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Lemons: Diversity and Relationships with Selected *Citrus* Genotypes as Measured with Nuclear Genome Markers

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Abstract. Inter-simple sequence repeats (ISSR), simple sequence repeats (SSR) and isozymes were used to measure genetic diversity and phylogenetic relationships among 95 *Citrus* L. accessions including 57 lemons [*C. limon* (L.) Burm. f.], related taxa, and three proposed ancestral species, *C. maxima* (Burm.) Merrill (pummelo), *C. medica* L. (citron), and *C. reticulata* Blanco (mandarin). The ancestry of lemons and several other suspected hybrids was also studied. Five isozyme and five SSR loci revealed relatively little variation among most lemons, but a high level of variation among the relatively distant *Citrus* taxa. Eight ISSR primers amplified a total of 103 polymorphic fragments among the 83 accessions. Similarity matrices were calculated and phylogenetic trees derived using unweighted pair-group method, arithmetic average cluster analysis. All lemons, rough lemons, and sweet lemons, as well as some other suspected hybrids, clustered with citrons. Most lemons (68%) had nearly identical marker phenotypes, suggesting they originated from a single clonal parent via a series of mutations. Citrons contributed the largest part of the lemon genome and a major part of the genomes of rough lemons, sweet lemons, and sweet limes. Bands that characterize *C. reticulata* and *C. maxima* were detected in lemons, suggesting that these taxa also contributed to the pedigree of lemon.

Although lemon (*Citrus limon*) was accepted as a species by the two most widely cited taxonomic systems (Swingle and Reece, 1967; Tanaka, 1977), many studies have suggested that lemon is likely to be of hybrid origin (Barrett and Rhodes, 1976; Green et al., 1986; Handa et al., 1986; Herrero et al., 1996; Malik et al., 1974; Torres et al., 1978). Many lemons have highly similar morphological and biochemical characters and some are known to have originated by mutation from other lemons. In germplasm collections, accessions with acid fruit that are similar in shape and color to lemon have generally been listed as *C. limon*. The origin, ancestry, and correct classification of these accessions are less well understood. Molecular markers show some diversity among lemons (Deng et al., 1995; Fang and Roose, 1997), but genetic diversity of a large sample of lemon cultivars from a wide range of geographic locations has not been reported.

Although nearly all cultivated *Citrus* are diploids, several other factors complicate the taxonomy of *Citrus*, including lemon. Nearly all *Citrus* taxa are interfertile, and hybridization of *Citrus* with several other genera is also possible (Iwamasa et al., 1988). Many *Citrus* taxa have a form of apomixis called nucellar embryony, in which embryos develop that are genetically identical to the mother. This permits hybrids to breed true. Seedlings originating from nucellar embryos differ from the maternal tree in increased thorniness and tree vigor, and may occasionally be named as distinct cultivars because of these traits. Another difficulty is that natural populations of *Citrus* have rarely been described in detail and it is likely that few now exist. The overall picture that has emerged from a variety of studies is that most cultivars derive directly, or by hybridization, from three ancestral species, *C. maxima* (pummelo), *C. medica* (citron), and *C. reticulata* (mandarin) (Barrett and Rhodes, 1976; Roose et al., 1995). Diversification in oranges [*C. sinensis* (L.) Osbeck], lemons, and grapefruit (*C. paradisi* Macf.) has occurred primarily through selection of bud sports or nucellar seedling variants (Roose et al., 1995). However, it is not always clear which cultivars have originated by mutation versus hybridization or selfing. Information on genetic diversity and phylogeny of cultivars can improve the efficiency of germplasm characterization and its use in breeding programs. Determination of the parentage of hybrid taxa such as lemon is also valuable to breeders attempting to resynthesize these types.

The following study examined heterozygosity and genetic diversity of molecular markers in 57 lemons, focusing on distinguishing lemon cultivars that originated by mutation from those that may have originated as unique hybrids. Relationships were also determined between lemons and some citrons, pummelos, mandarins, other *Citrus* taxa, and three related genera.

Since morphological characters are subject to environmental modifications and may not always unambiguously distinguish or correctly cluster closely related taxa, molecular markers are often used to clarify phylogenetic relationships. Several molecular methods are widely applied in plant systematics, including isozymes, restriction fragment-length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD), techniques based on dispersed repetitive DNA such as inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) or microsatellites, and restriction site variation of chloroplast DNA (Whitkus et al., 1994).

In this study, we used ISSR markers which target divergence in regions containing dispersed repetitive DNA and can rapidly differentiate closely related individuals (Fang and Roose, 1997; Zietkiewicz et al., 1994). The technique involves polymerase chain reaction (PCR) amplification of DNA using a mixture of primers composed of a microsatellite sequence such as $(TG)_7$ anchored at the 3' or 5' end by two to four arbitrary, often

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degenerate (mixed) nucleotides. The advantage of this technique is that the multiband profile per gel and high frequency of polymorphism result in relatively low cost per marker compared to RAPD and RFLP. ISSR markers are dominant markers that cannot detect zygosity (Fang and Roose, 1999).

