The Origin of Cultivated Citrus as Inferred from Internal Transcribed Spacer and Chloroplast DNA Sequence and Amplified Fragment Length Polymorphism Fingerprints

Xiaomeng Li and Rangjin Xie

College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China; and Citrus Research Institute, Chinese Academy of Agricultural Sciences-Southwest University, Chongqing 400712, China

Zhenhua Lu

College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China; and Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences, Zhengzhou 450009, China

Zhiqin Zhou¹

Key Laboratory of Horticulture for Southern Mountain Regions, Ministry of Education, Chongqing 400715, China; College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China; and Citrus Research Institute, Chinese Academy of Agricultural Sciences-Southwest University, Chongqing 400712, China

ADDITIONAL INDEX WORDS. phylogeny, maximum parsimony, neighbor-joining, hybrid, parent

ABSTRACT. *Citrus* species are among the most important fruit trees in the world and have a long cultivation history. However, until now, the exact genetic origins of cultivated *Citrus* such as sweet orange (*Citrus sinensis*), lemon (*C. limon*), and grapefruit (*C. paradisi*) have remained unidentified. In the present study, amplified fragment length polymorphism (AFLP) fingerprints, nuclear internal transcribed spacer (ITS), and three plastid DNA regions (*psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG*) of 30 accessions of the cultivated citrus and their putative wild ancestors were analyzed in an attempt to identify their paternal and maternal origins. Molecular phylogenetic trees were constructed based on the AFLP data, and chloroplast DNA and ITS sequences using the genus *Poncirus* as the outgroup. Our results indicated that bergamot (*C. aurantifolia*) and lemon were derived from citron (*C. medica*) and sour orange (*C. aurantium*), and grapefruit was a hybrid that originated from a cross between pummelo (*C. grandis*) and sweet orange. Rough lemon (*C. limon*) was probable as a parent of rangpur lime (*C. limonia*) and guangxi local lemon (*C. limonia*). Our data also demonstrated that sweet orange and sour orange were hybrids of mandarin (*C. reticulata*) and pummelo, while rough lemon was a cross between citron and mandarin. For mexican lime (*C. aurantifolia*), our molecular data confirmed a species of *Papeda* to be the female parent and *C. medica* as the male. These findings provide new information for future study on the taxonomy, evolution, and genetic breeding of *Citrus*.

Citrus species are among the most important fruit crops in the world. They are widely grown in the tropical, subtropical, and borderline subtropical/temperate areas of the world. The genus *Citrus* was established by Carl Linnaeus in 1753 (Swingle and Reece, 1967). Since the establishment of the genus, *Citrus* has attracted much research interest for more than 250 years (Bayer et al., 2009; Nicolosi et al., 2000; Swingle and Reece, 1967; Tanaka, 1977). The genus *Citrus* has been variously described as consisting of 1 to 162 species (Malik, 1973; Tanaka,

1977). The most widely accepted taxonomic systems today are those of Swingle (1943) and Tanaka (1977) who recognized 16 and 162 species, respectively. Scora (1975) and Barrett and Rhodes (1976) suggested that there were only three true species within the subgenus *Eucitrus* of Swingle's system (i.e., *C. medica*, *C. reticulata*, and *C. grandis*). The other genotypes were derived from hybridization between these true species.

Citrus has a long history of cultivation—more than 4000 years (Scora, 1988; Webber et al., 1967). However, the huge controversy over the phylogeny of key wild species, and the genetic relationship between the cultivated species and their putative wild progenitors have remained unresolved, mainly due to the sexual compatibility between *Citrus* and related genera, the high frequency of bud mutations, the long history of cultivation, and wide dispersion. Previous studies on the genetic origin of cultivated citrus were mainly based on morphological, biochemical, and isozyme data (Barrett and Rhodes, 1976; Fang, 1993; Scora, 1988; Torres et al., 1978). Recently, DNA markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA

Received for publication 1 Apr. 2010. Accepted for publication 4 May 2010. This study was supported by the National Natural Science Foundation of China (NSFC grant no. 30671450).

We thank Timothy Schubert (Florida Department of Agriculture and Consumer Service, Division of Plant Industry) and Robert R. Krueger (USDA-ARS, National Clonal Germplasm Repository for Citrus and Dates) for supplying leaf materials. We are grateful to Prof. Daming Zhang from the Institute of Botany, Chinese Academy of Sciences, for providing PCR amplification primers. We would also like to thank Prof. Guangyan Zhong and Mr. Dong Jiang for their help with the experiments.

¹Corresponding author. E-mail: zqzhoubj@yahoo.com.

(RAPD), and sequence-characterized amplified regions (SCARs) were widely used to investigate the phylogenetic relationship of *Citrus* and its close relatives to infer into the genetic origins of the cultivated types (Federici et al., 1998; Nicolosi et al., 2000). More recently, DNA sequence data gave some clues about the progenitors of cultivated citrus (Bayer et al., 2009). Although these studies provided useful information for the origin of citrus and its taxonomy, 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.

Amplified fragment length polymorphism is a whole-genome approach to studying genetic variation at lower-level systematics (Bussell et al., 2005). The AFLP technique has been successfully applied in phylogenetic studies with many important crops such as wheat [*Triticum aestivum* (Heun et al., 1997)] as well as fruit tree species (Coart et al., 2003; Pang et al., 2007). In particular, the AFLP technique has been used successfully to assess the origin of landrace cultivars of cultivated potato [*Solanum tuberosum* (Spooner et al., 2005)]. Chloroplast DNA (cpDNA) sequence analysis is especially useful in plant phylogenetic study because of its evolutionary conservatism, the relative abundance in plant tissue, small size (135–160 kb), and predominant uniparental inheritance (Olmstead and Palmer, 1994). The utility of cpDNA to examine maternal lineages of hybrids has been documented in *Hippophae gonocarpa* (Wang et al., 2008). ITS sequences are biparentally inherited in most angiosperms (Álvarez and Wendel, 2003; Baldwin, 1992). Because of high-frequency gene conversion, conserved size, and great variation, ITS sequences were widely used in phylogenetic tree construction for inferring into the hybrid origin of species (Sang et al., 1995; Siripun and Schilling, 2006).

In the present study, we analyzed three plastid genome regions (*psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG*), nuclear ITS sequences, and AFLP fingerprints of 30 citrus accessions in an attempt to construct the cytoplasmic phylogeny of the cultivated citrus and infer into their maternal origins using the cpDNA sequence data, and to reconstruct the nuclear phylogeny of the cultivated citrus and infer into their paternal origins using AFLP and nuclear ITS data. The combination of nuclear DNA and cpDNA data allowed us to identify the exact genetic origin of the cultivated citrus.

