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Phylogenetic Relationships among Selected *Citrus* Germplasm Accessions Revealed by Inter-simple Sequence Repeat (ISSR) Markers

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Abstract. **ISSR markers were analyzed to study phylogenetic relationships among 46 *Citrus* L. accessions representing 35 species. A dendrogram based on the unweighted pair-group method, arithmetic average cluster analysis was constructed using a similarity matrix derived from 642 polymorphic ISSR fragments generated by 10 primers. These 46 accessions could be classified into five major groups: 1) *C. indica* Tan.; 2) *C. maxima* (Burm.) Merrill; 3) lemon [*C. limon* (L.) Burm.] or lime [*C. aurantifolia* (Christm.) Swingle] type accessions; 4) *C. halimii* B.C. Stone; and 5) sour orange (*C. aurantium* L.), mandarins and their hybrids. Group 5 was further divided into three subgroups. Although some previous work had grouped it with mandarins, *C. indica* appeared to be a distinct genotype or species that was not close to mandarins. *C. tachibana* Tan. grouped closely to mandarins. *C. vulgaris* Risso was not related to sour orange but was similar to accessions usually classified in the lime or lemon group. Sour orange and its hybrids, *C. nippokoreana* Tan., *C. hanayu* Hort. ex Shirai, *C. sudachi* Hort. ex Shirai, and *C. yuko* Hort. ex Tan. had close phylogenetic relationships with mandarins. Although the mandarin accessions studied were divergent in morphology, the genetic distances among them were relatively small. Relationships among these *Citrus* accessions revealed by ISSR markers were generally in agreement with previous taxonomic classifications.**

Study of *Citrus* taxonomy and phylogeny is complicated by wide cross-compatibility among species and apomixis in some taxa. Selection and propagation of many natural or man-made hybrids and many mutants during a long history of cultivation makes *Citrus* classification even more difficult. The most widely accepted taxonomic systems for *Citrus* are those of Swingle (1946) and Tanaka (1977) who recognized 16 and 162 species, respectively. Many studies of the taxonomy and phylogeny of cultivated *Citrus* species, encompassing a variety of techniques (Barrett and Rhodes, 1976; Green et al., 1986; Handa et al., 1986; Herrero et al., 1996; Torres et al., 1978), have generally supported the three-species concept within the subgenus *Citrus* first proposed by Scora (1975) and Barrett and Rhodes (1976). *C. maxima* (Burm.) Merrill, *C. medica* L., and *C. reticulata* Blanco have been considered the ancestral species in the subgenus *Citrus* while other "forms" in this subgenus might have derived from hybridization among these species, with other species in the subgenus *Papeda,* or with related genera (Fang et al., 1993).

Swingle (1946) recognized 10 species in the subgenus *Citrus*, whereas Tanaka (1977) claimed 147 species. Except for *C. tachibana* (Mak.) Tan. and *C. indica* Tan., there were many disagreements between these two systems concerning the taxonomy of the remaining eight species recognized by Swingle. A major conflict concerned the treatment of mandarins. Swingle placed all mandarins except *C. tachibana* and *C. indica* in *C. reticulata*, whereas Tanaka recognized 36 species. The differences in *Citrus* taxonomy are mainly due to researchers employing different species concepts.

In addition, some of the characters used in taxonomic determinations are difficult to evaluate since they are affected by subjective interpretations. Many morphological traits expressed at various developmental stages are required to assign an individual to a specific taxon; however, some of these traits are influenced by the environment. DNA markers are phenotypically neutral, abundant, and less subject to environmental effects. Thus, DNA markers have been extensively used to study phylogenetic relationships in many plants (Whitkus et al., 1994).

Simple sequence repeats (SSRs), also called microsatellites, are tandem repeats of di-, tri-, or tetra-nucleotides which are abundant in eukaryotic genomes (Hamada et al., 1982). SSRs have been recognized as good sources of genetic markers in many plants including *Citrus* (Akkaya et al., 1992; Kijas et al., 1997). Standard polymerase chain reaction (PCR) analysis of microsatellites requires a knowledge of genomic sequences flanking the SSR to design primers that amplify the microsatellite region and reveal polymorphisms resulting from variation in repeat length. Sequencing and primer development are time consuming and expensive for a single marker, and only a few sequence-tagged microsatellite markers have been developed for *Citrus* (Kijas et al., 1997).

