGGG-3′ | 1600 | 11.63 |
| *psb*C | 5′-GGTCGTGACCAAGAAACCAC-3′ | *trn*S | 5′-GGTTCGAATCCCTCTCTCTC-3′ | 1650 | 12.06 |
| *trn*S | 5′-GAGAGAGAGGGATTCGAACC-3′ | *trnf*M | 5′-CATAACCTTGAGGTCACGGG-3′ | 200 | 0 |
| *trn*S | 5′-CGAGGGTTCGAATCCCTCTC-3′ | *trn*T | 5′-AGAGCATCGCATTTGTAATG-3′ | 1300 | 9.54 |
| *trn*M | 5′-TGCTTTCATACGGCGGGACT-3′ | *rbc*L | 5′-GCTTTAGTCTCTGTTTGTGG-3′ | 2700 | 10.67 |
| Total | | | | 9000 | 10.7 |

sis has been widely used for phylogeny studies, no report of this technique being used in *Citrus* has been found.

As mentioned above, numerous studies have been carried out on the origin of citrus and its taxonomy; however, the results have not been always in agreement. A clearer understanding of the citrus genetic background is necessary for better a characterization and utilization of citrus germplasm. Molecular markers have been widely employed to clarify phylogenetic relationships in many plants, and they could be equally useful in citrus taxonomy studies. Therefore, we investigated the phylogenetic relationships among *Citrus* and its relatives and studied the origin of some important citrus species using molecular markers, namely, RAPDs, SCARs and cpDNA analysis.

## Materials and methods

### Plant materials and DNA extraction

Thirty genotypes belonging to subgenus *Cirus* and six to subgenus *Papeda* were sampled for RAPD and SCAR analysis; 23 of these were also used for cpDNA analysis. Four related genera were included in both analyses for comparison (Table 1). The plant materials were provided by germplasm collection of Istituto di Coltivazioni arboree, University of Catania (Italy), the Istituto Sperimentale per l'agrumicoltura (Italy) and the Hunan Horticultural Research Institute, Changsha (China).

For genetic characterization of RAPDs and SCARs, two crosses were made using a pummelo as the female parent and "Etrog" citron and "Dancy" mandarin as pollen parents. Individuals from the two $F_1$ progenies were analyzed for the segregation of the molecular markers.

Total DNA was extracted from young leaves according to the protocol of Doyle and Doyle (1987) as modified by Deng et al. (1995).

### RAPD analysis

Initially, 200 arbitrary decamer primers (Operon Technologies) were tested on pummelo, citron and mandarin, and those that generated a higher degree of polymorphisms were then selected to analyze all the genotypes. Amplification was performed in a Cetus GenAmp PCR System 9600 (Perkin Elmer) using the reaction mixtures and PCR parameters described by Deng et al. (1995). Amplification products were separated on a 1.5% agarose gel in 0.5 × TBE buffer, stained with ethidium bromide and detected under UV light.

### SCAR analysis

The chosen RAPD fragments were purified from the gel with a Gel-Purification Kit (Qiagen GmbH). The purified DNA was re-

**Table 4** SCAR polymorphisms[a] among 40 genotypes of *Citrus* and related genera generated with 8 primers[b] and compared with their original RAPDs[c]

| Genotypes | R1 | SC1 | R2 | SC2 | | | R3 | SC3 | | R4 | SC4 | | R5 | SC5 | R7 | SC7 | | | R8 | SC8 | R9 | SC9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 900 | | 250 | 450 | 1500 | | 500 | 600 | | 300 | 750 | | 800 | | 350 | 500 | 1000 | | 800 | | 500 |
| *C. medica* | – | – | – | + | – | + | + | – | + | + | – | – | – | + | – | – | – | + | – | – | – | – |
| *C. deliciosa* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. poonensis* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. tangerina* Dancy | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. chuana* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. succosa* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. tangerina* Nan Ju | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. erythrosa* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. grandis* | + | + | + | + | + | – | – | – | – | – | – | + | – | + | – | – | – | – | – | – | – | – |
| *C. paradisi* | + | + | + | – | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. aurantium* | + | + | + | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | + | + | – | – |
| *C. sinensis* | – | – | + | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. limon* | – | – | – | + | – | + | + | – | + | + | – | + | + | + | – | – | – | + | + | + | – | – |
| *C. aurantifolia* | – | – | – | + | + | + | + | – | + | + | – | + | – | + | – | – | – | + | – | – | + | + |
| *C. limettioides* | – | – | + | – | + | + | + | – | + | + | – | + | + | + | + | – | + | – | – | – | – | – |
| *C. tachibana* | – | – | – | + | – | – | – | + | – | – | – | + | – | + | – | – | – | – | – | – | – | – |
| *C. indica* | – | – | – | – | – | + | – | + | – | – | – | – | – | + | – | – | – | + | + | – | – | – |
| *C. nobilis* | – | – | – | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. unshiu* | – | – | – | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. reshni* | – | – | – | + | – | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. macrophylla* | – | – | – | + | + | + | + | – | + | + | – | + | – | + | + | – | + | – | – | – | – | + |
| *C. jambhiri* | – | – | – | + | – | – | + | – | + | + | – | + | + | + | + | – | + | + | – | – | – | – |
| *C. limonia* | – | – | – | + | – | + | + | – | + | + | – | + | + | + | – | – | – | – | – | – | – | – |
| Murcott | – | – | – | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. clementina* | – | – | – | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. tankan* | – | – | + | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. volkameriana* | – | – | – | + | – | + | + | + | + | + | – | + | + | + | – | – | + | – | – | – | – | – |
| *C. junos* | – | + | – | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| Gou Tou Cheng | – | + | + | + | + | – | – | + | – | – | – | + | + | + | – | – | – | – | – | – | – | – |
| *C. bergamia* | – | + | – | + | – | + | + | – | + | + | – | + | + | + | – | – | – | + | + | + | – | – |
| *C. latipes* | – | – | – | + | + | – | – | + | – | – | – | + | – | + | – | – | – | – | – | – | – | – |
| *C. macroptera* | – | – | – | + | + | – | – | – | – | – | – | + | – | + | – | – | – | – | – | – | – | + |
| *C. micrantha* | – | – | – | + | + | – | – | – | – | – | – | + | – | + | – | – | – | – | – | – | + | + |
| *Poncirus* | – | – | – | – | – | – | – | – | – | – | – | + | – | – | – | – | – | – | – | – | – | – |
| *Fortunella* | – | – | – | + | + | – | – | – | – | – | – | + | – | + | – | – | – | – | – | – | – | – |
| *Microcitrus* | – | – | – | + | – | – | – | – | – | – | – | + | – | + | – | + | – | – | – | – | – | – |
| *Eremocitrus* | – | – | – | – | – | – | – | – | – | – | + | + | – | – | – | – | – | – | – | – | – | – |