We also used isozymes and SSRs, which are codominant markers (Staub et al., 1996). SSR markers typically have many different alleles and therefore are quite informative about possible parents of hybrids. For PCR amplification, a pair of specific primers that flank a rapidly evolving repetitive DNA sequence are required. Allelic variation is predominantly determined by differences in the number of repeat units.

Materials and Methods

Plant materials. The number of accessions studied was 86 for isozymes, 72 for SSRs, and 83 for ISSRs. These included 57 lemons (*C. limon*), six citrons (*C. medica*), four pummelos (*C.*

Table 1. Accessions used in isozyme, ISSR, and SSR studies are marked with X and are identified by Tanaka species name and CRC identification number (Citrus Research Center, University of California, Riverside).

Species	CRC no.	Cultivar or common name	Isozyme	SSR	ISSR
C. aurantifolia (Christm.) Swing.	1710	'Mexican' lime	X	X	–
C. aurantium L.	0628	'Standard' sour orange	X	X	–
C. aurantium L.	2438	'Tunisian' sour orange	X	X	–
C. bergamia Risso and Poit.	2881	Bergamot orange	X	X	–
C. clementina Hort. ex Tan.	0279	'Algerian Clementine' mandarin	X	X	X
C. halimii B. C. Stone	3900	Unnamed	–	X	–
C. indica Tan.	3163	Indian wild orange	X	X	–
C. jambhiri Lush.	3386	'Estes' rough lemon	X	–	X
C. jambhiri Lush.	3385	'Florida' rough lemon	X	X	X
C. jambhiri Lush.	1222	'Mazoe' rough lemon	X	–	X
C. jambhiri Lush.	2325	'South African' rough lemon	–	–	X
C. jambhiri Lush.	3060	Unnamed rough lemon	–	–	X
C. limetta Risso	2695	'Faris' sweet lemon	X	–	X
C. limetta Risso	3492	Iraq sweet lemon	X	X	X
C. limetta Risso	0569	'Millsweet' sweet lemon	X	X	X
C. limetta Risso	3093	Iran sweet lemon	X	X	X
C. limettioides Tan.	3051	'Mitha-Tulia' sweet lime	X	X	X
C. limon (L.) Burm. f.	3496	'Allen Newman Eureka' lemon	X	–	X
C. limon (L.) Burm. f.	3007	'Allen Variegated' lemon	X	X	X
C. limon (L.) Burm. f.	2429	'Amber' lemon	X	X	X
C. limon (L.) Burm. f.	3387	'Arancino' lemon	X	–	X
C. limon (L.) Burm. f.	3506	'Bergamotto' lemon	X	–	X
C. limon (L.) Burm. f.	3590	'Berna' lemon	X	X	X
C. limon (L.) Burm. f.	3265	'Bitrouni' o.p. seedling lemon	X	X	X
C. limon (L.) Burm. f.	3499	'Blanchard' Eureka lemon	X	–	X
C. limon (L.) Burm. f.	0710	Chinese lemon	X	X	X
C. limon (L.) Burm. f.	3498	'Cascade Eureka' lemon	X	X	X
C. limon (L.) Burm. f.	3837	'Cook Eureka' lemon	X	–	X
C. limon (L.) Burm. f.	3043	'Corona Old Line Eureka' lemon	X	–	X
C. limon (L.) Burm. f.	3591	'Corpaci' lemon	X	X	X
C. limon (L.) Burm. f.	3500	'Femminello Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3388	'Femminello Ovale' lemon	X	X	X
C. limon (L.) Burm. f.	3389	'Femminello Sfusato' lemon	X	X	X
C. limon (L.) Burm. f.	3836	'Foothill Lisbon' lemon	X	–	X
C. limon (L.) Burm. f.	3005	'Frost Eureka' lemon	X	X	X
C. limon (L.) Burm. f.	3176	'Frost Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3835	'Galligan Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	0565	'Genoa' lemon	X	X	X
C. limon (L.) Burm.f.	3737	'Improved Meyer' lemon	X	X	X
C. limon (L.) Burm. f.	2323	'India' lemon	X	X	X
C. limon (L.) Burm. f.	3593	'Interdonato' lemon	X	X	X
C. limon (L.) Burm. f.	3885	Iran local lemon	X	X	X
C. limon (L.) Burm. f.	2899	'Italian Pink Fleshed' lemon	X	–	X
C. limon (L.) Burm. f.	3010	'Kaweah Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3045	'Kulu' lemon	X	X	X
C. limon (L.) Burm. f.	3194	'Kusner' lemon	X	–	X
C. limon (L.) Burm. f.	4005	'Lapithiotiki' lemon	–	X	X
C. limon (L.) Burm. f.	2317	'Limon Real' lemon	X	X	X

maxima), nine rough lemons (*C. jambhiri* Lush.), one lime (*C. aurantifolia* (Christm.) Swing.), one mandarin (*C. clementina* Hort. ex Tanaka or *C. reticulata*), and 18 other *Citrus* genotypes and three related genera, *Fortunella* Swingle, *Eremocitrus* Swingle, and *Microcitrus* Swingle (Table 1). All accessions were sampled from the Citrus Variety Collection at the University of California, Riverside. The accessions sampled included economically and genetically important lemon cultivars, and known and suspected lemon hybrids. Fresh leaves were used for both enzyme and DNA extraction.