Materials and Methods

MATERIALS. A total of 30 accessions was analyzed in the present study, including 24 belonging to the subgenus *Citrus*, three from the subgenus *Papeda*, and three from the genus *Poncirus* that were used as the outgroups. Detailed information on the materials studied is provided in Table 1.

DNA ISOLATION, AMPLIFICATION, AND SEQUENCING. Total DNA was isolated from silica gel-dried leaves following the cetyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1987). The chloroplast spacer regions were amplified using primers 7U (5'-GGCTACACAAACCGTTGAAG-3') and 7L (5'-ATGTTGACATGAGGAGGAAC-3') for *psbH* – *petB* intergenic spacer (Reverdatto et al., 1989), *trnLc* (5'-CGAAATCGGTAGACGCTACG-3') and *trnLf* (5'-ATTTGAAGTGGTGACACGAG-3') for the *trnL* intron and *trnL* – *trnF* intergenic spacer (Taberlet et al., 1991), and *trnS* (GCU) (5'-GAACGAATCACACTTTTACCAC-3') and *trnG* (UCC)

(5'-GCCGCTTTAGTCCTCAGC-3') for the *trnS* – *trnG* intergenic spacer (Hamilton, 1999). The polymerase chain reaction (PCR) reactions were carried out in a total volume of 20 µL containing 2.5 to 5 ng of DNA template, 0.3 µmol·L⁻¹ of each primer, 0.2 mmol·L⁻¹ of each dNTP, 2 mmol·L⁻¹ MgCl₂, 0.65 U of EX *Taq* DNA polymerase, and 10×*Taq* polymerase buffer (TaKaRa Biotechnology, Shiga, Japan). Amplifications were conducted in a T3 Thermocycler (Whatman Biometra, Göttingen, Germany). Polymerase chain reaction cycling parameters for the three cpDNA regions (*psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG*) were similar, with an initial denaturation for 4 min at 94 °C, followed by 30 cycles of 50 s at 94 °C, 30 s at 57 °C, 58 °C, 59 °C for *psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG*, respectively, and 1.5 min at 72 °C, with a final extension time of 10 min at 72 °C. PCR products were electrophoresed on and excised from a 1.5% agarose gel, then purified using DNA Fragment Quick Purification / Recover kit (Dingguo Biotechnology, Beijing, China). Sequencing was done on an ABI 3730 (Applied BioSystems, Foster City, CA) by Invitrogen (Invitrogen, Carlsbad, CA).

The ITS fragments including ITS1, ITS2 and 5.8S were amplified with primers AB101 (5'-ACGAATT CATGGTCCGGTGAAGTGTCG-3') and AB102 (5'-TAGAATTCCTCCGGTTCG CTCGCCGTTAC-3') (Robinson et al., 2001). The PCR reaction was carried out in a total volume of 25 µL containing 5 to 50 ng of template DNA, 0.15 µmol·L⁻¹ of each primer, 0.16 mmol·L⁻¹ of each dNTP, 1.5 mmol·L⁻¹ MgCl₂, 0.75 U of *Taq* DNA polymerase and 10×*Taq* polymerase buffer (TakaRa). Amplification was performed in a T3 Thermocycler (Whatman Biometra) using the following protocol: 2 min at 96 °C, followed by 30 cycles of 1 min at 96 °C, 1 min at 60 °C and 2 min at 72 °C, and finally 10 min at 72 °C. Polymerase chain reaction products were electrophoresed on 1.5% agarose gels and were purified using a DNA Fragment Quick Purification/ Recover kit (Dingguo). The purified ITS templates were ligated to the pGEM-T Easy vector (Promega, Madison, WI) following the manufacturer's protocol and were subsequently transformed into competent cells. At least 14 clones with correct inserts (determined through amplification of PCR products from an entire ITS of each individual) were screened for different ITS types using restriction enzymes *EcoRII* (/CCWGG), *AccBSI* (GAG/CGG), *AsuC2I* (CC/SGG), and *MluI* (A/CGCGT), which could detect species-specific ITS types among the three true species. Selected positive clones were sequenced in both directions using T7 and SP6 plasmid primers. Sequencing was done on an ABI 3730 (Applied Biosystems) by Invitrogen.

AFLP FINGERPRINTS. The AFLP analyses were performed according to Vos et al. (1995). Genomic DNA (≈500 ng) was digested with restriction enzymes *MseI* and *EcoRI* (Fermentas Canada, Burlington, ON, Canada). Digestion and ligation was performed in a single reaction for 6 h at 37 °C. The preamplification was performed with *MseI*-0 and *EcoRI*-0 primer pairs. Selective amplifications were performed with 15 primer combinations (PCs): E-AAC/M-CAA, E-AAC/M-CAC, E-AAC/M-CAT, E-AAC/M-CTA, E-AAC/M-CTT, E-AAG/M-CAC, E-ACT/M-CTT, E-ACG/M-CTA, E-AGC/M-CA A, E-AGC/M-CAC, E-AGC/M-CAG, E-AGC/M-CTA, E-AGC/M-CTC, E-AGG/M-CAA, and E-AGG/M-CTG. Selective amplification products were run in a 6% denaturing polyacrylamide gel. Fragments were scored manually as present (1) or absent (0) in the readable region of bands from 50 to 600 bp in length and were used to construct a binary data matrix with individual plants in rows and bands in columns.

Table 1. Taxonomy and collection sites of materials used in the study of phylogenetic relationships of the cultivated citrus and their putative wild ancestors using ITS and cpDNA sequence and AFLP fingerprints.