ISSR amplification is a technique that can rapidly differentiate closely related individuals (Zietkiewicz et al., 1994). ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(CA)_8$ anchored at the 3′ or 5′ end by two to four arbitrary, often degenerate (mixed) nucleotides. The sequences of repeats and anchor nucleotides are arbitrarily selected. Coupled with separation of amplification products on a polyacrylamide gel, ISSR amplification can reveal

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many more fragments per primer than random amplified polymorphic DNA (RAPD). ISSR has been used to investigate the genomic origins of the genus *Eleusine* (Salimath et al., 1995) to assess genetic diversity in dent corn (*Zea mays* L.), popcorn (*Zea mays* L.) (Kantety et al., 1995), Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco], and sugi (*Cryptomeria japonica* D. Don) (Tsumura et al., 1996). Recently, we used ISSR markers to fingerprint and cluster 48 trifoliate orange [*Poncirus trifoliata* (L.) Raf.] germplasm accessions (Fang et al., 1997) and to identify mutationally derived citrus cultivars (Fang and Roose, 1997). We found that some mutationally derived citrus cultivars had one to four unique ISSR fragments of ≈1200 scorable fragments, while two trifoliate orange accessions derived from selfing or hybridization had different fingerprint patterns but no unique fragments.

Knowledge of relative genetic relationships among genotypes is useful in a breeding program to organize germplasm resources. If germplasm collections could be systematically organized based on genetic relationships, then the efficiency of sampling and use of

Table 1. *Citrus* materials used for ISSR analysis and the number of unique ISSR fragments.

Scientific name (Tanaka's system)	Scientific name (Swingle's system)	Common name	Accession no.	Unique fragments (no.)
C. amblycarpa Ochse	*C. reticulata* Blanco	Nasnaran mandarin	2485	7
C. aurantium L.	*C. aurantium*	Standard sour orange	0628	11
C. deliciosa Ten.	*C. reticulata*	Willowleaf mandarin	3843	3
C. depressa Hay.	*C. reticulata*	Shekwasha mandarin	2448	3[y]
C. depressa	*C. reticulata*	Shekwasha mandarin	2710	3[y]
C. erythrosa Hort. ex Tan.	*C. reticulata*	Fukushu mandarin	3292	4
C. halimii B. C. Stone[z]	None	Mountain citron	3780	23
C. hanayu Hort. ex Shirai	*C. ichangensis* Swingle hybrid	None	3469	5
C. indica Tan.	*C. indica.*	Indian wild orange	3163	16
C. intermedia Hort. ex Tan	*C. aurantium* hybrid	Yuma mikan	3474	4
C. keraji Hort. ex Tan.	*C. reticulata*	Keraji mandarin	3144	1
C. kinokuni Hort. ex Tan.	*C. reticulata*	Kinokuni mandarin	0696	1
C. kinokuni	*C. reticulata*	Mukaku kishu mandarin	3887	2
C. leiocarpa Hort. ex Tan.	*C. reticulata*	Koji orange	3147	0
C. lycopersicaeformis Hort. ex Tan.	*C. reticulata*	Monkey orange	3564	3
C. maderaspatana Hort. ex Y. Tan.	*C. aurantium*	None	3225	5
C. maxima (Burm.) Merrill	*C. maxima*	Kao Phuang pummelo	3926	7
C. maxima	*C. maxima*	New Guinea pummelo	3282	4
C. megaloxycarpa Lush.	*C. limon* (L.) Burm. f.	None	3241	14
C. miaray Wester	*C. aurantium*	None	3574	15
C. nippokoreana Tan.	*C. reticulata*	Korai tachibana mandarin	3228	4
C. nobilis Lour.	*C. reticulata*	King mandarin	0303	1
C. oleocarpa Hort. ex Tan.	*C. reticulata*	Tim Kat mandarin	2692	0
C. pennivesiculata Tan.	*C. aurantifolia* (Christ.) Swing.	None	2434	11
C. reshni Hort. ex Tan.	*C. reticulata*	Cleopatra mandarin	3844	2
C. reticulata Blanco	*C. reticulata*	Canton mandarin	3576	1
C. reticulata	*C. reticulata*	Changsha mandarin	3577	1
C. reticulata	*C. reticulata*	Huang Yen Man Chieh mandarin	3897	3
C. reticulata	*C. reticulata*	PI433932	3813	0
C. reticulata	*C. reticulata*	Ponkan mandarin	3849	2
C. reticulata	*C. reticulata*	Seedling	3239	1
C. reticulata	*C. reticulata*	Som Kaeo II mandarin	3852	1
C. reticulata	*C. reticulata*	Tien Chieh mandarin	2376	0
C. rokugatsu Hort. ex Y. Tan.	*C. aurantium*	Za daidai	3473	10
C. succosa Hort. ex Tan.	*C. reticulata*	Jimikan mandarin	3280	2
C. sudachi Hort. ex Shirai	*C. ichangensis* hybrid	None	3471	1
C. sunki Hort. Ex Tan.	*C. reticulata*	Mandarin sunki	3143	1
C. tachibana (Mak.) Tan.	*C. tachibana*	Tachibana orange	3150	3
C. taiwanica Tan. & Shim.	*C. aurantium*	Nansho daidai	2588	5
C. tangerina Hort.ex Tan.	*C. reticulata*	Dancy mandarin	3026	1
C. tardiva Hort. ex Shirai.	*C. reticulata*	Giri-mikan mandarin	3297	0
C. unshiu Marc.	*C. reticulata*	Okitsu satsuma mandarin	3820	5
C. vulgaris Risso[z]	None	None	0760	8
C. yatsushiro Hort. ex Tan.	*C. reticulata*	Yatsushiro mikan mandarin	3466	0
C. yatsushiro	*C. reticulata*	Yatsushiro mikan mandarin	3880	0
C. yuko Hort. Ex Tan.	*C. ichangensis* hybrid	None	3146	0