DNA EXTRACTION. Total DNA for the ISSR and SSR study was extracted from young leaves according to the protocol of Webb and Knapp (1990) modified as described by Fang et al., 1997. DNA pellets were redissolved in 250 mL of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

ISOZYME ANALYSIS. Isozymes were analyzed according to Xiang and Roose (1988). Denotation of alleles and loci was according to Torres et al. (1982). A total of four enzyme systems were analyzed; GOT (glutamate oxaloacetate transaminase), IDH (isocitrate dehydrogenase), MDH (malate dehydrogenase), and SkDH (shikimate dehydrogenase). One locus was scored in each system, except for MDH where two were scored.

Table 1. Continued.

Species	CRC no.	Cultivar or common name	Isozyme	SSR	ISSR
C. limon (L.) Burm. f.	3970	'Limonero Fino' lemon	X	X	X
C. limon (L.) Burm. f.	3501	'Limoneira 8A Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3200	'Limoui Sangui' lemon	X	X	X
C. limon (L.) Burm. f.	3390	'Lo Porto' lemon	X	X	X
C. limon (L.) Burm. f.	3159	'Lunario' lemon	X	X	X
C. limon (L.) Burm. f.	3013	'Lupe Lisbon' lemon	X	–	X
C. limon (L.) Burm. f.	3892	'Mesero' lemon	X	X	X
C. limon (L.) Burm. f.	3009	'Messina' lemon	X	X	X
C. limon (L.) Burm. f.	3392	'Monachello' lemon	X	X	X
C. limon (L.) Burm. f.	3839	'Monroe Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3841	Nicaraguan lemon	X	X	X
C. limon (L.) Burm. f.	3924	'Peretta' lemon	X	X	X
C. limon (L.) Burm. f.	3505	'Prior Lisbon' seedling lemon	X	X	X
C. limon (L.) Burm. f.	3491	'Primofiore' lemon	X	X	X
C. limon (L.) Burm. f.	3893	'Ricote' lemon	X	–	X
C. limon (L.) Burm. f.	3840	'Rosenberger Lisbon' lemon	X	–	X
C. limon (L.) Burm. f.	3838	'Ross Eureka' lemon	X	X	X
C. limon (L.) Burm. f.	3894	'Santa Teresa' lemon	X	X	X
C. limon (L.) Burm. f.	3001	'Seedless Lisbon' lemon	X	X	X
C. limon (L.) Burm. f.	3199	'Soh Long' lemon	–	–	X
C. limon (L.) Burm. f.	3261	'Soh Synteng' lemon	X	X	X
C. limon (L.) Burm. f.	4014	'Taylor Eureka' lemon	X	X	X
C. limon (L.) Burm. f.	0599	'Variegated Eureka' lemon	X	–	X
C. limon (L.) Burm. f.	0280	'Villafranca' lemon	–	–	X
C. limon (L.) Burm. f.	0390	'Villafranca' lemon	X	X	X
C. limon (L.) Burm. f.	3300	Wild lemon	X	X	X
C. limonia Osbeck	3932	'Hangleson' Rangpur lime	X	X	X
C. lumia Risso and Poit	3925	'Lumia' lemon	X	X	X
C. maxima (Burm.) Merrill	2355	Unnamed Thai pummelo	X	X	–
C. maxima (Burm.) Merrill	1224	Chinese pummelo seedling	X	X	X
C. maxima (Burm.) Merrill	2240	'Siamese Acidless' pummelo	X	X	X
C. maxima (Burm.) Merrill	2346	'African' pummelo	X	X	X
C. maxima (Burm.) Merrill	2340	Unnamed pummelo	X	X	X
C. reticulata x *C. maxima*	3555	'Cocktail' grapefruit	–	–	X
C. medica L.	3819	Unnamed citron	X	X	X
C. medica L.	3527	'Hiawassie' citron	X	X	X
C. medica L.	3532	'Papuan' citron	X	X	X
C. medica L.	3768	'Buddha`s Hand' citron	X	X	X
C. medica L.	3891	'Ethrog' citron	X	X	X
C. medica L.	3523	'Diamante' citron	X	X	–
C. micrantha Wester	3605	'Samuyao' papeda	X	X	–
C. sinensis (L.) Osbeck	2750	'Olinda Valencia' orange	X	X	X
C. spp. (hybrid)	1462	'Cuban' shaddock	–	–	X
C. tengu Hort. ex Tan.	3464	Unnamed pummelo hybrid	–	–	X
Eremocitrus glauca (L.) Swing.	3463	Australian desert lime	X	X	–
Fortunella polyandra (Ridl.) Tan.	3901	Malayan kumquat	X	X	–
Microcitrus australis (Planch.) Swing.	3666	Australian round lime	X	X	–

Table 2. ISSR primers used to study diversity and phylogeny of lemon and the number of fragments observed and scored for each primer.