Common name	Swingle system	Tanaka system	Location ^z	Voucher
Fumin trifoliolate orange	<i>Poncirus trifoliata</i>	<i>P. trifoliata</i>	CRI	J. Dong LT0129
Flying Dragon trifoliolate orange	<i>P. trifoliata</i> var. <i>monstrosa</i>	<i>P. trifoliata</i> var. <i>monstrosa</i>	CRI	J. Dong LT0083
Trifoliolate orange	<i>P. trifoliata</i>	<i>P. trifoliata</i>	CRI	J. Dong LT0021
Mauritius papeda	<i>Citrus hystrix</i>	<i>C. hystrix</i>	CRI	J. Dong LP0009
Melanesian papeda	<i>Citrus macroptera</i>	<i>C. macroptera</i>	CRI	J. Dong LP0095
Small-flowered papeda	<i>Citrus micrantha</i>	<i>C. micrantha</i>	CAL	R.R. Krueger accession no. PI 539694
Etrog citron	<i>Citrus medica</i>	<i>C. medica</i>	CRI	J. Dong LP0012
Buddha's hand citron	<i>C. medica</i> var. <i>sarcodactylis</i>	<i>C. medica</i>	CRI	J. Dong LM0019
Femminello lemon	<i>Citrus limon</i>	<i>C. limon</i>	CRI	J. Dong LM0078
Rough lemon	<i>C. limon</i>	<i>Citrus jambhiri</i>	CRI	J. Dong LM0094
Eureka lemon	<i>C. limon</i>	<i>C. limon</i>	CRI	J. Dong LM0084
Bergamot	<i>Citrus aurantifolia</i>	<i>Citrus bergamia</i>	CRI	J. Dong LM0069
Mexican lime	<i>C. aurantifolia</i>	<i>C. aurantifolia</i>	CRI	J. Dong LM0003
Rangpur lime	<i>C. limon</i>	<i>Citrus limonia</i>	CRI	J. Dong LM0001
Guangxi local lemon	<i>C. limon</i>	<i>C. limonia</i>	CRI	J. Dong LM0032
Sour orange	<i>Citrus aurantium</i>	<i>C. aurantium</i>	CRI	J. Dong LA0003
Goutoucheng	<i>C. aurantium</i>	<i>C. aurantium</i>	CRI	J. Dong LA0008
Sweet orange	<i>Citrus sinensis</i>	<i>C. sinensis</i>	CRI	J. Dong LS0306
Valencia sweet orange	<i>C. sinensis</i>	<i>C. sinensis</i>	CRI	J. Dong LS0049
Marsh grapefruit	<i>Citrus paradisi</i>	<i>C. paradisi</i>	CRI	J. Dong LG0093
Sha tian you pummelo	<i>Citrus grandis</i>	<i>C. grandis</i>	CRI	J. Dong LG0002
Guan xi mi you pummelo	<i>C. grandis</i>	<i>C. maxima</i>	CRI	J. Dong LG0038
Wan bai you pummelo	<i>C. grandis</i>	<i>C. grandis</i>	CRI	J. Dong LG0026
Indian wild orange	<i>Citrus indica</i>	<i>C. indica</i>	CRI	J. Dong LR0419
Tachibana orange	<i>Citrus tachibana</i>	<i>C. tachibana</i>	CRI	J. Dong LR0215
Daoxian wild mandarin	<i>Citrus reticulata</i>	<i>Citrus daoxianensis</i>	CRI	J. Dong LR0050
Hongju	<i>C. reticulata</i>	<i>Citrus tangerina</i>	CRI	J. Dong LR0633
Tuchu	<i>C. reticulata</i>	<i>Citrus chuana</i>	CRI	J. Dong LR0347
Suanju	<i>C. reticulata</i>	<i>Citrus sunki</i>	CRI	J. Dong LR0039
Mankieh mandarin	<i>C. reticulata</i>	<i>Citrus tardiferax</i>	CRI	J. Dong LR0029

^zCAL = U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA; CRI = Citrus Research Institute, Chinese Academy of Agricultural Sciences, Chongqing, China.

DATA ANALYSIS. Pairwise genetic distance (D) between all individuals was estimated according Nei and Li's (1979) similarity coefficient and was implemented in the PAUP*, version 4.0b10, software (Swofford, 2000) using the formula: $D = 1 - (2n_{ij}/[n_i + n_j])$, where n_{ij} is the number of shared fragments between two individuals i and j, and n_i and n_j are the total number of fragments in individual i and j, respectively. From these values, mean intra- and interspecific distance values were obtained. The cpDNA and ITS sequence data were aligned using ClustalX, version 1.81 (Thompson et al., 1997) with manual adjustments as necessary. Boundaries of *psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG* regions were determined by comparison with the *C. sinensis* cpDNA sequence (GenBank no. DQ864733). Boundaries of the ITS1 and ITS2 regions and the coding sequences of 5.8S rDNA were determined using available ITS sequence from *C. maxima* (*C. grandis*; GenBank no. AM398229). The MEGA program, version 3.0 (Kumar et al., 2004), was employed to estimate GC content and nucleotide substitution. The three cpDNA fragments were combined in the maximum parsimony (MP) and neighbor-joining (NJ) analyses. The MP and NJ analyses were conducted using computer software PAUP*, version 4.0b10 (Swofford,

2000). All analyses were performed with uninformative characters excluded. In the MP analysis, all characters were assigned equal weight, gaps were treated as missing data, and character states were treated as unordered. A heuristic search was performed with the MULPARS option, tree bisection-reconnection (TBR) branch swapping, and random stepwise addition with 1000 replicates. Topological robustness was assessed by bootstrap analysis with 1000 replicates using simple taxon addition (Felsenstein, 1985).

Results

PHYLOGENETIC ANALYSIS OF CHLOROPLAST SEQUENCE. The sequences of *psbH* – *petB* varied from 1089 to 1111 bp in length. The final alignment of *psbH* – *petB* was 1111 bp, among which 19 sites were variable and 19 sites were parsimony informative. The sequences of *trnL* – *trnF* varied from 967 to 979 bp in length. The final alignment of *trnL* – *trnF* was 979 bp, and 13 sites were variable and 11 sites were parsimony informative. The amplified *trnS* – *trnG* region length varied from 577 to 647 bp, and the final alignment of *trnS* – *trnG* was 649 bp, including 17 variable sites and 16 informative sites.

Among the three regions, the informative site of *trnS* – *trnG* was the highest (2.47%), *psbH* – *petB* was 1.71%, and *trnL* – *trnF* was the lowest (1.12%). Nucleotide sequences from the three noncoding regions, *psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG* were combined for this analysis. The combined data set consisted of 2739 characters, of which 2689 sites were constant, and 46 variable characters were phylogenetically informative.