[a] Polymorphisms are indicated with + for the presence and – for absence of SCAR fragments

[b] The primer SC6 did not generate any PCR product, so it is not listed in the table

[c] The original RAPDs are indicated as follows: R1 = K10-900, R2 = H14-450, R3 = S07-600, R4 = H07-500, R5 = G11-800, R7 = U11-500, R8 = G04-800, R9 = V05-500

**Table 5** Site mutations detected on amplified cpDNA fragments

| Primer | Enzymes | Mutations sites (bp) | | Primer | Enzymes | Mutation sites (bp) | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | | | 0 | 1 |
| *trn*H/*trn*K | *Alu* I | 600 | 290 + 310 | *trn*D/*trn*T | *Alu* I | 590 | 250 + 340 |
| | *Alu* I | 490 | 450 + 40[a] | | *Alu* I | 490 | 390 + 100 |
| | *Alu* I | 490 | 400 + 100 | | *Hae*III | 1620 | 1170 + 450 |
| | *Alu* I | 490 | 250 + 150 | | *Hae*III | 1570 | 1060 + 510 |
| | *Taq* I | 220 | 180 + 40[a] | | *Hae*III | 1170 | 650 + 520 |
| | *Hae*III | 800 | 7500+ 50[a] | | *Hae*III | 1170 | 670 + 500 |
| | *Xba* I | 600 | 540 + 60[a] | | *Mva* I | 1450 | 930 + 520 |
| | *Xba* I | 200 | 180 + 20[a] | | *Mva* I | 1550 | 1000 + 550 |
| | *Hinf* I | 880 | 240 + 640 | | *Mva* I | 1550 | 930 + 620 |
| | *Hinf* I | 880 | 450 + 430 | | *Mva* I | 550 | 450 + 100 |
| | *Hinf* I | 750 | 570 + 180 | | *Taq* I | 380 | 360 + 20[a] |
| | *Tru*9 I | 1150 | 950 + 200 | | *Taq* I | 290 | 280 + 10[a] |
| | *Tru*9 I | 300 | 270 + 30[a] | | *Taq* I | 190 | 180 + 10[a] |
| | *Tru*9 I | 200 | 190 + 10[a] | | *Sau*3 A I | 310 | 300 + 10[a] |
| | *Tru*9 I | 120 | 80 + 40[a] | | *Sau*3 A I | 250 | 240 + 10[a] |
| | *Hha* I | 1500 | 1300 + 200 | | *Sau*3 A I | 250 | 230 + 20[a] |
| | *Hha* I | 300 | 250 + 50[a] | | *Sau*3 A I | 180 | 150 + 30[a] |
| | *Hha* I | 300 | 280 + 20[a] | | *Hinf* I | 300 | 280 + 20[a] |
| | *Sau*3 A I | 300 | 160 + 140 | | *Hinf* I | 240 | 230 + 10[a] |
| | *Sau*3 A I | 260 | 240 + 20[a] | | *Hinf* I | 140 | 120 + 20[a] |
| | *Sau*3 A I | 230 | 190 + 40[a] | | *Sty* I | 520 | 400 + 120 |
| *psb*C/*trn*S | *Sau*3 A I | 300 | 280 + 20 | | *Sty* I | 400 | 380 + 20[a] |
| | *Alu* I | 120 | 80 + 40 | | *Rsa* I | 1620 | 1000 + 620 |
| *trn*S/*trn*T | *Alu* I | 900 | 500 + 400 | | *Rsa* I | 1000 | 650 + 350 |
| *trn*M/*rbc*L | *Hinf* I | 200 | 190 + 10[a] | | *Rsa* I | 1000 | 800 + 200 |
| | *TaqI* | 220 | 180 + 40[a] | | *Sma* I | 1620 | 1420 + 200 |
| | *Taq* I | 220 | 160 + 60[a] | | *Sma* I | 1620 | 1400 + 220 |
| | *Rsa* I | 750 | 720 + 30[a] | | *Tru9* I | 1020 | 560 + 460 |
| | *Sau*3 A I | 800 | 780 + 20[a] | | *Tru*9 I | 1020 | 580 + 440 |
| | *Sau*3 A I | 530 | 500 + 30[a] | | *Tru*9 I | 1020 | 600 + 420 |
| | *Sty* I | 750 | 700 + 50[a] | | *Hpa* II | 1400 | 1200 + 200 |
| | *Hha* I | 500 | 490 + 10[a] | | | | |

[a] Small fragments were not visible on agarose gel

amplified using the same primer and inserted into TA Cloning plasmid (Invitrogen). The cloned RAPD products were sequenced using an automated DNA sequencer (310 ABI Prism Genetic Analyzer, Perkin Elmer).