Primer[z]	Fragments (no.)	Polymorphic fragments
$BDB(CA)_7C$	50	15
$DBDA(CA)_7$	55	14
$(GT)_8YA$	30	4
$HVH(CA)_7T$	55	18
$VHVG(TG)_7$	50	16
$HVH(TCC)_7$	37	13
$(GA)_8YG$	62	12
$(TCC)_5RY$	35	11
Total	374	103

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

SSR ANALYSIS. Five pairs of primers (Kijas et al., 1997) were used to amplify genomic DNA. The forward fluorescent labeled primers were purchased from LI-COR (Lincoln, Nebr.) and the reverse (unlabelled) ones from Cruachem (Dulles, Va.) or Genosys (Woodlands, Texas). PCR amplifications were conducted in a 10 mL volume containing 5 ng of template DNA, 2-4 mM $MgCl_2$, 0.2 mM dNTP, 0.2 units of Taq polymerase, 0.025 μM forward and reverse primer, 10 mM Tris-HCl pH 9.0, 50 mM KCl and 0.1% TritonX-100. A 96-well thermocycler (RoboCycler Gradient 96, STRATAGENE, La Jolla, Calif.) was used for PCR amplifications. PCR conditions varied among primer pairs: primers AGG9, CAC23, and CAC33 were amplified in reactions containing 2 mM $MgCl_2$ for 32 cycles with 55 °C annealing temperatures. Primer CAC39 was amplified in reactions containing 3 mM $MgCl_2$ for 35 cycles with 45 °C annealing temperature. Primer TAA41 was amplified in reactions containing 4 mM $MgCl_2$ for 38 cycles with 45 °C annealing temperature. LI-COR Stop Solution was added to each PCR product. After denaturation of fluorescent PCR products at 92 °C for 3 min, they were separated on an automated LI-COR 4200LR Sequencer using 18 cm plates with a 7% Long Ranger gel containing 7 M urea and 1× TBE buffer, then scored and sized using RFLPSCAN software (Scanalytics, Billerica, Mass.).

ISSR ANALYSIS. A total of 8 primers (Table 2) evaluated previously (Fang and Roose, 1997) were used to amplify DNA. Primers were purchased from the University of British Colombia (Vancouver, British Colombia, Canada) or Cruachem (Dulles, Va.). Each 15 μL amplification reaction consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% TritonX-100, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1.2 μM primer, 0.01% gelatin, 2% formamide, 0.75 unit of Taq polymerase (Promega, Madison, Wis.), and 25 ng of template DNA. The PCR conditions did not vary among primers. Each reaction mixture was overlaid with 50 μL of mineral oil to prevent evaporation during the amplification. A 96-well thermal cycler (Ericomp, San Diego, Calif.) was used for amplification under the following conditions: 3 min at 94 °C for 1 cycle, 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 final extension. Amplification products were separated on 320 × 380 × 0.40 mm (thickness) 6% nondenaturing polyacrylamide gels containing 3 M urea and 1× TBE buffer (Zietkiewicz et al. 1994). DNA was detected by silver staining (Bassam et al. 1991).

We tested the repeatability of ISSR markers. Nine samples were reextracted and re-amplified with all eight primers. When these duplicate samples were analyzed on the same gel, all patterns were identical. Two trees of the same variety, 'Faris' sweet lemon, were also compared. No difference was found between them. Repeatability of patterns among gels run on different dates is lower, perhaps due to variation in PCR and staining conditions.

DATA ANALYSIS. To increase the proportion of the genome sampled and the total number of polymorphic bands scored, isozyme and SSR allelic data were combined for analysis. Separate similarity matrices based on the proportion of shared bands or alleles were constructed for the ISSR and combined isozyme-SSR bands using Dice's coefficient (Dice, 1945). Cluster analysis was performed with NTSYS-PC version 1.80 (Rohlf, 1993) using the unweighted pair-group method, arithmetic average (UPGMA). The similarity matrices are available to readers upon request.

Results and Discussion

ISOZYMES AND SSRS. Twelve isozyme alleles from five loci and 36 polymorphic SSR fragments amplified with five pairs of SSR primers were scored as allelic characters, making a total of 48 scored characters. In the SSR study, 16 different alleles were detected at the locus amplified by primer TAA41. The fewest number of SSR alleles, three, was amplified with primer CAC33. Seventy-two *Citrus* taxa that had both isozyme and SSR data were placed in the phylogenetic tree.

For each accession, the total number of alleles at 10 isozyme and SSR loci was counted as a measure of heterozygosity level (Table 3). Lemons had the highest number of alleles with most accessions having 19 alleles at 10 loci, whereas the three related genera and three basic *Citrus* species averaged only 13 and 12 alleles, respectively, suggesting that lemons are highly heterozygous. There is a close correlation between the proportion of homozygous loci and the apparent evolutionary origin of *Citrus* genotypes. Taxa with a higher proportion of homozygous loci are primitive types, while those with a lower proportion of homozygous loci are of hybrid origin (Fang et al., 1994). However, because we sampled only 10 isozyme and SSR loci, the estimate of heterozygosity reported herein is likely to have a rather large sampling error so that some accessions of probable hybrid ancestry (e.g., sweet orange) have relatively low allele numbers.