Parsimony analysis of the chloroplast data resulted in a single most parsimonious tree of length 51 steps [Fig. 1; consistency index (CI) = 0.980, retention index (RI) = 0.995]. The topologies of the phylogenetic trees based on MP and NJ (data not shown) analyses were identical, differing in statistic support values. From Fig. 1, we can see that the genus *Citrus* formed a strongly supported monophyletic group (bootstrap support [BS] 100%). Within the group, there were two major clades, designated A and B. Clade A comprised all accessions of *C. medica*, *C. reticulata*, two accessions of *C. limonia*, and *C. jambhiri* (BS 65). Clade B consisted of all accessions of *Papeda*, *C. grandis*, *C. sinensis*, *C. aurantium*, and *C. paradisi* ‘Marsh’, plus two accessions of *C. limon* and *C. aurantifolia* (BS 95). Within clade A, two accessions of *C. medica* formed a strongly supported monophyletic group (BS 100), and *C. jambhiri*, *C. limonia* ‘Guangxi’, and *C. limonia* ‘Rangpur’ nested in the mandarin group, forming a strongly supported monophyletic group (BS 99). Within the latter group, *C. jambhiri* had a close relationship with *C. limonia* ‘Rangpur’, *C. reticulata* ‘Hongju’, and *C. reticulata* ‘Mankieh’ (BS 64), while *C. limonia* ‘Guangxi’, *C. reticulata* ‘Daoxian’, and *C. reticulata* ‘Suanju’ grouped together (BS 63). Clade B branched into two subclades, with the first subclade containing three accessions of *Papeda* and *C. aurantifolia* ‘Mexican’ (BS 100), which was sister to the second subclade containing all accessions of *C. grandis*, *C. sinensis*, *C. aurantium*, *C. paradisi* ‘Marsh’, and two accessions of *C. limon* and *C. aurantifolia* (BS 95). In the second subclade, there was a polytomy of seven minor clades: *C. limon* ‘Femminello’, *C. limon* ‘Eureka’, *C. bergamia*, and *C. aurantium* ‘Daidai’ formed a clade (BS 64), two accessions of *C. sinensis* were found in another clade (BS 64), and the remaining five accessions separately formed a clade.

Fig. 1. Single most parsimonious tree for 30 genotypes of *Citrus* and *Poncirus* obtained from combined *psbH* – *petB*, *trnL* – *trnF*, and *trnS* – *trnG* sequence data. (tree length = 51, consistency index = 0.980, retention index = 0.995). The numbers above branches are bootstrap support percentages higher than 50% in parsimony and neighbor-joining analyses.

CLUSTER ANALYSIS OF AFLP DATA. Initially, 64 AFLP primer combinations were screened while 15 displayed clear stable polymorphisms. The 15 primer combinations generated a total of 233 fragments in the materials studied, of which 187 (80.3%) were polymorphic. On average, 15.5 bands were amplified with one primer combination, of which 12.5 were polymorphic (Table 2). Intra- and interspecific values derived from AFLP data are presented in Table 3. When interspecific mean distances within genus *Citrus* were compared, *C. medica* and *C. paradisi* appeared to be the most distant pair (0.0592, Table 3), while the least

interspecific distance was between *C. sinensis* and *C. paradisi* (0.0108, Table 3). When comparing intraspecific mean distances, *C. sinensis* appeared to be the least distant (0.0024, Table 3).

Of the 233 binary characters, 186 were parsimony informative. A heuristic search resulted in 19 most parsimonious trees with a length of 539 (CI = 0.345, RI = 0.674) (data not shown). The tree constructed by the NJ analysis is shown in Fig. 2. The topologies of the trees constructed by MP and NJ methods were virtually in congruence. The phylogenetic trees based on both methods strongly supported delineation of the genus *Citrus* as a monophyletic group (BS 100). The ingroup consisted of a ladder of four clades (designated C–F). Clade C, containing *C. hystrix*, *C. macropetala*, and *C. micrantha* was strongly supported (BS 98). Clade D was made up of three accessions of *C. grandis* (BS 74). Clade E consisted of all accessions of *C. medica*, *C. limon*, *C. aurantifolia*, and *C. limonia* (BS 67). Clade F composed of all accessions of *C. reticulata*, *C. sinensis*, *C. aurantium*, and *C. paradisi* ‘Marsh’ (with less than 50% bootstrap support). Within clade E, two accessions of *C. medica* clustered together with *C. aurantifolia* ‘Mexican’ (BS 66), which all together grouped with the clade containing *C. limon* ‘Femminello’ and *C. limon* ‘Eureka’. *C. jambhiri* and *C. limonia* ‘Rangpur’ formed a strongly supported clade (BS

97), which clade was sister to *C. limonia* ‘Guangxi’. Clade F divided into two subclades (with less than 50% bootstrap support), with seven accessions of *C. reticulata* forming a strongly supported subclade (BS 83), which became sister to the subclade including all accessions of *C. sinensis*, *C. aurantium*, and *C. paradisi* ‘Marsh’. Within the latter subclade, *C. paradisi* ‘Marsh’ was close to *C. sinensis* (with less than 50% bootstrap support).

ITS PHYLOGENY. Length variation for the entire ITS region (including the 5.8S coding region) ranged from 642 to 676 bp. The ITS1 region ranged from 227 to 257 bp, and the ITS2 region ranged from 251 to 256 bp. As expected, the 5.8S sequence was identical in size (164 bp) in all the taxa, and no gaps were found when aligning the sequences. The G + C content of the sequences ranged from 63.7% to 67.2%. Aligned ITS sequences were 679 bp long and 614 sites were constant, while 44 of the variable characters were phylogenetically informative. Two species of *Poncirus* had a unique 4-bp insertion in ITS2. There was a 29-bp deletion in ITS1 of *C. medica* and its putative hybrids. Twelve cultivated accessions, including sour orange, sweet orange, grapefruit, lemon, and lime, had at least two major different ITS types.

Parsimony analysis of the ITS data set resulted in a single most parsimonious tree of length 71 steps (CI = 0.930, RI = 0.982). The topologies of the phylogenetic trees based on MP and NJ (data not shown) analyses were identical, differing in statistic support values. The most parsimonious tree is shown in Fig. 3. From Fig. 3, it is apparent that the genus *Citrus* is supported as a monophyletic group (BS 88). The group divided into two large clades (designated G and H). Clade G was a polytomy containing all accessions of *C. reticulata* and one ITS repeat type of all accessions of *C. sinensis*, *C. aurantium*, *C. limon*, *C. aurantifolia*, *C. limonia*, and *C. paradisi* ‘Marsh’, except *C. aurantifolia* ‘Mexican’ (BS 91). Clade H consisted of all accessions of *Papeda*, *C. medica*, *C. grandis*, and the other ITS repeat types of all accessions of *C. sinensis*, *C. aurantium*, *C. limon*, *C. aurantifolia*, *C. limonia*, and *C. paradisi* ‘Marsh’ (BS 95). Within clade H, there was a polytomy of three minor subclades. The first subclade contained one ITS repeat type of *C. limon* ‘Eureka’ and *C. aurantium* ‘Goutoucheng’ (BS 100). The second subclade was also a polytomy consisting of three accessions of *C. grandis*, and one ITS repeat type of two accessions of *C. sinensis*, *C. aurantium*, and *C. paradisi* ‘Marsh’ (BS 95). Within this subclade, *C. paradisi* ‘Marsh’ was close to *C. sinensis* (BS 65). The third clade comprised all accessions of *Papeda*, *C. medica*, and all of the other ITS repeat types of *C. limon*, *C. limonia*, and *C. aurantifolia*, except *C.*

Table 2. AFLP polymorphism among 30 genotypes of *Citrus* and *Poncirus* generated with 15 primer combinations.