Two specific oligonucleotides were then synthesized for each RAPD fragment (Gibco Brl Life Technologies). The SCAR primers were composed of the original arbitrary primer (10-mer) followed by 10–14 immediately adjacent nucleotides. These primers were used to amplify DNA of all the genotypes tested. PCR was performed for 30 cycles and an annealing temperature ranging from 60°C to 68°C was set up for each pair of SCAR primers (Table 2).

### Chloroplast DNA analysis

The cpDNA of the 27 genotypes was amplified using 6 pairs of "unversal" primers (Table 3) (Demesure et al. 1995). The PCR consisted of 5 ng of template DNA, 1× buffer [1.0 ml TRIS-HCl, 500 m*M* KCl (pH 8.3)], 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, 2 µ*Taq* polymerase, 1 µ*M* of each primer. The parameters of the amplification reaction were: 1 cycle of 2 min at 92°C; 10 cycles of 5 s at 92°C, 15 s at 55°C, 1 min at 72°C, and 20 cycles of 5 s at 92°C, 15 s at 55°C, 1 min at 72°C, plus 10 s for each cycle and an end cycle of 10 min at 72°C.

The cpDNA fragments obtained were digested with restriction endonucleases (*Alu*I, *Bam*HI, *Bcl*I, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hin*dIII, *Hinf*I, *Hpa*II, *Msp*I, *Mva*I, *Not*I, *Pst*I, *Pvu*II, *Rsa*I, *Sau*3AI, *Sca*I, *Sma*I, *Sty*I, *Taq*I, *Tru*9I, *Xba*I) and then electrophoresed on 2% agarose gels.

### Data analysis

The polymorphic bands were scored as 1 for presence and 0 for absence. Two similarity matrices were then constructed, one based on RAPD and SCAR markers and another based on cpDNA markers. PAUP ver.3.1 (Swofford 1993) was used for phylogenetic analysis and tree construction. Bootstrap estimates were calculated for 500 re-samplings, and a tree was constructed for each set of data with 50% majority rule consensus. To provide higher confidence in the phylogenetic analysis, we also performed Polymorphism Parsimony (PHYLIP: Phylogeny Inference Package 3.57; Felsestein 1995).

## Results

### RAPD polymorphisms

Of the 200 primers used, 192 generated amplification products in the 3 genotypes tested (pummelo, citron and mandarin). From these 192 primers, the 70 that produced a greater number of polymorphic bands and ones that were quite evident were chosen for analyzing all 40 genotypes. With each selected primer, 3–15 polymorphic fragments were obtained and a total of 262 useful markers were revealed. Among the polymorphic RAPDs,

those present only in 1 genotypes were considered to be unique fragments. A search for unique bands was made for all the species tested, and it was found that *Poncirus trifoliata* possessed 10 unique fragments, *Microcitrus australis* 13 and *Eremocitrus glauca* 11 fragments, while, *Fortunella margarita*, belonging to the fourth related genera analyzed, had only 3 unique markers. Within *Citrus* species, few unique bands were revealed, e.g. 7 for *C. indica*, 2 for *C. macroptera*, *C. limonia* and *C. ichangensis* and 1 for *C. grandis*, *C. celebica*, *C. latipes*, *C. aurantium* and *C. succosa* ("Zhu Ju" mandarin).

## SCAR analysis

Nine RAPD markers (K10-900, H14-450, S07-600, H07-500, G11-800, U11-500, G04-800, V05-500, W01-550) were chosen for converting into SCARs. All of them were successfully cloned into plasmid and sequenced. Correspondent SCAR primers were synthesized and successively used to amplify the DNA of all genotypes examined (Table 2). Among them, one pair of primers (SC6 derived from W01-550) did not generate any PCR product, while the other 8 produced 14 polymorphic fragments (Table 4). A fragment of the same base-pair as in the original RAPD was amplified with all of primers, with the exception of SC4. However, differences were observed between SCAR and RAPD polymorphisms. In addition, 1 or 2 new polymorphic bands were obtained with 4 SCAR primers (SC2, SC3, SC4 and SC7).

## cpDNA analysis

For the cpDNA analysis, six pairs of universal primers were used as reported in Table 3. Each primer generated only 1 monomorphic fragment, each ranging in size from 1300 to 2700 bp except for one (*trn*S/*trnf*M), which produced a short fragment (200 bp) that was subsequently excluded from successive analyses. In order to generate polymorphisms from the monomorphic bands obtained, we used 25 restriction enzymes. Among these, 20 were able to recognize at least 1 restriction site. A total of 210 sites were found corresponding to 964 bp. From these recognized sites, 63 mutations (insertion/deletion and substitutions) were detected, and 140 cpDNA markers were generated (Table 5). Most of the mutations were detected from the fragment amplified by primer *trn*D/*trn*T (31) and *trn*H/*trn*K (21).

## Phylogenetic analysis

Based on the RAPD and SCAR data, the genetic distance between each pair of genotypes was calculated by PAUP analysis. The mean genetic distances ranged from 0.004 among various mandarins to 0.456 between *C. aurantifolia* and *C. aurantium*. Of course, higher distances were also found between genera ranging from 0.322 to 0.329 (matrix available on request).