Genetic variation among lemon cultivars (*C. limon*) was very low, which is consistent with previous studies (Deng et al., 1995; Herrero et al., 1996; Torres et al., 1978). Most (43 of 53) lemons (*C. limon*) had identical genotypes for the five isozyme loci studied, but 'Bitrouni', Chinese, 'Interdonato', 'Kulu', 'Limon Real', 'Limoui Sangui', 'Improved Meyer', Nicaraguan, 'Peretta', and Wild lemons differed from the common genotype at one or more loci. Three of the four sweet lemons (Iran, Iraq, and 'Millsweet') differed from lemon for *Idh*.

The dendogram (not presented) based on isozyme and SSR data was similar to that from the larger number of loci studied for ISSR markers (see below). Most (28 of 41) lemons (*C. limon*) were identical, having similarity values of 1.0. The main group of 28 lemons had higher similarity coefficients with citrons (0.59 to 0.69) than with pummelos (0.48 to 0.62), although they clustered slightly closer to pummelos than to citrons. High similarity values, ranging between 0.82 and 0.88 were found among lemons, sweet lemons (*C. limetta* Risso), and rough lemons (*C. jambhiri*), and Bergamot orange (*C. bergamia* Risso and Poit.), while similarity values were lower (0.64 to 0.72) among lemons, Mexican lime (*C. aurantifolia*), and sweet limes (*C. limettioides* Tan.). One difference from the ISSR tree was that the isozyme and

SSR data clustered 'African' pummelo nearer to lemons than to other pummelos. However, deletion of any one of three loci from the data placed all pummelos together, an indication that clustering with this dataset is not always robust. The details of relationships among *Citrus* samples are discussed below. A few additional accessions were studied only for isozymes and SSRs, and are therefore discussed herein. Sour orange (*C. aurantium* L.) clustered with sweet orange (*C. sinensis*) and 'Algerian Clementine' (*C. clementina*). Indian wild orange (*C. indica* Tan.) clustered with 'African pummelo', and near the lemons and limes. *Citrus micrantha* Wester clustered with the lemons and mandarin-orange group, but with a similarity of only 0.60. Among the related genera, *Fortunella polyandra* (Ridl.) Tan. was a sister group to all of the *Citrus* taxa, and *Eremocitrus glauca* (L.) Swing. clustered with *Microcitrus australis* (Planch.) Swing. as the most divergent group. Because relatively few loci were studied, relationships among accessions indicated by the isozyme-SSR data should be viewed cautiously.

ISSR MARKERS. Amplification of DNA resulted in multiple banding profiles with all eight ISSR primers tested. The number of fragments per primer ranged from 30 [$(GT)_8YA$] to 55 [$DBDA(CA)_7$]. The number of polymorphic fragments scored ranged from 4 with $(GT)_8YA$ to 18 with $HVH(CA)_7$ (Table 2). Bands were assigned codes ranging from 1 to 4 that indicated band intensity. Computer analyses conducted with alternative datasets such as 1–2–3–4, 2–3–4, and 3–4 gave similar trees, so all 103 polymorphic bands scored were used for analysis.

The total number of ISSR bands scored in each accession is shown in Table 3. We expect more bands in hybrids for codominant markers because, if parents are homozygous for different alleles at the same locus, then the hybrid will have both alleles (bands). For dominant markers, we expect an increased number of bands in hybrids because it is likely that the parental taxa are homozygous for different (present versus absent) alleles at different loci and the hybrid is expected to have the band present (dominant) phenotype at all such loci. For example if parent 1 has genotype $1^+1^+2^-2^-$ and parent 2 has genotype $1^-1^-2^+2^+$, then the hybrid will have genotype $1^+1^-2^+2^-$ and have more bands than either parent. Taxa with high levels of heterozygosity included most lemons, sweet lemon, and rough lemon, while citron,

Table 3. Number of ISSR bands and isozyme-SSR alleles scored for each accession or taxon arranged in descending order by number of ISSR bands.