[z]Not recorded in both Swingle's and Tanaka's systems.
[y]These three unique fragments present in both accessions of *C. depressa*.

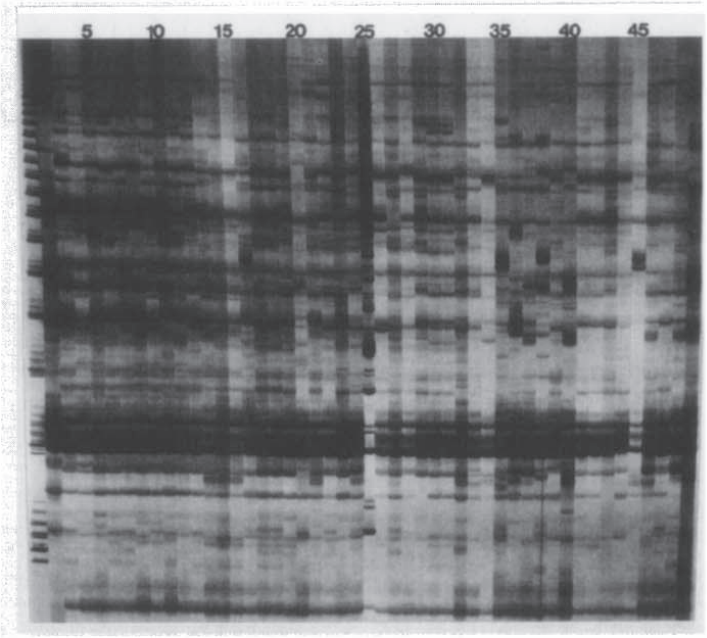

Fig. 1. ISSR profiles amplified from DNA of *Citrus* accessions using primer $(TCC)_5RY$. Lane order: 1) 123 bp ladder (the smallest band was 369 bp), 2) *Poncirus trifoliata*-Flying Dragon, 3) *C. tangerina*, 4) *C. nobilis*, 5) *C. reticulata*-Changsha, 6) *C. kinokuni*-Kinokuni, 7) *C. reticulata*-Ponkan, 8) *C. unshiu*, 9) *C. reticulata*-Som Kaeo II, 10) *C. deliciosa*, 11) *C. reticulata*-Seedling, 12) *C. reticulata*-PI433932, 13) *C. succosa*, 14) *C. yatsushiro*-3466, 15) *C. yatsushiro*-3880, 16) *C. amblycarpa*, 17) *C. depressa*-2448, 18) *C. depressa*-2710, 19) *C. erythrosa*, 20) *C. indica*, 21) *C. keraji*, 22) *C. leiocarpa*, 23) *C. lycopersicaeformis*, 24) *C. nippokoreana*, 25) 100 bp ladder (the smallest band was 400 bp), 26) *C. sunki*, 27) *C. tachibana*, 28) *C. tardiva*, 29) *C. reshni*, 30) *C. reticulata*-Tien Chieh, 31) *C. oleocarpa*, 32) *C. hanayu*, 33) *C. sudachi*, 34) *C. yuko*, 35) *C. pennivesiculata*, 36) *C. halimii*, 37) *C. intermedia*, 38) *C. megaloxycarpa*, 39) *C. miaray*, 40) *C. maxima*-New Guinea, 41) *C. reticulata*-Huang Yen Man Chieh, 42) *C. kinokuni*-Mukaku kishu, 43) *C. reticulata*-Canton, 44) *C. maderaspatana*, 45) *C. vulgaris*, 46) *C. taiwanica*, 47) *C. rokugatsu*, 48) *C. aurantium*, and 49) *C. maxima*-Kao Phuang.