Primer combinations	Fragments (no.)	Polymorphic fragments (no.)	Polymorphism rate (%)
E-AAC/ M-CAA	18	16	88.9
E-AAC/ M-CAC	14	13	92.9
E-AAC/ M-CAT	7	4	57.1
E-AAC/ M-CTA	13	12	92.3
E-AAC/ M-CTT	17	14	82.4
E-AAG/ M-CAC	9	7	77.8
E-ACT/ M-CTT	17	14	82.4
E-ACG/ M-CTA	23	20	87.0
E-AGC/ M-CAA	17	14	82.4
E-AGC/ M-CAC	17	12	70.6
E-AGC/ M-CAG	17	13	76.5
E-AGC/ M-CTA	19	15	78.9
E-AGC/ M-CTC	15	11	73.3
E-AGG/ M-CAA	20	15	75.0
E-AGG/ M-CTG	10	7	70.0
Sum/mean	233/15.5	187/12.5	NA/80.3

Table 3. Intra- and interspecific mean distance values (Nei and Li, 1979) for *Citrus* taxa based on AFLP data.

Taxa	<i>Papeda</i>	<i>C. medica</i>	<i>C. limon</i>	<i>C. aurantifolia</i>	<i>C. limonia</i>	<i>C. aurantium</i>	<i>C. sinensis</i>	<i>C. paradisi</i>	<i>C. grandis</i>	<i>C. reticulata</i>
<i>Papeda</i>	0.0222									
<i>C. medica</i>	0.0517	0.0055								
<i>C. limon</i>	0.0494	0.0288	0.0126							
<i>C. aurantifolia</i>	0.0427	0.0314	0.0202	0.0244						
<i>C. limonia</i>	0.0530	0.0335	0.0144	0.0241	0.0109					
<i>C. aurantium</i>	0.0433	0.0536	0.0311	0.0326	0.0305	0.0163				
<i>C. sinensis</i>	0.0467	0.0551	0.0343	0.0370	0.0309	0.0159	0.0024			
<i>C. paradisi</i>	0.0507	0.0592	0.0389	0.0390	0.0375	0.0166	0.0108			
<i>C. grandis</i>	0.0513	0.0579	0.0533	0.0483	0.0549	0.0356	0.0348	0.0293	0.0247	
<i>C. reticulata</i>	0.0571	0.0567	0.0351	0.0481	0.0288	0.0256	0.0199	0.0276	0.0496	0.0131

Fig. 2. A neighbor-joining dendrogram for 30 genotypes of *Citrus* and *Poncirus* derived from AFLP data. The numbers above branches are bootstrap support percentages higher than 50% in parsimony and neighbor-joining analyses.

limon 'Eureka'. The third clade (BS 68) branched into two subclades, one being a polytomy containing *C. hystrix*, *C. macroptera*, *C. micrantha*, and *C. aurantifolia* 'Mexican' (BS 78). The other was also a polytomy consisting of all accessions of *C. medica* and one ITS repeat type of *C. limon* 'Femminello', *C. jambhiri*, *C. bergamia*, *C. aurantifolia* 'Mexican', *C. limonia* 'Rangpur', and *C. limonia* 'Guangxi' (BS 98).

Discussion

Because of the widely accepted belief that some or even most *Citrus* species have been derived from hybridization, in

the present study, we used AFLP data to determine paternal origin, cpDNA data to identify maternal origin, and ITS data to assess the full parentage of the cultivated citrus. A combination of nuclear and chloroplast genome information permitted us to make a more concrete conclusion about the exact genetic origin of the cultivated citrus. In the following discussion, we discuss the origins of 12 cultivated citrus types based on previous findings in combination with the new data generated in this study.

SWEET ORANGE (*C. SINENSIS*).

Sweet orange is one of the most widely grown and consumed *Citrus* types. Based on morphological data, Barrett and Rhodes (1976) suggested that sweet orange was a hybrid of pummelo and mandarin. The suggestion was supported by RAPD and SCAR markers (Nicolosi et al., 2000). Protein data also supported that *C. reticulata* was a parent of sweet orange (Handa et al., 1986; Malik et al., 1974). Chloroplast DNA analyses revealed that the chloroplast genome of sweet orange was derived from pummelo (Barkley et al., 2006; Nicolosi et al., 2000). However, Torres et al. (1978) proposed that sweet orange was improbable as an F1 result of a cross between pummelo and mandarin. In the present study, two accessions of *C. sinensis* grouped in the pummelo cluster with a strong support (BS 100) in the cpDNA tree (Fig. 1). This result strongly supported that pummelo was a maternal parent of sweet orange. On the other hand, our AFLP tree (Fig. 2) clearly indicated that sweet orange was most closely related to mandarin paternally. Most importantly, on our ITS tree (Fig. 3), *C. sinensis*04 and *C. sinensis* 'Valencia'06 grouped in the mandarin cluster (BS 91), while

*C. sinensis*06 and *C. sinensis* 'Valencia'03 clustered with pummelo (BS 95). These results for the first time simultaneously revealed the maternal and paternal origins of sweet orange and provided the concrete molecular evidences that sweet orange was a hybrid of pummelo and mandarin, which acted as the female and male parents, respectively.