		No. of		
	Common	ISSR	Isozyme and SSR[y]	
Species	name	bands[z]	Alleles	Loci
C. limon	All others (41 cvs.)	61–71	19	10
C. limetta	Sweet lemon (4 cvs.)	54–69	18	10
C. limon	'Limoui Sangui' lemon	60	18	10
C. limon	'Italian Pink Fleshed' lemon	59	---	---
C. *lumia*	'Lumia' lemon	58	13	9
C. jambhiri	Rough lemon (5 cvs.)	55–57	17	8
C. limettioides	'Mitha-Tulia' sweet lime	57	16	8
C. limon	'Bergamotto' lemon	56	---	---
C. limon	'Limon Real' lemon	55	---	---
C. limon	'Peretta' lemon	54	16	10
C. limon	'Soh Synteng' lemon	52	17	10
C. limon	'Improved Meyer' lemon	52	19	10
C. micrantha	'Samuyao' papeda	---	14	10
C. aurantifolia	'Mexican' lime	---	15	9
Fortunella polyandra	Malayan kumquat	---	13	9
C. limon	'Interdonato' lemon	51	13	10
C. aurantium	Sour orange (2 cvs.)	---	11	8
*C. limon*ia	'Hangleson' Rangpur lime	50	18	10
C. limon	'Soh Long' lemon	50	---	---
C. limon	'Kulu' lemon	48	19	10
C. limon	Chinese lemon	47	11	10
C. limon	Nicaraguan lemon	45	12	10
C. medica	Citron (6 cvs.)	36–44	10–12	10
C. limon	'Bitrouni' seedling lemon	43	---	---
C. reticulata x *C. maxima*	'Cocktail' grapefruit	41	---	---
C. limon	Wild lemon	39	9	9
C. clementina	'Algerian Clementine' mandarin	37	14	10
C. sinensis	'Olinda Valencia' orange	37	13	9
C. tengu	Unnamed pummelo hybrid	32[x]	---	---
C. maxima	Pummelo (5 cvs.)	24–30	11–13	10
Microcitrus australis Australian round lime		---	13	10
Eremocitrus glauca Australian desert lime		---	9	8

[z]Range of band numbers shown for taxa with multiple cultivars.
[y]In taxa with multiple accessions, one locus may have missing data for one or more accessions. Value shown is maximum observed.
[x]One band missing due to amplification or staining errors.