germplasm resources could be greatly improved. At the inception of a breeding program, it would be advantageous to complement available phenotypic data with information on genetic similarity to maximize genetic diversity of parents. Later in a breeding program, information regarding genetic similarity between genotypes could influence the choice of individuals to cross in hybrid combinations to optimize expression of heterosis (Nienhuis et al., 1994; Smith et al., 1990).

The Citrus Variety Collection at the Univ. of California, Riverside, includes many mandarin accessions and other *Citrus* species that are rarely used as scion cultivars or in breeding programs. Little work has been done toward determining the phylogenetic relationships among them or between them and other species since Tanaka defined their taxonomic status. In the present paper, we report the application of ISSR markers to study the phylogenetic relationships among mostly uncultivated *Citrus* species in order to better use them in our breeding programs. Accessions that are relatively divergent and have more unique molecular markers will be given a higher priority in screening for disease and pest resistance . Moreover, the collection can be more efficiently maintained by identifying and eliminating duplicate accessions through analysis of large number of molecular markers.

Materials and Methods

Plant materials. Forty-six *Citrus* accessions representing thirty-five species (Table 1) in the Citrus Variety Collection at the Univ. of California, Riverside, were sampled for ISSR analysis. The scientific names in both Tanaka's and Swingle's systems are given for each accession. However, only Tanaka's scientific names are used hereafter.

DNA extraction and ISSR analysis. Total DNA was extracted from young leaves using hexadecyltrimethylammonium bromide (CTAB) according to protocols described previously (Fang et al., 1997). Ten ISSR primers (Table 2) that produced clear banding patterns were selected based on our previous work (Fang and Roose, 1997). These primers were either synthesized by Cruachem Inc. (California) or purchased from the Biotechnology Laboratory, University of British Columbia, Canada. Each 20 µL amplification reaction consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 200 µM each of dNTP, 1 µM primer, 0.01% gelatin, 2% formamide, 1 unit of *Taq* polymerase (Promega, Wisconsin), and 25 ng of template DNA. Each reaction mixture was overlaid with 50 µL of mineral oil. Amplification was performed in an thermal cycler (Ericomp Inc., Calif.) using the following temperature profile: 4 min at 94 °C, followed by 30 s at 94 °C, 45 s at 52 °C, and 2 min at 72 °C for 27 cycles, and 7 min at 72 °C for a final extension. Amplification products were separated on 320 × 380 × 0.4 mm 6% nondenaturing polyacrylamide gels containing 3 M urea and 1× TBE buffer for 12 h at 450 v. DNA was detected by silver staining (Bassam et al., 1991).

Data analysis. Polymorphic fragments were scored as present (coded as 1) or absent (coded as 0) by one researcher, and rechecked by another person. The weak (those which could not be seen clearly on a light box) and smeared fragments were not scored. Only fragments between 150 and 2500 bp were scored because the ISSR–PCR products beyond this range were generally not clear and reproducible. A similarity matrix with the similarity coefficient of Dice (1945) was constructed based on the presence or absence of polymorphic fragments for each primer. The Dice coefficient is identical to that of Nei and Li (1979) according to Dudley (1994). Cluster analysis was performed with NTSYS-pc version 1.80, a numerical taxonomy and multivariate analysis software package (Rohlf, 1993) using unweighted pair-group method, arithmetic average (UPGMA). The similarity matrix is available to readers upon request.