LEMON (*C. LIMON*). Lemon is one of the most important cultivated citrus, mostly used for juice or flavoring. Although lemon was accepted as a species by the Swingle and Tanaka systems (Swingle and Reece, 1967; Tanaka, 1977), the hypotheses on the origin of lemon are divergent. Based on morphological characteristics, Swingle (1943) and Malik et al. (1974) considered

Fig. 3. Single most parsimonious tree for 30 genotypes of *Citrus* and *Poncirus* obtained from ITS sequence data (tree length = 71, consistency index = 0.930, retention index = 0.982). The numbers above branches are bootstrap support percentages higher than 50% in parsimony and neighbor-joining analyses. The numbers following the Latin names are the clone numbers.

it to be a hybrid of citron and lime. Barrett and Rhodes (1976) suggested that lemon was a trihybrid of citron, pummelo, and a species of *Microcitrus* of Tanaka's system. Based on RAPD, SCAR, and cpDNA analysis, Nicolosi et al. (2000) proposed that lemon originated from citron and sour orange, with sour orange being the maternal parent. Citron was a parent of lemon supported by other molecular markers (Barkley et al., 2006; Federici et al., 1998). ISSR data showed that pummelo was a close relative, or

a direct or indirect ancestor of lemon (Gulsen and Roose, 2001b). Sour orange as a parent is supported by Hirai and Kozaki (1981) and Torres et al. (1978). In the present study, *C. limon* 'Femminello', *C. limon* 'Eureka', and *C. bergamia* clustered with *C. aurantium* 'Daidai' within the pummelo group (BS 64) based on the cpDNA tree (Fig. 1). This result clearly demonstrated that sour orange was probable a maternal parent of lemon. On the other hand, in our AFLP tree, *C. limon* 'Femminello', *C. limon* 'Eureka', and *C. jambhiri* grouped in the citron cluster (BS 53) (Fig. 2). This result suggested that citron was most likely a parent of lemon. Moreover, on our ITS tree, *C. jambhiri*08 and *C. limon* 'Femminello'03 grouped in the citron cluster (BS 97), while *C. limon* 'Eureka'15 clustered with *C. aurantium* 'Goutoucheng'25 (BS 100). These results confirmed that sour orange and citron were parents of lemon. Furthermore, other ITS repeat types of three accessions of lemon (*C. jambhiri*14, *C. limon* 'Femminello'04, and *C. limon* 'Eureka'18) clustered with mandarin (BS 91). This finding suggested that mandarin could be a parent of lemon. However, sour orange was also mandarin hybrid, which could account for the contribution of mandarin gene. Our data provides convincing evidence in supporting the origin of lemon, with sour orange being the maternal parent and citron being the paternal parent.

Webber (1943) thought rough lemon to be a hybrid of lemon and citron. Based on RAPD, RFLP, and SCAR markers, Nicolosi et al. (2000) suggested that citron and mandarin were the ancestors of rough lemon. Citron as a parent of rough lemon was supported by SSR data (Barkley et al., 2006). In the present study, *C. jambhiri*, *C. limonia* 'Rangpur', *C. reticulata* 'Hongju', and *C. reticulata* 'Mankieh' formed a monophyletic clade (BS 64) in the cpDNA tree (Fig. 1). This

result was in agreement with DNA sequence analysis of Bayer et al. (2009), and suggested that mandarin was probably a maternal parent of rough lemon. On the other hand, our AFLP tree showed that *C. jambhiri* first clustered with *C. limonia* 'Rangpur' and *C. limonia* 'Guangxi', and then together they grouped with *C. limon* and *C. aurantifolia* 'Mexican' in the citron group (BS 53) (Fig. 2). This result indicated that citron was likely to be a paternal parent of rough lemon. In addition,

two ITS repeat types of rough lemon (*C. jambhiri*08 and *C. jambhiri*14) grouped in the mandarin and citron clusters with strong support, respectively. These molecular pieces of evidence have more clearly revealed that rough lemon was a hybrid of mandarin and citron, with mandarin being the maternal parent and citron being the paternal parent.

LEMON-LIKE CITRUS (*C. LIMONIA*). Webber (1943) believed that rangpurs were hybrids of limes and mandarins. The hypothesis was supported by SSR data (Barkley et al., 2006). Nicolosi et al. (2000) suggested that the hybrid origin of rangpur lime was a cross of citron and mandarin hybrid. That mandarin was a parent of rangpur lime was supported by chloroplast data (Gulsen and Roose, 2001a). In the present study, our cpDNA data showed that *C. limonia* 'Rangpur', *C. jambhiri*, and two accessions of *C. reticulata* formed a monophyletic group within the mandarin group (BS 64), while *C. limonia* 'Guangxi' clustered with other two accessions of *C. reticulata* (Fig. 1). This result suggested that mandarin was most likely a maternal parent of rangpur lime and Guangxi local lemon. On the other hand, our AFLP data demonstrated that *C. limonia* 'Rangpur' clustered together with *C. jambhiri* and *C. limonia* 'Guangxi' (BS 73) (Fig. 2). This result suggested that rough lemon was probably a paternal parent of rangpur lime and Guangxi local lemon. Our results provide additional molecular evidence for the origins of lemon-like citrus. In particular, our ITS analysis demonstrated that *C. limonia* 'Rangpur'02 and *C. limonia* 'Guangxi'03 clustered in the mandarin group, while *C. limonia* 'Rangpur'06 and *C. limonia* 'Guangxi'04 grouped in the citron cluster. The finding confirmed the hypothesis that mandarin and citron were parents of lemon-like citrus.

GRAPEFRUIT (*C. PARADISI*). Grapefruit is the one *Citrus* biotype in which a hybrid origin and subsequent selection for mutants are well documented (Moore, 2001). It was considered to have originated most probably from a hybrid between pummelo and sweet orange (Barrett and Rhodes, 1976), perhaps an introgression back to pummelo (Gmitter, 1995; Moore, 2001). Isozyme data (Fang, 1993; Herrero et al., 1996), SSR data (Barkley et al., 2006), and AFLP data (Pang et al., 2007) clustered grapefruit with pummelo, supporting the suggestion of a backcross with pummelo. However, SCAR (Nicolosi et al., 2000), RFLP plus RAPD (Federici et al., 1998), and other SSR data (Pang et al., 2003), which clustered grapefruit with sweet orange, disagreed with this suggestion. In the present study, *C. paradisi* 'Marsh' grouped in the pummelo cluster with strong support (BS 100) in the cpDNA tree (Fig. 1). This result suggested that pummelo was most likely a maternal parent of grapefruit. On the other hand, in our AFLP tree, two accessions of *C. sinensis*, *C. aurantium*, and *C. paradisi* 'Marsh' formed a monophyletic clade with weak support (Fig. 2), and *C. paradisi* 'Marsh' was closer to *C. sinensis* than *C. aurantium*. This result suggested that sweet orange was possibly a paternal parent of grapefruit. Furthermore, on our ITS tree (Fig. 3), *C. paradisi* 'Marsh'04, *C. sinensis*04, and *C. sinensis* 'Valencia'06 clustered together, indicating a close relationship between sweet orange and grapefruit. In particular, *C. paradisi* 'Marsh'10 grouped in the mandarin cluster (BS 91). This suggested that mandarin could be a parent of grapefruit. However, sweet orange was also a mandarin hybrid, which could account for the contribution of mandarin gene. Our data confirmed that grapefruit was a hybrid of sweet orange and pummelo.