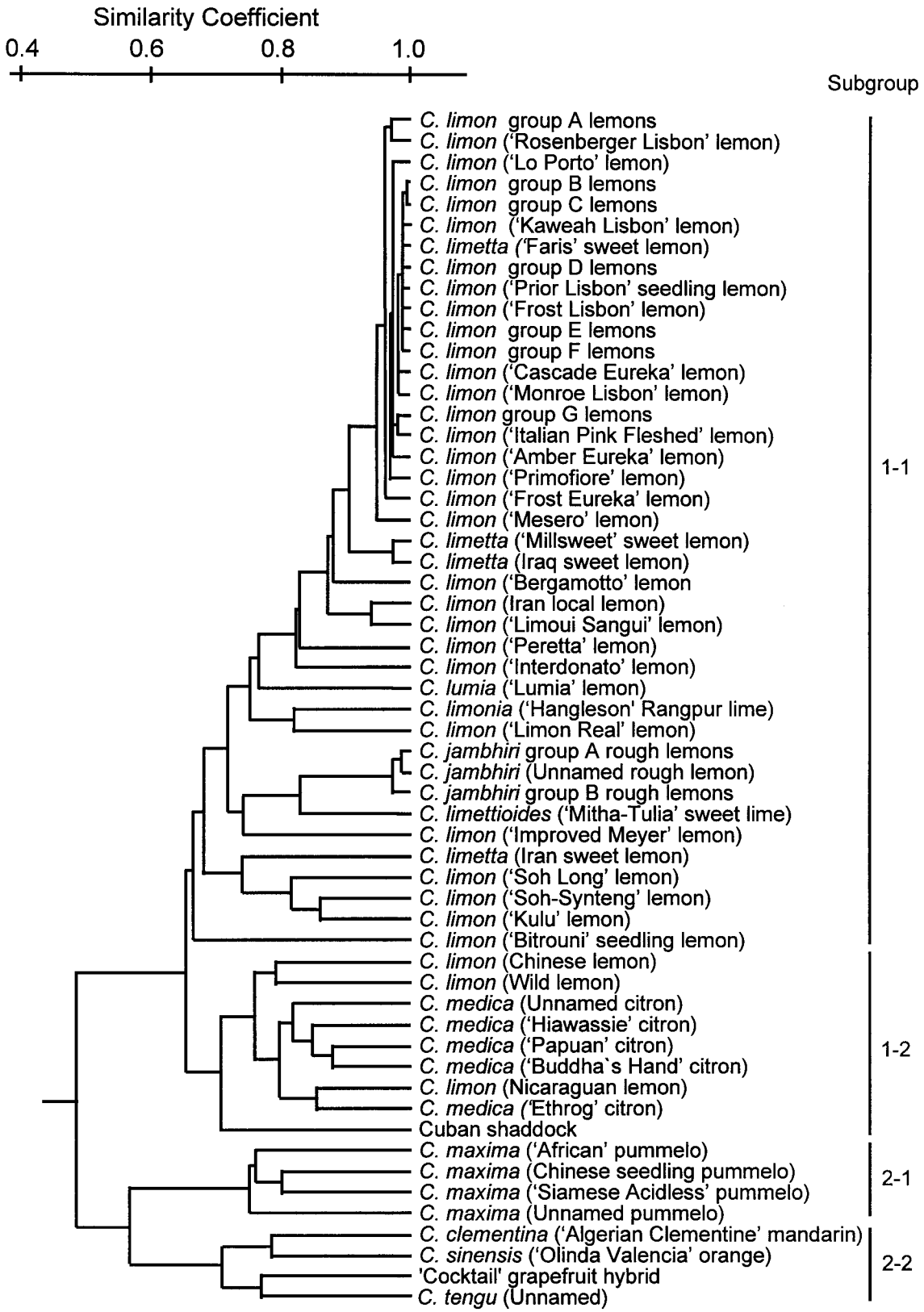

Fig. 1. UPGMA dendogram of 83 accessions of *Citrus* from ISSR data. Similarity values are shown on top of the dendogram. *Citrus limon* group A includes 'Berna' and 'Lapithiotiki'; *C. limon* group B includes 'Femminello Lisbon', 'Foothill Lisbon', 'Villafranca' (CRC 0390), 'Cook Eureka', 'Allen Variegated', 'Messina', 'Femminello Sfusato', 'Femminello Ovale', 'Corona Eureka', 'Arancino', 'Kusner', 'Seedless Lisbon', 'Genoa Eureka', 'Ross Eureka', 'Ricote Eureka', 'Blanchard Eureka', 'Lupe Lisbon' and 'Lunario'; *C. limon* group C includes 'Villafranca' (CRC 0280), 'Monachello' and 'Allen Newman Eureka'; *C. limon* group D includes 'Corpaci' and 'Santa Teresa'; *C. limon* group E includes 'Galligan Lisbon' and 'Limoneira 8A Lisbon'; *C. limon* group F includes 'Limonero Fino' and 'Taylor Eureka'; *C. limon* group G includes 'India' and 'Variegated Eureka'; *C. jambhiri* group A includes 'Mazoe' and 'Florida' rough lemons; *C. jambhiri* group B includes 'South African' and 'Estes' rough lemons.

Table 4. Number of polymorphic ISSR bands shared by various pairs of groups. The second number shows the number of potentially scorable bands in that pair of taxa. Six additional bands were monomorphic in all taxa studied.

	Rough lemon	Citron	Pummelo	'Algerian Clementine' mandarin	'Olinda Valencia' orange	'Improved Meyer' lemon
Lemons (main group)	45/98	40/103	25/98	30/103	28/103	45/103
Rough lemons (*C. jambhiri*)	---	37/96	19/96	25/96	22/96	40/98
'Improved Meyer' lemon	40/98	34/103	15/101	26/103	24/103	---
'Interdonato' lemon	39/98	40/103	17/98	20/103	17/103	38/103
Sweet lemons (*C. limetta*)	42/98	35/103	20/101	20/103	20/103	37/103
'Hangleson' Rangpur lime	41/98	35/103	14/102	23/103	19/103	37/103
'Olinda Valencia' orange	22/96	19/103	25/102	35/103	---	24/103

'Algerian Clementine', and pummelo had low levels. The number of bands in sweet orange was similar to that in 'Algerian Clementine' and citrons, a surprising result considering that it is generally considered to be a pummelo x mandarin hybrid. In their classic paper, Barrett and Rhodes (1976) postulate that sweet orange is "predominantly the *C. reticulata* genotype introgressed with genes from *C. grandis*" [*C. grandis* (L.) Osbeck = *C. maxima*]. This suggests that heterozygosity in sweet orange should be only slightly higher than that of *C. reticulata*, and somewhat lower than that of 'Cocktail' grapefruit, a known *C. reticulata* x *C. maxima* hybrid. The observed values for these accessions (Table 3) are fairly consistent with this expectation, but the low value for sweet orange might also be attributed to sampling error. It is also possible that the number of ISSR bands differs between taxa due to differential expansion or loss of microsatellite sequences. In this case, we would expect that the heterozygosity index based on the isozyme-SSR data would not be affected. The isozyme-SSR data and ISSR data generally ranked taxa similarly by heterozygosity and the correlation between the number of ISSR bands and the average number of isozyme and SSR alleles per locus was only moderately strong ($r = 0.63$; $P = 0.0035$).

Based on 103 polymorphic ISSR fragments, a similarity matrix was generated using the Dice coefficient of Nei and Li (1979). The dendrogram constructed by UPGMA cluster analysis is illustrated in Fig. 1. Based on this dendrogram, the genotypes can be separated into two major groups with a similarity value of 0.47.

Group 1 consists of all citrons and lemon types. There are two subgroups within this class with the similarity value of 0.65. Subgroup 1–1 includes true lemons, rough lemons, lemon hybrids, sweet lemons, and one sweet lime. Although 103 bands were scored, 18 lemon types, including some commercially important cultivars such as 'Eureka' and 'Lisbon' types, could not be distinguished from each other. This was strong evidence that differences among these accessions originated by mutation, not by sexual recombination. We refer to this group as the main lemon group in the text. This group included 'Femminello Lisbon', 'Foothill Lisbon', 'Villafranca' (CRC 0390), 'Cook Eureka', 'Allen Variegated', 'Messina', 'Femminello Sfusato', 'Femminello Ovale', 'Corona Old Line Eureka', 'Arancino,' 'Kusner', 'Seedless Lisbon', 'Genoa Eureka', 'Ross Eureka', 'Ricote Eureka', 'Blanchard Eureka', 'Lupe Lisbon' and 'Lunario' lemons. Three cultivars identical to each other, 'Monachello', 'Villafranca' (CRC 0280), and 'Allen Newman Eureka', were almost (over 99%) identical to the lemons of the main group. Based on an RFLP study, Albanese et al., (1992) suggested that 'Monachello' did not originate as a zygotic seedling because it was identical to other lemons, which is consistent with our result. Of two 'Villafranca' lemons, accession 0280 had one additional band of 140 base pairs (bp) for $(GA)_8YG$ primer. Either it must have a mutation or the first must have lost a band due to mutation. Four other groups (D–G in Fig. 1) each included two indistinguishable cultivars, but were slightly divergent from the main lemon group.