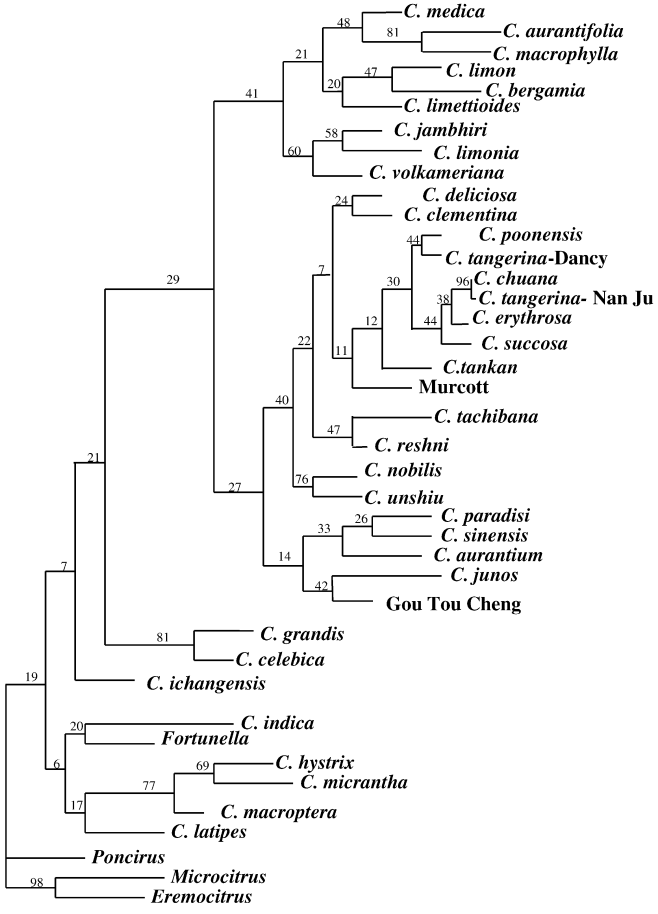


**Fig. 1** A 50% majority rule consensus tree (PAUP tree) for 40 genotypes of *Citrus* and related genera derived from bootstrap analysis (500 replications) of RAPD and SCAR data with confidence levels for arms

Ten bootstrap trees were obtained in the cluster analysis by PAUP, and a consensus tree with 50% majority rule was constructed (Fig. 1). This dendrogram indicated that the genus *Citrus* is quite distant from the related genera *Poncirus*, *Microcitrus* and *Eremocitrus* but not from *Fortunella*.

Eight clusters were separated as follows:

1) Citron cluster: *C. medica*, *C. aurantifolia*, *C. macrophylla*, *C. limon*, *C. bergamia*, *C. limettioides*, *C. jambhiri*, *C. limonia* and *C. volkameriana*;
2) Mandarin cluster: all mandarin and mandarin-like accessions as well as *C. tachibana* in the first sub-cluster; *C. paradisi*, *C. aurantium*, *C. sinensis*, *C. junos* and Gou Tou Cheng in the second sub-cluster;
3) Pummelo cluster: *C. grandis* and *C. celebica*, a Papeda species;
4) Ichang cluster: *C. ichangensis*;
5) Fortunella cluster: *F. margarita* and *C. indica*;
6) Micrantha cluster: *C. hystrix*, *C. micrantha*, *C. macroptera* and *C. latipes*, all belonging to subgenus *Papeda*;

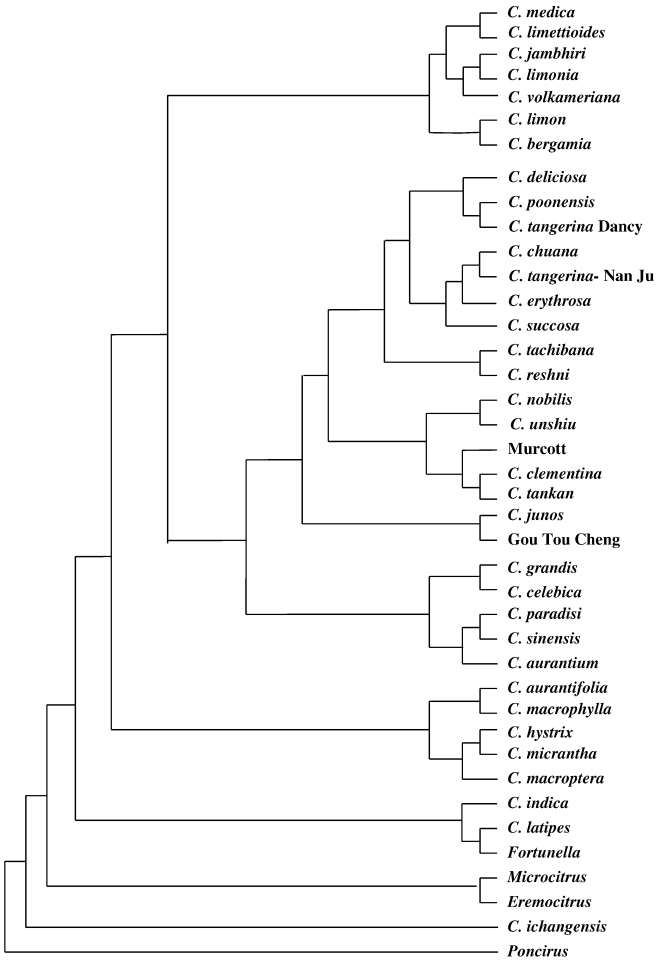


**Fig. 2** Dendrogram (PP tree) for 40 genotypes of *Citrus* and related genera obtained by Polymorphism Parsimony analysis of RAPD and SCAR data