Results and Discussion

ISSR amplification. ISSR amplification from all DNA samples resulted in multibanded profiles with all 10 primers. Representative banding patterns observed with primer $(TCC)_5RY$ are shown in Fig. 1. The amplified fragment size ranged from 80 to 3000 bp

Table 2. ISSR primers used in this experiment and polymorphic fragments obtained

Primer[z]	Polymorphic fragment
$HVH(CA)_7T$	80
$BDB(CA)_7C$	60
$DBDA(CA)_7$	63
$HVH(TG)_7T$	64
$VHVG(TG)_7$	34
$(GA)_8YG$	74
$HVH(TCC)_5$	65
$(TCC)_5RY$	67
$BDB(TCC)_5$	30
$(AG)_8YT$	105
Total	642

[z]R = purine, Y = pyrimidine, B = non-A, D = non-C, H = non-G, V = non-T.

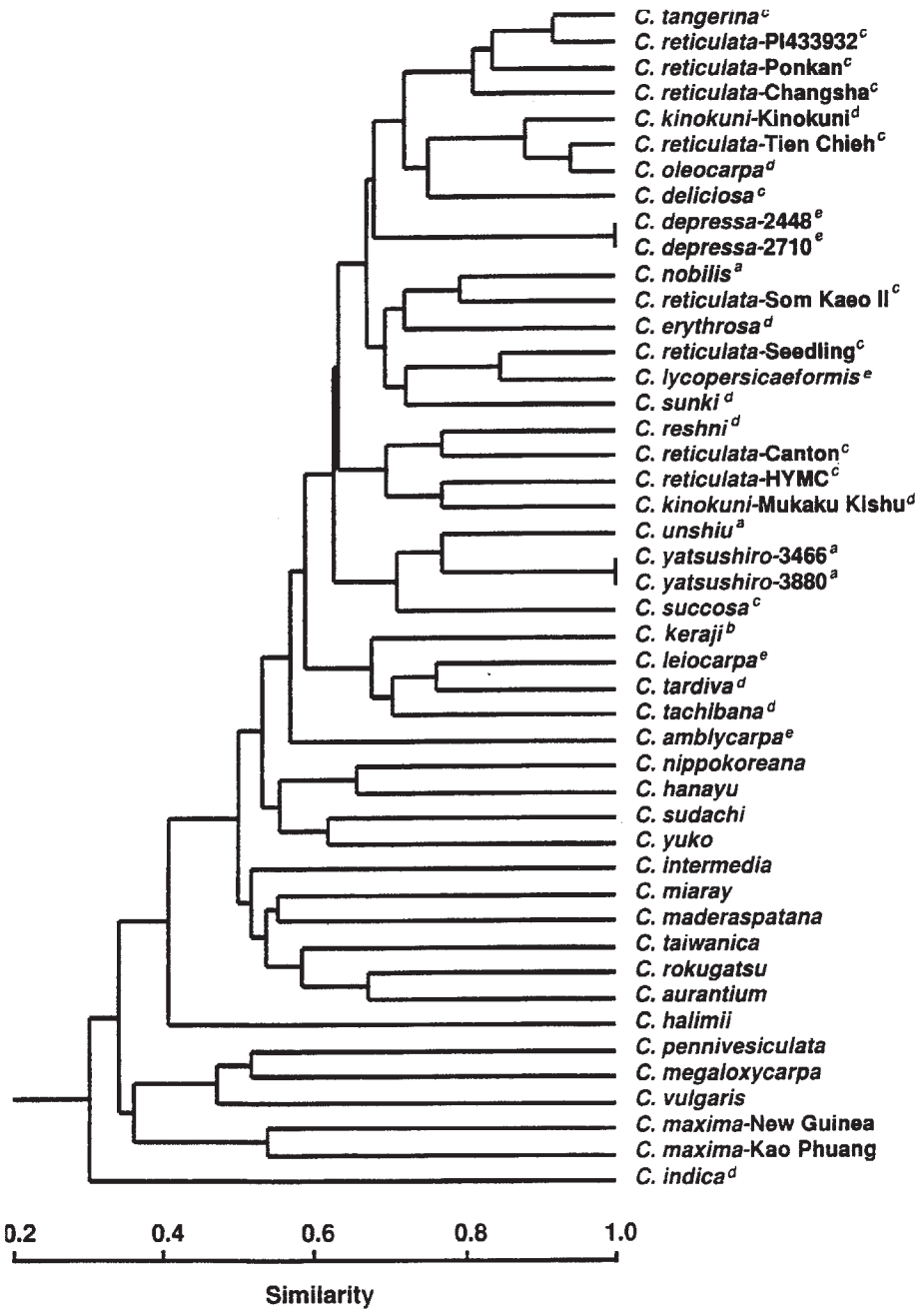

Fig. 2. Dendrogram illustrating the phylogenetic relationships among 46 *Citrus* accessions. Dendrogram was drawn based on UPGMA cluster analysis, using the similarity matrix derived from 642 polymorphic ISSR fragments. In Tanaka's system (1977), 29 mandarin accessions and *C. indica* were classified into: [a]Subsection Euacrumen, [b]Subsection Microacrumen-Group Anisodora, [c]Subsection Microacrumen-Group Citriodora-Subgroup Megacarpa, [d]Subsection Microacrumen-Group Citriodora-Subgroup Microcarpa-Microgroup Angustifolia, and [e]Subsection Microacrumen-Group Citriodora-Subgroup Microcarpa-Microgroup Latifolia.

with the scorable region being from 150 to 2500 bp. Nearly all fragments were polymorphic among accessions. The number of polymorphic fragments per primer ranged from 30 [BDB(TCC)$_5$] to 105 [(AG)$_8$YT] with an average of 64 (Table 2).

Phylogenetic relationships among accessions. Based on 642 polymorphic ISSR fragments generated by 10 primers, a similarity dendrogram (Fig. 2) was constructed using UPGMA cluster analysis. From this dendrogram, the 46 accessions could be classified into five major groups having within-group similarity values of ≥0.45. Although differences between these groups were relatively large, it should be noted that this grouping criterion was somewhat arbitrary.

Group 1 consisted of only *C. indica*. This species was indigenous to India. Swingle (1946) and Tanaka (1977) recognized it as a wild species. Tanaka considered it closely related to mandarins. ISSR data also suggested that *C. indica* is a distinct species, but not closely related to mandarins. The similarity value between *C. indica* and other accessions was low (≈0.30). The morphology of the two *C. indica* trees of this accession in