SOUR ORANGE (*C. AURANTIUM*). Fruits of sour orange are bitter and acidic, thus they were primarily used for their ornamental value and for extracting neroli oil in the past. Because the species is tolerant to cold temperatures and various soil conditions (Castle, 1987), currently, most of them are used principally as a rootstock for sweet orange and other *Citrus* biotypes. Scora (1975) and Barrett and Rhodes (1976) proposed that sour orange originated from *C. reticulata* and *C. grandis*. The suggestion was supported by isozyme study (Fang, 1993), and RAPD and SCAR markers (Nicolosi et al., 2000). Nicolosi et al. (2000) further revealed that pummelo acted as the female parent of sour orange, which was supported by Barkley et al. (2006) and Bayer et al. (2009). However, Malik et al. (1974) ruled out the possibility that sour orange was a hybrid of sweet orange and mandarin. In the present study, *C. aurantium* 'Daidai' clustered with two accessions of *C. limon* and *C. bergamia*, and then together they formed monophyletic group with three accessions of *C. grandis*, *C. aurantium* 'Goutoucheng', *C. sinensis*, and *C. paradisi* 'Marsh', with strong support (BS 100) in the cpDNA tree (Fig. 1). This result strongly supported that pummelo was probably a maternal parent of sour orange. On the other hand, in our AFLP tree, two accessions of *C. aurantium* and *C. sinensis* grouped with *C. paradisi* 'Marsh', and then together they formed monophyletic group with all accessions of *C. reticulata*, with weak support (Fig. 2). This result suggested that mandarin was possibly a paternal parent of sour orange. In addition, on our ITS tree (Fig. 3), *C. aurantium* 'Daidai'14 and *C. aurantium* 'Goutoucheng'05 grouped in the mandarin cluster (BS 91), while *C. aurantium* 'Daidai'13 clustered with pummelo (BS 95). Our data confirmed that sour orange was a hybrid of pummelo and mandarin, with pummelo being the maternal parent and mandarin being the paternal parent. In particular, our data have more clearly identified the maternal and paternal origins of sour orange than all other previous data.

Nicolosi et al. (2000) suggested that bergamot was a hybrid of citron and sour orange. That citron was a parent of bergamot was supported by SSR data (Barkley et al., 2006). However, chloroplast data suggested that pummelo was a parent of bergamot (Gulsen and Roose, 2001a; Bayer et al., 2009). In the present study, *C. bergamia*, *C. aurantium* 'Daidai', *C. limon* 'Femminello', and *C. limon* 'Eureka' formed a monophyletic clade (BS 64) in the cpDNA tree (Fig. 1). This result suggested that sour orange was most likely a maternal parent of bergamot. On the other hand, our AFLP tree showed that *C. bergamia*, *C. limon*, *C. aurantifolia*, and *C. limonia* grouped in the citron cluster (BS 67) (Fig. 2). This result indicated that citron was probably a paternal parent of bergamot. In addition, on our ITS tree (Fig. 3), *C. bergamia*06 grouped in the citron cluster (BS 96), supporting the affinitive relation between bergamot and citron, while *C. bergamia*07 grouped with mandarin (BS 91). This result suggested that mandarin was probable a parent of bergamot. However, sour orange was also mandarin hybrid, which could account for the contribution of the mandarin gene. Our data confirmed that bergamot was a hybrid of sour orange and citron, with sour orange being the maternal parent and citron being the paternal parent.

LIME (*C. AURANTIFOLIA*). Barrett and Rhodes (1976) suggested that the lime probably arose from a trihybrid cross involving *C. medica*, *C. grandis*, and a *Microcitrus* species. Handa et al. (1986) thought that mandarin was a parent of lime, and another parent may be one of citron, pummel, and *Papeda*.

Nicolosi et al. (2000) found that all RAPD and SCAR markers present in Mexican lime were also present in citron or in *C. micrantha*, suggesting that these two species were the parents. That citron was a parent of limes was supported by isozyme data (Torres et al., 1978) and RFLP data (Federici et al., 1998). *Papeda* was one parent of lime, supported by SSR data (Barkley et al., 2006). In the present study, *C. aurantifolia* 'Mexican' clustered with three accessions of *Papeda*, with strong support (BS 100) in the cpDNA tree (Fig. 1). This result strongly supported that *Papeda* was most likely a maternal parent of Mexican lime. On the other hand, our AFLP tree indicated that *C. aurantifolia* 'Mexican' and two accessions of *C. medica* formed a monophyletic clade (BS 66) (Fig. 2). This result suggested that citron was probable a paternal parent of Mexican lime. Meanwhile, on our ITS tree, *C. aurantifolia* 'Mexican'03 and *C. aurantifolia* 'Mexican'10 clustered with *Papeda* and citron groups, respectively. These results strongly supported that Mexican lime was a hybrid of *Papeda* and citron. Our molecular evidence presented more convincing data than all other previous studies in supporting the origin of lime.