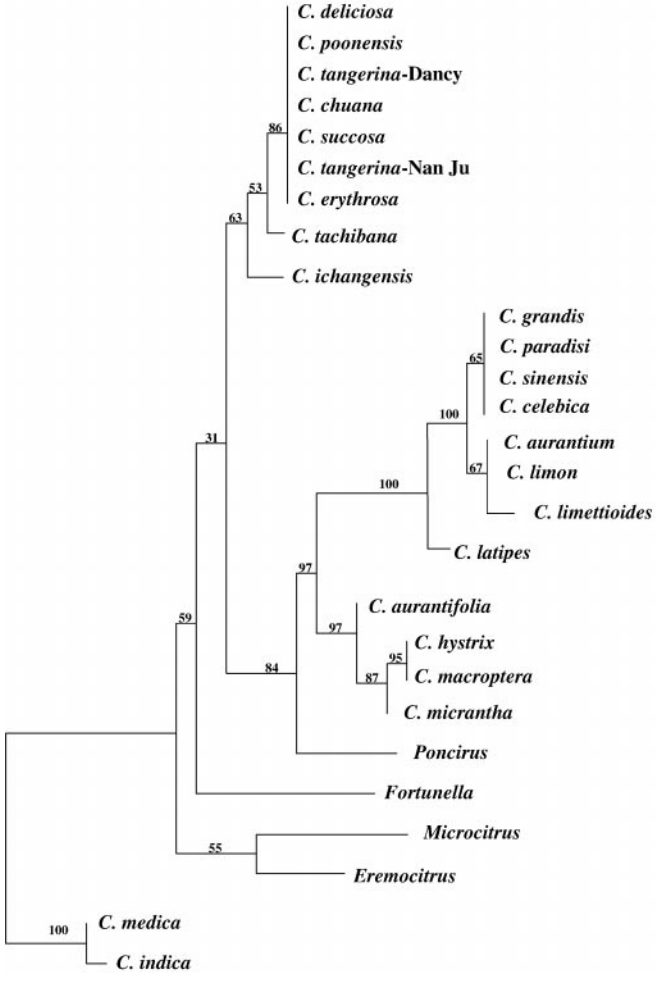


**Fig. 3** A 50% majority rule consensus tree for 27 genotypes of *Citrus* and related genera derived from bootstrap analysis (500 replications) of cpDNA data with confidence level for arms

7) Poncirus cluster: *P. trifoliata*
8) Microcitrus cluster: *M. australis* and *E. glauca.*

Another phylogenetic tree was obtained with Polymorphism Parsimony (PP) (Fig. 2), which was quite similar to the PAUP tree with the same eight clusters. The main differences from the PAUP tree are the following:

1) *C. aurantifolia* and *C. macrophylla* moved from the Citron cluster to the Micrantha cluster;
2) *C. paradisi*, *C. sinensis* and *C. aurantium* are located in Pummelo cluster instead of the Mandarin cluster;
3) *C. latipes* changed its position from the Micrantha cluster to the Fortunella cluster.

Based on cpDNA markers we calculated a genetic distance between each pair of the 27 analyzed genotypes by PAUP analysis. The mean genetic distances ranged from 0 to 0.531 (matrix available on request).

In the cluster analysis, a 50% majority rule consensus tree was constructed (Fig. 3) and eight clusters were formed:

1) Citron cluster: *C. medica* and *C. indica*;
2) Microcitrus cluster: *M. australis;*
3) Eremocitrus cluster: *E. glauca*;
4) Fortunella cluster: *F. margarita*;
5) Mandarin cluster: with all the mandarin accesions (identical among them), and *C. tachibana* and *C. ichangensis*;
6) Pummelo cluster: *C. grandis*, *C. paradisi*, *C. sinensis*, *C. celebica* (identical among them); *C. aurantium* and *C. limon* (identical); *C. limettioides* and *C. latipes*;
7) Micrantha cluster: *C. hystrix* and *C. macroptera* (identical); *C. aurantifolia* and *C. micrantha*;
8) Poncirus cluster: *P. trifoliata*

The dendogram obtained using Polymorphism Parsimony cluster analysis was very similar to the PAUP tree (Fig. 4).

## Origin

To investigate the origin of cultivated citrus species, we examined the genotypes included in the first four clus-

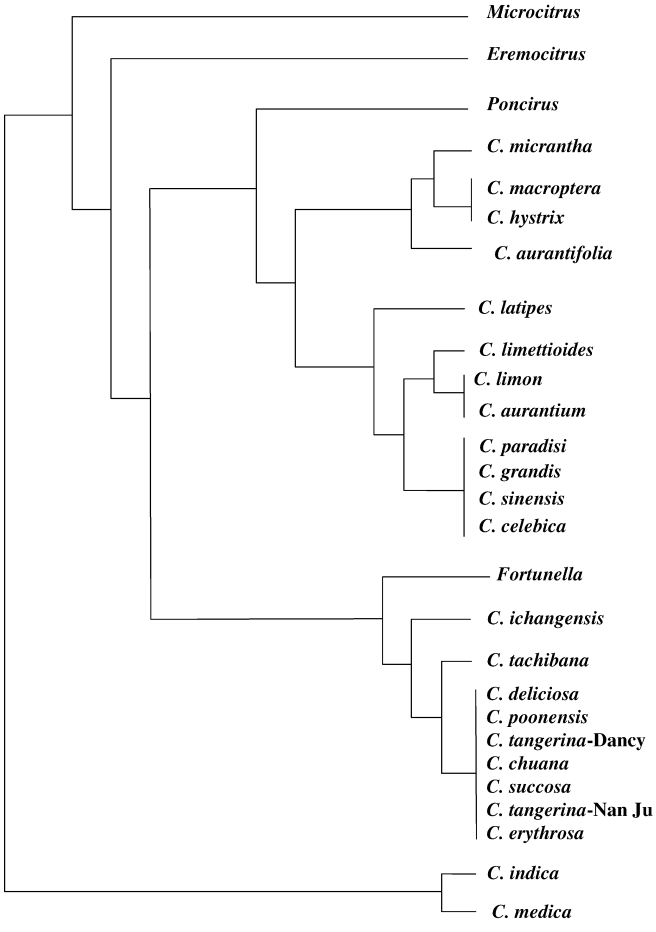


**Fig. 4** Dendrogram of 27 genotypes and related genera obtained by Polymorphism Parsimony analysis of cpDNA data

ters of the PP tree (Fig. 2). Those RAPDs and SCARs present in at least 1 genotype of a cluster but absent in all other clusters were designated as "unique markers" to the cluster. In such a way, unique markers were ascertained in the clusters analyzed; namely, 26 in the Citron, 7 in the Mandarin, 13 in the Pummelo and 22 in the Micrantha.