our collection is generally consistent with that described by Swingle (1946). This *C. indica* accession may be a hybrid of *C. indica* and other *Citrus* species as speculated by R.W. Scora (personal communication). However, this accession had 16 unique of ≈650 scorable ISSR fragments, suggesting that it is unlikely to have derived by hybridization or mutation from other accessions in this study. Our RFLP markers (Federici et al., 1998) showed that this accession had a low level of heterozygosity and was closely related to *C. medica*. If it is a hybrid, this accession must have derived from hybridization between two very closely related genotypes.

Group 2 included two pummelo (*C. maxima*) accessions. 'Kao Phuang' pummelo is an ordinary cultivar, whereas 'New Guinea' pummelo is an accession introduced from New Guinea in 1956. In the original files of the Citrus Variety Collection, this accession was recorded as a *Citrus* species from New Guinea, but was not given a taxonomic classification. It is similar to pummelo in morphology. The ISSR markers clustered 'New Guinea' pummelo with 'Kao Phuang' pummelo, which suggested that 'New Guinea' pummelo should be placed into *C. maxima*. These accessions had four and seven unique ISSR fragments, respectively. The relatively low similarity value (0.53) between these two pummelo accessions suggests considerable diversity in *C. maxima*.

Group 3 was composed of three accessions, i.e., *C. pennivesiculata* Tan., *C. megaloxycarpa* Lush., and *C. vulgaris* Risso. Swingle and Reece (1967) considered *C. pennivesiculata* as lime or its hybrid, and *C. megaloxycarpa* as lemon. *C. vulgaris* was not listed in either Swingle's or Tanaka's system. Risso (1813) (also see Swingle, 1946) granted *C. vulgaris* a species status but considered it to be similar to sour orange. However, the fruit of *C. vulgaris* in our collection is similar to lime. The ISSR data indicate that *C. vulgaris* is not related to sour orange but is close to *C. pennivesiculata* and *C. megaloxycarpa*, suggesting that *C. vulgaris* belongs to the acid fruit group which contains lemon, lime, and citron (*C. medica*) (Scora, 1988). Though *C. vulgaris*, *C. pennivesiculata* and *C. megaloxycarpa* were clustered in the same group, the similarity value between them was only 0.47. Moreover, each had eight or more unique ISSR fragments, indicating that these three taxa are quite different.