Literature Cited

- Álvarez, I. and J.F. Wendel. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Mol. Phylogenet. Evol.* 29:417–434.
- Baldwin, B.G. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example from the compositae. *Mol. Phylogenet. Evol.* 1:3–16.
- Barkley, N.A., M.L. Roose, R.R. Krueger, and C.T. Federici. 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theor. Appl. Genet.* 112:1519–1531.
- Barrett, H.C. and A.M. Rhodes. 1976. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. *Syst. Bot.* 1:105–136.
- Bayer, R.J., D.J. Mabberley, C. Morton, C.H. Miller, I.K. Sharma, B.E. Pfeil, S. Rich, R. Hitchcock, and S. Sykes. 2009. A molecular phylogeny of the orange subfamily (Rutaceae: Aurantioideae) using nine cpDNA sequences. *Amer. J. Bot.* 96:668–685.
- Bussell, J.D., M. Waycott, and J.A. Chappill. 2005. Arbitrarily amplified DNA markers as characters for phylogenetic inference. *Perspect. Plant Ecol. Evol. Syst.* 7:3–26.
- Castle, W.S. 1987. Citrus rootstock, p. 361–399. In: R.C. Rom and R.F. Carlson (eds.). *Rootstocks for fruit crops*. Wiley, New York.
- Coart, E., X. Vekemans, M.J.M. Smulders, I. Wagner, J.V. Huylensbroeck, E.V. Bockstaele, and I. Roldán-Ruiz. 2003. Genetic variation in the endangered wild apple (*Malus sylvestris* (L.) Mill.) in Belgium as revealed by amplified fragment length polymorphism and microsatellite markers. *Mol. Ecol.* 12:845–857.
- Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bul.* 19:11–15.
- Fang, D.Q. 1993. Intra- and intergeneric relationships of *Poncirus polyandrus*: Investigation by leaf isozymes. *J. Wuhan Bot. Res.* 11:34–40 (In Chinese with English abstract).
- Federici, C.T., D.Q. Fang, R.W. Scora, and M.L. Roose. 1998. Phylogenetic relationships within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. *Theor. Appl. Genet.* 96:812–822.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783–791.
- Gmitter, F.G. 1995. Origin, evolution and breeding of the grapefruit. *Plant Breed. Rev.* 13:345–363.
- Gulsen, O. and M.L. Roose. 2001a. Chloroplast and nuclear genome analysis of the parentage of lemons. *J. Amer. Soc. Hort. Sci.* 126: 210–215.
- Gulsen, O. and M.L. Roose. 2001b. Lemons: Diversity and relationships with selected *Citrus* genotypes as measured with nuclear genome markers. *J. Amer. Soc. Hort. Sci.* 126:309–317.
- Hamilton, M.B. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Mol. Ecol.* 8:521–523.
- Handa, T., Y. Ishizawa, and C. Oogaki. 1986. Phylogenetic study of Fraction I protein in the genus *Citrus* and its close related genera. *Jpn. J. Genet.* 61:15–24.
- Herrero, R., M.J. Asins, E.A. Carbonell, and L. Navarro. 1996. Genetic diversity in the orange subfamily Aurantioideae. I. Intraspecific and intragenus genetic variability. *Theor. Appl. Genet.* 92:599–609.
- Heun, M., R. Schafer-Pregl, D. Klawan, R. Castagna, M. Accerbi, B. Borghi, and F. Salamini. 1997. Site of einkorn wheat domestication identified by DNA fingerprinting. *Science* 278:1312–1314.
- Hirai, M. and I. Kozaki. 1981. Isozymes of citrus leaves. *Proc. Intl. Soc. Citriculture* 1:10–13.
- Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5:150–163.
- Malik, M.N. 1973. A new concept in *Citrus* classification. *Pakistan J. Sci. Res.* 25:268–271.
- Malik, M.N., R.W. Scora, and R.K. Soost. 1974. Studies on the origin of the lemon. *Hilgardia* 42:361–382.
- Moore, G.A. 2001. Oranges and lemons: Clues to the taxonomy of *Citrus* from molecular markers. *Trends Genet.* 17:536–540.
- Nei, M. and W.H. Li. 1979. A mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 75:5269–5273.
- Nicolosi, E., Z.N. Deng, A. Gentile, S. La Malfa, G. Continella, and E. Tribulato. 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theor. Appl. Genet.* 100:1155–1166.
- Olmstead, R.G. and J.D. Palmer. 1994. Chloroplast DNA systematics: A review of method and data analysis. *Amer. J. Bot.* 81:1205–1224.
- Pang, X.M., C.G. Hu, and X.X. Deng. 2003. Phylogenetic relationships among *Citrus* and its relatives as revealed by SSR markers. *Acta Genetica Sinica* 30:81–87.
- Pang, X.M., C.G. Hu, and X.X. Deng. 2007. Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. *Genet. Resources Crop Evol.* 54:429–436.
- Reverdatto, S.V., A.V. Andreeva, A.A. Buryakova, O.G. Chakhmakhcheva, and V.A. Efimov. 1989. Nucleotide sequence of the 5.2 kbp barley chloroplast DNA fragment, containing *psbB-psbH-perB-perD* gene cluster. *Nucleic Acids Res.* 17:2859–2860.
- Robinson, J.P., S.A. Harris, and B.E. Juniper. 2001. Taxonomy of the genus *Malus* Mill. (Rosaceae) with emphasis on the cultivated apple, *Malus domestica* Borkh. *Plant Syst. Evol.* 226:35–58.
- Sang, T., D.J. Crawford, and T.F. Stuessy. 1995. Documentation of reticulate evolution in peonies (Paeonia) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evolution. *Proc. Natl. Acad. Sci. USA* 92: 6813–6817.
- Scora, R.W. 1975. On the history and origin of *Citrus*. *Bull. Torrey Bot. Club* 102:369–375.
- Scora, R.W. 1988. Biochemistry, taxonomy and evolution of modern cultivated *Citrus*, p. 277–289. In: R. Goren and K. Mendel (eds.). *Proc. 6th Intl. Citrus Congr. Vol. 1*. Margraf, Weikersheim, Germany.
- Siripun, K.C. and E.E. Schilling. 2006. Molecular confirmation of the hybrid origin of *Eupatorium godfreyanum* (Asteraceae). *Amer. J. Bot.* 93:319–325.
- Spooner, D.M., K. Mclean, G. Ramsay, R. Waugh, and G.J. Bryan. 2005. A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. *Proc. Natl. Acad. Sci. USA* 102:14694–14699.
- Swingle, W.T. 1943. The botany of *Citrus* and its wild relatives of the orange subfamily, p. 129–474. In: H.J. Webber and L.D. Batchelor (eds.). *The citrus industry. Vol. 1*. Univ. of California, Berkeley.

- Swingle, W.T. and P.C. Reece. 1967. The botany of *Citrus* and its wild relatives, p. 190–423. In: W. Reuther, H.J. Webber, and L.D. Batchelor (eds.). The citrus industry. Vol. 1. Univ. of California, Berkeley.
- Swofford, D.L. 2000. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4b. Sinauer, Associates, Sunderland, MA.
- Taberlet, P., L. Gielley, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* 17:1105–1109.
- Tanaka, T. 1977. Fundamental discussion of *Citrus* classification. *Studia Citrologica* 14:1–6.
- Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The Clustal-X Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882.
- Torres, A.M., R.K. Soost, and U. Diederhufen. 1978. Leaf isozymes as genetic markers in *Citrus*. *Amer. J. Bot.* 65:869–881.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T.d. Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* 23: 4407–4414.
- Wang, A., F. Schluetz, and J. Liu. 2008. Molecular evidence for double maternal origins of the diploid hybrid *Hippophae goniocarpa* (Elaeagnaceae). *Bot. J. Linn. Soc.* 156:111–118.
- Webber, H.J. 1943. Cultivated varieties of *Citrus*, p. 475–668. In: H.J. Webber and L.D. Batchelor (eds.). The citrus industry, Vol. I. Univ. of California, Berkeley.
- Webber, H.J., W. Reuther, and H.W. Lawton. 1967. History and development of the citrus industry, p. 1–39. In: W. Reuther, H.J. Webber, and L.D. Batchelor (eds.). The citrus industry, Vol. I. Univ. of California, Berkeley.