The presence of unique markers in every species within each cluster was scored. The taxon that possessed the highest number of unique markers was considered to be the possible ancestor of the other genotypes of the cluster, and they were identified as follows: *C. medica* with 19 of the 26 unique markers for the Citron cluster, *C. grandis* with 11/13 for the Pummelo cluster and *C. micrantha* with 18/22 for the Micrantha cluster. No evident differences were found among the mandarins tested, so no one species could be considered as an ancestor of the others.

Another approach we used to obtain a clearer comprehension of the origin of cultivated citrus was to generate dominant homozygotic markers. Two crosses, therefore, were made between the 3 true species: 80 individuals were obtained from *C. grandis* × *C. medica* and 72 individuals from *C. grandis* × *C. reticulata*. For each parent, 15 RAPDs and all of the SCARs obtained were checked out through the two progenies, and several dominant-homozygotic markers were discovered: 2 RAPDs (A18-1100 and S07-500) and 2 SCARs (SC2-250 and SC3-500) for mandarin, 1 RAPD (H14-450) and 1 SCAR for pummelo (SC2-450) and 4 RAPDs (H01-450, H12-400, S07-600, U11–1000) and 3 SCARs (SC2-1500, SC3-600, SC7-1000) for citron.

Finally, all the molecular markers present in 1 genotype were compared with those of the others, so that shared markers were identified, making it possible to formulate hypotheses on the origin of 17 citrus (Table 6).

In sweet orange, 71 RAPDs and SCARs were revealed, of which 35 were shared with pummelo and 36 with mandarin. For sour orange, a similar result was obtained with 42 out of 84 markers from pummelo and 36 from mandarin. Sour orange had 6 "extra markers" (C05-1000, C07-600, G04-800, G06-600, W09-360, W09-600), 1 of which (C05-1000) was even unique to sour orange. In addition the 6 dominant-homozygotic markers of pummelo (H14-450, SC2-450) and mandarin (A18-1100, S07-500, SC2-250, SC3-500) were all present in the two oranges except for the absence of a mandarin marker (A18-1100) in sour orange. Therefore, we can assume that pummelo and mandarin were the ancestors of sweet and sour oranges.

Grapefruit should derive from a backcross between sweet orange and pummelo as 45 of its 72 markers were shared with pummelo and 27 with mandarin-hereditary sweet orange markers. Among the latter ones, 6 (A18-1100, C02-620, C08-600, G11–1250, J04-750 and Z13-750) were absent in sour orange. Nevertheless, 1 mandarin dominant-homozygotic marker (SC-250) present in sweet orange was absent in grapefruit, indicating that mandarin could not have been its direct parent.

The results of cpDNA analysis suggested that pummelo acted as the female parent of sour orange, sweet orange and grapefruit.

Of the 78 lemon markers, 45 were shared with citron and 31 with sour orange, among which 14 were pummelo-hereditary, 13 mandarin-hereditary and 4 (G04-800, G06-600, W09-360, W09-600) shared with sour orange "extra markers". We did not consider sweet orange to be involved in lemon ancestry because it did not have 6 (C05-700, C19-1000, V03-700, V04-1600, V05-900 and Z13-550) of the 27 pummelo – or mandarin – hereditary markers. Moreover, a sour orange unique marker (W09-360) and 3 citron dominant-homozygotic SCARs (SC2-1500, SC3-600, SC7-1000) were present in lemon. This suggested that lemon originated from citron and sour orange, even though lemon had 2 "extra markers", 1 (C05-1250) shared with bergamot and *Fortunella* and another (K10-625) with mandarin. Based on cpDNA analysis citron can be considered to be the male parent of lemon, while sour orange cpDNA, which was identical with lemon, can be considered the female parent.

Among the 73 markers revealed in Palestine sweet lime, 48 were present in citron and 20 were pummelo (9) and mandarin (11) hereditary orange markers, of which

**Table 6** RAPD markers identified in the 17 genotypes undertaken for origin analysis and shared with the candidate ancestors

| Genotypes | Markers identified | Markers shared with | | | | | | Extra markers |
|---|---|---|---|---|---|---|---|---|
| | | Pummelo | Mandarin | Citron | *C. micrantha* | Sweet orange | Sour orange | |
| Sweet orange | 71 | 35 | 36 | | | | | |
| Sour orange | 84 | 42 | 36 | | | | | 6 |
| Grapefruit | 72 | 45 | | | | 27 | | |
| Lemon | 78 | | | 45 | | | 31 | 2 |
| Palestine lime | 73 | | | 48 | | | 21 | 4 |
| Volkamer lemon | 56 | | 6 | 27 | | | 22 | 1 |
| Bergamot | 66 | | | 28 | | | 37 | 1 |
| Rough lemon | 79 | | 32 | 42 | | | | 5 |
| Rangpur lime | 85 | | 32 | 46 | | | | 7 |
| Mexican lime | 78 | | | 46 | 32 | | | |
| Alemow | 82 | | | 45 | 26 | | | 11 |
| Cleopatra | 56 | | 56 | | | | | |
| Clementine | 57 | | 52 | | | 5 | | |
| Tankan | 65 | | 60 | | | 5 | | |
| King | 44 | | 39 | | | 4 | 1 | |
| Murcott | 70 | | 62 | | | 3 | 5 | |
| Satsuma | 49 | | 46 | | | 2 | 1 | |