Group 4 had only one accession, *C. halimii* B. C. Stone. This is

a relatively recently recognized species indigenous to the hill forests of Malaya and the adjacent peninsular region of Thailand (Stone et al., 1973). Investigation of essential oils, isoenzymes, and epicuticular leaf waxes supported the species standing of *C. halimii* (Gulz et al., 1987; Scora et al., 1976). The ISSR marker data showed that *C. halimii* was clustered as a single group that was not closely linked with other accessions. This species had 23 unique ISSR fragments, the most of any accession studied.

Group 5, the largest group, included the remaining 39 accessions. Overall, the phylogenetic relationships among accessions in this group were close. The similarity value within this group was ≈0.50. This group could be divided into three subgroups described below based on similarity value, number of unique fragments, as well as works of Swingle (1946) and Tanaka (1977).

Subgroup 1 comprised six accessions, namely *C. intermedia* Hort. ex Tan., *C. miaray* Wester, *C. maderaspatana* Hort. ex Y. Tan., *C. taiwanica* Tan. & Shim., *C. rokugatsu* Hort. ex Y. Tan., and *C. aurantium*. Each had four or more unique fragments. Swingle and Reece (1967) considered the first five accessions as *C. aurantium* or its hybrids. The present study supported this grouping in that the genetic similarity (0.51) was similar to that between two pummelo accessions. The clustering among these six accessions was consistent with Tanaka's system that placed *C. intermedia* in Section Cephalocitrus and the others in Section Aurantium (Tanaka, 1977).

Subgroup 2 included *C. nippokoreana* Tan., *C. hanayu* Hort. ex Shirai, *C. sudachi* Hort ex Shirai, and *C. yuko* Hort. ex Tan. Swingle and Reece (1967) classified *C. nippokoreana* within *C. reticulata*; however, Tanaka (1977) did not recognize it as a mandarin species, but placed it into Section Osmocitrus that contained *C. hanayu, C. sudachi*, and *C. yuko* as well. The ISSR data supported Tanaka's treatment of *C. nippokoreana* and the other three species. However, subgroup 2 was very closely related to mandarins in subgroup 3 as shown in Fig. 2, indicating that this subgroup has fairly close phylogenetic relationships with mandarins.

Subgroup 3 consisted of 29 mandarin accessions including *C. tachibana*. In this subgroup, *C. amblycarpa* Ochse was the most distinct. Moreover, *C. amblycarpa* had seven unique ISSR fragments, the most for any of these 29 mandarin accessions. *C. tachibana* was one of ten species in the subgenus *Citrus* recognized by Swingle (1946). Later, Swingle and Reece (1967) suspected that *C. tachibana* might be a satellite species of *C. reticulata*. Tanaka (1977) considered *C. tachibana* a mandarin species and placed it in Section Acrumen that consisted of 36 mandarin species. Our present study indicated that *C. tachibana* is closely allied to mandarins and should be regarded as a mandarin accession. Although Swingle and Reece (1967) considered *C. depressa* Hay. a hybrid of *C. tachibana*, the ISSR data showed that two *C. depressa* accessions were relatively distant from *C. tachibana*. The two *C. depressa* accessions were identical for all ISSR markers. In addition, they were indistinguishable in morphology. They may be duplicate accessions. Likewise, no ISSR marker differences were observed between two *C. yatsushiro* Hort. ex Tan. accessions. Although these 29 mandarin accessions were distributed in five microgroups in Tanaka's system (Fig. 2), they were scattered in the dendrogram. For example, *C. tachibana* and *C. erythrosa* Hort. ex Tan. were in the same microgroup in Tanaka's system, while *C. keraji* Hort. ex Tan. was in a different group. However, in our dendrogram, *C. tachibana* was closer to *C. keraji* than to *C. erythrosa*. Eight *C. reticulata* accessions distributed in several small clusters. Although these mandarin accessions were morphologically divergent, their phylogenetic relationships as measured by ISSR markers were approximately equal to that between two pummelo accessions.

In summary, many ISSR markers can be generated rapidly to assess phylogenetic relationships among *Citrus* accessions. Accessions with many unique ISSR fragments could be useful for expanding the germplasm base of breeding programs. The relationships among the *Citrus* accessions studied were generally in agreement with Swingle's (1946) classification system.

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