3 (A04-400, C08-600, G11-1230) were present only in sweet orange and 1 (V06-600) solely in sour orange; meanwhile another marker (W09-600) was shared with a sour orange "extra marker". From these results, one ancestor should be an orange, but it was difficult to determine whether it should be sour orange or sweet orange. However, 2 (SC2-1500, SC3-600) of the 3 citron dominant-homozygotic SCARs were found in Palestine sweet lime, thereby confirming that citron was the ancestor, but also suggesting that Palestine sweet lime might not be an $F_1$ hybrid, as it did not have 1 dominant-homozygotic marker (SC7-1000). Four "extra markers" were also found in this lime. The cpDNA results indicated that citron was the male parent, and an orange should be the female parent.

In the Volkamer lemon, 56 markers were detected, 27 of them shared with citron, 22 with sour orange and 6 with mandarin. Among the 22 sour orange markers, 16 were mandarin-hereditary, 3 pummelo-hereditary and 3 were shared with the sour orange "extra markers". Sweet orange did not have 1 of the pummelo-hereditary and 1 of the mandarin-hereditary bands. However, we found that 5 of the 6 mandarin bands were in common with 1 or 2 sour orange genotypes, such as Yuzu and Gou Tou Cheng. One mandarin hereditary sour orange SCAR (SC3-500) and 2 citron dominant-homozygotic SCARs (SC2-1500 and SC3-600) were found in Volkamer lemon, but a citron dominant-homozygotic was absent. It is clear that citron and sour orange were ancestors, but they could not be the direct parents.

All of the 66 markers present in bergamot were shared with citron (28) and sour orange (37). Of the 37 markers shared with sour orange, 20 were pummelo-hereditary, 14 mandarin-hereditary and 3 shared with sour orange "extra markers". Of the 34 pummelo- and mandarin-hereditary markers, 8 were present in sour orange but not in sweet orange. All of the citron dominant-homozygotic SCARs and 1 sour orange specific marker (SC8-800) were found in bergamot.

Most of the 79 markers of Rough lemon were shared with citron (42) and mandarin (32). One (SC3-600) of the three citron dominant-homozygotic fragments and 2 (A18-1100, SC2-250) of the 4 mandarin ones were present in Rough lemon. Five additional markers (U11-450, V15-1300, V16-570, V17-400, W09-600) were "extra markers". Similar results were obtained for Rangpur lime: of the 85 markers observed, 46 were shared with citron and 32 with mandarin. Moreover, 2 dominant-homozygotic SCARs for citron (SC2-1500 and SC3-600) and 1 for mandarin (SC4-750) were found. Seven "extra markers" were also detected. We can assume that citron and mandarin are the ancestors both of Rough lemon and Rangpur lime, but they can not be considered to be direct parents.

All of the markers found in Mexican lime (78) were present either in citron (46) or in *C. micrantha* (32). Similar results were obtained for alemow, namely, 45 out of 82 markers were in common with citron and 26 with *C. micrantha*; however, there were 11 "extra markers". All the dominant-homozygotic SCARs for citron were found in Mexican lime, while only 2 (SC2-1500 and SC3-600) were found in alemow. A *C. micrantha* SCAR fragment (SC9-500) was present in both genotypes. The cpDNA analysis of Mexican lime indicated *C. micrantha* as the female parent.

Cleopatra appears mandarin-like as its 57 markers were shared with mandarins.

Clementine, Tankan and Satsuma had most of their markers shared with mandarin (52/57, 60/65 and 46/49, respectively), but they had 3–5 pummelo-hereditary sweet orange markers.

Similar results were obtained for Murcott and King with 62/70 and 39/44 markers, respectively, in common with mandarin. In King, 5 pummelo-hereditary markers

were found, among which 4 were shared with sweet orange and 1 with sour orange. In Murcott, out of 8 pummelo-hereditary markers, 3 were in common with sweet orange and 5 with sour orange.

## Discussion

Based on the phylogenetic analysis with RAPD and SCAR data, *Poncirus, Microcitrus* and *Eremocitrus* are distant from *Citrus* but *Fortunella* can not be separated from *Citrus*, as described by Herrero et al. (1996) and Federici et al. (1998).

According to Swingle, *Citrus* is divided into two subgenera, *Citrus* and *Papeda*. The molecular phylogeny based on total DNA analysis indicated a clear separation between the two subgenera. However, *C. indica* is clustered with papeda, although Federici et al. (1998) placed it in the lemon/lime/citron group, and *C. celebica* is tightly linked with *C. grandis*.

Almost all of the cultivated citrus belong to the subgenus *Citrus*, and Barrett and Rhodes (1976) suggested that they should be derived from 3 true species. Our results support this hypothesis since citron, pummelo and mandarin can be placed into three distinct clusters. Nevertheless, *C. aurantifolia* and *C. macrophylla* are included in the Micrantha cluster, therefore, *C. micrantha* may be another species which contributed to the origin of the cultivated citrus.

The clustering differences between the two phylogenetic trees with respect to several genotypes may possibly be explained by their hybrid origin. *C. aurantifolia* and *C. macrophylla* are placed in the Citron cluster by PAUP analysis but in Micrantha by Polymorphism Parsimony. *C. sinensis, C. aurantium* and *C. paradisi* form a subcluster of Mandarin cluster in the PAUP tree and stay together with Pummelo in the PP tree.

CpDNA data resulted in a different phylogenetic tree. The related genera, including *Fortunella*, form a single cluster each and are distant from each other and from *Citrus*, as observed by Green et al. (1986). Within *Citrus*, 4 clusters are formed but the subgenera *Citrus* and *Papeda* of Swingle disappears. Most of the *Citrus* genotypes analyzed, however, fall into the subgenera described by Tanaka, *Archicitrus* and *Metacitrus*, except for the Citron cluster, including *C. indica* and *C. medica*, which belong to 2 separate subgenera. Meanwhile, these 2 species are very distant from other *Citrus* spp. even more distant than the related genera, unlike that observed with RFLP data by Federici et al. (1998).

Tanaka (1961) hypothesised that citrus originated in Asia about 30 million years ago from *C. hystrix, C. latipes, C. macroptera* and *C. combara*. The cpDNA data support this hypothesis despite the fact that the last species was not included in the present study. The presence of *C. latipes* in the Pummelo cluster and *C. hystrix* and *C. macroptera* (identical) in the Micrantha cluster might indicate that the ancient maternal relationship is in the cluster.

With respect to the Mandarin cluster, Li et al. (1992), on the basis of isozyme data, suggested that *C. ichangensis* could be the ancestor of mandarins through an intermediate genotype, *C. mangshanensis* (a wild mandarin). In the present cpDNA analysis, *C. ichangensis* is clustered with all the mandarin accessions and *C. tachibana*; this supports this hypothesis.

The differences between total DNA and cpDNA results, due to the nature of the two genomes, lead to a better understanding of the divergences between Tanaka's and Swingle's classification. In comparison with nuclear DNA, cpDNA is conservative and maternally inherited; its analysis permits us to trace backwards to the original type. In fact, each cluster included accessions ranging from cultivated types to the ancient ones. Nuclear DNA, in contrast, contains the genetic information of a given species that originated by the combination of the male and female parents. In addition, natural and artificial selection lead to the loss of remote genetic traces and, consequently, the separation of cultivated genotypes from the wild. Therefore, total DNA analysis data separated the subgenus *Citrus* containing all the cultivated species from the *Papeda*, all species not edible, as classified by Swingle.

The origin of citrus has interested many researchers using a variety of methods. The hypotheses formulated have provided the basis for further clarification of the origin of some important citrus, although some assumptions are still questionable. For sweet orange and grapefruit, full agreement has been achieved among different researchers (Barrett and Rhodes 1976; Torres et al. 1978; Scora 1988), and the present molecular analysis supports the hypotheses.

Sour orange is considered a hybrid between pummelo and mandarin. Our molecular data indicates the same origin but the 6 "extra markers" suggests that it originated by a cross different from that of sweet orange, and the pummelo and/or mandarin accessions analyzed were not sufficient to reveal the specific parents.

The hypotheses on the origin of lemon are divergent. Based on morphological characteristics, Swingle (1943) and Malik et al. (1974) considered it to be a hybrid of citron and lime. Barrett and Rhodes (1976) suggested that lemon may be a trihybrid of citron, pummelo and a species of *Microcitrus*, but one carrying a greater proportion of citron genes acquired by further introgression from citron. As lemon possesses the *W* allele in the isozyme phosphoglucose isomerase (absent in citron and lime) Torres et al. (1978) excluded the possibility of a hybrid between citron and lime, indicating the origin to be sour orange × lime. Sour orange was also suggested as a candidate parent by Hirai and Kozaki (1981). In the present work, molecular markers indicated that lemon may be a hybrid between citron and sour orange. In the cpDNA analysis, only 1 polymorphic band was found between sour orange and the rest of Pummelo cluster, but lemon even had this sour orange cpDNA marker.

Mexican lime was also considered a trihybrid by Barrett and Rhodes (1976). Torres et al. (1978) indicated

that this lime may be an hybrid between citron and a papeda after having analysed three isozyme systems. Our molecular data made no doubt that citron was the male parent and the most probably female was *C. micrantha* or something very similar.

Webber (1943) assumed that Palestine sweet lime might be a hybrid of Mexican lime with a sweet lemon or a sweet citron. Barrett and Rhodes (1976) thought that this lime probably arose from a cross of Mexican lime by sweet orange. We found that citron was the male parent, but Palestine sweet lime might be a $F_2$ or a backcross hybrid, as 48 out of 73 markers were present in citron and one citron dominant-homozygotic SCAR was absent in it. The female parent may be a sweet orange. The cpDNA profile was not identical to that of either sweet or sour orange and it had 4 "extra markers"; thus, the probable parent might be a genotype similar to orange but one not included in the present study.

Citron, as an important true species, took part in the origin of many citrus species, but our cpDNA data indicates that citron always acted as the male parent.

In a previous work (Deng et al. 1996) we found that bergamot and Volkamer lemon are hybrids of citron and sour orange, and the present data confirms this assumption. In Volkamer lemon, however, a greater proportion of mandarin genes was observed, probably gained by backcrossing with mandarin.

A hybrid origin of citron and mandarin was found for Rough lemon and Rangpur lime, but 5 and 7, respectively, "extra markers" present in the 2 genotypes imply that one or both direct parents were not included in the present analysis.

Clementine was previously assumed to be a hybrid between mandarin and sweet orange (Deng et al. 1996). Here, data on this genotype, together with Tankan and Satsuma, supports this assumption. The uncertainty of sweet or sour orange as a parent of Murcott and King could be caused by the insufficient number of mandarin or orange accessions analyzed. In the above 5 genotypes, most of their markers were in common with mandarins, but about 70–80% of these markers were also present in orange. This may be a result from backcross events between orange and mandarin.

In summary, the combination of different kinds of molecular markers proved to be a powerful tool in carrying out a more complete analysis of citrus phylogeny and origin. Obviously, if more genotypes, especially wild types, were included in the study, it would have been possible to determine more precisely the parents of these selections.

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