We consider it likely that all lemons with more than 97% similarity for ISSR markers are clonally derived from a single ancestor. By this criterion, 39 of the cultivars studied are included in this group. 'Mesero' lemon, 'Lo Porto' lemon, and the group composed of 'Berna', 'Lapithiotiki', and 'Rosenberger Lisbon' lemons were closely related to the main lemon group with similarity values of about 0.96, differing from the main lemon group for several fragments. These accessions were identical to the main group of lemons for the isozymes and SSR markers studied. Because of the differentiation between this group and the main group, it is less likely that these cultivars originated by mutation.

We find it surprising that the major horticultural groups of 'Lisbon' and 'Eureka' lemon cultivars do not form discrete clusters. Most of these 'Eureka' and 'Lisbon' cultivars originated as selections by growers, supposedly from other cultivars of the same type. One possible explanation for this lack of concordance is that a few ISSR markers evolve so quickly that they are not useful for analysis of even recently diverged genotypes. Alternative explanations are that a few of the bands scored are artifacts that do not represent genetic differences between cultivars, that some cultivars are not correctly classified as 'Eureka' or 'Lisbon' types, or that these horticultural groups are polyphyletic.

The main group of lemon cultivars shared 18 ISSR bands with citron that were not found in mandarin or pummelo. These lemons shared six and eight bands (that were not present in the other ancestral taxa) with mandarin and pummelo, respectively. Therefore, lemons display evidence of citron, pummelo and mandarin parentage, but the largest portion of their nuclear genome apparently derives from citron. The contribution of mandarin to lemon may be poorly estimated in our data because we studied only one mandarin, *C. clementina*, and it may have a small portion of sweet orange ancestry (Nicolosi et al., 2000).

Multiple accessions were sampled from two other lemon taxa within Subgroup 1–1. The four rough lemons (*C. jambhiri*) clustered together, but there were few polymorphic fragments within the rough lemon group. The isozyme genotypes of four rough lemons were identical ($Skdh^{FS}$, $Mdh\text{-}1^{FS}$, $Mdh\text{-}2^{FF}$, Idh^{MI}, and $Got1^{FS}$). This is the only other lemon group in which differ-

entiation has occurred only by mutation. 'Mazoe' and 'Florida' rough lemons were identical for all 103 ISSR bands, and the other two accessions studied were only slightly divergent. Of the four *C. limetta* accessions, Iraq and 'Millsweet' sweet lemons were nearly identical. 'Faris' sweet lemon appears to be an acidless mutant of *C. limon* and clustered with the main lemon group. Its morphology is also virtually identical that of the acid lemons. The fourth *C. limetta* accession, CRC 3093, was only distantly related to the 'Iraq'-'Millsweet' group, with a similarity value of 0.64. This *C. limetta* accession probably has a different origin from the other three *C. limetta* accessions, which is consistent with an RFLP study (Federici et al., 1998) in which it clustered with two *C. limettioides* accessions not included in this study. It had a 0.64 similarity value with 'Mitha-Tulia' sweet lime, the only *C. limettioides* accession included in this study. The main group of *C. limetta* accessions and the *C. limettioides* accession had two ISSR bands not found in the three ancestral species, suggesting that these two groups may have some common parentage not represented among the accessions we studied.

The rest of the lemons were easily distinguishable, with similarity values of about 0.8. This suggests that almost one third of accessions currently classed as lemons have origins different from the "true lemons". Lemons having independent origins were 'Interdonato', 'Limon Real', 'Iran' lemon, 'Limoui Sangui', 'Peretta', 'Lumia' *(C. lumia* Risso and Poit), 'Improved Meyer', 'Soh Long', 'Soh Synteng', 'Kulu', 'Bergamotto' and 'Bitrouni'. All of these lemons also were distinct for the isozyme-SSR data. Deng et al. (1995) also found that 'Interdonato' was quite distinct from other lemon cultivars, including 'Lisbon', 'Eureka', 'Messina', and 'Santa Teresa' for RAPD markers.

'Hangleson' Rangpur lime (*C. limonia* Osbeck) has a similarity value of 0.76 with lemons. Previous studies (Swingle and Reece, 1967) suggested that *C. limonia* was close to mandarins (*C. reticulata*) and to *C. aurantium*, but it had more bands in common with citrons and rough lemons than with the single mandarin studied herein (35%, 41%, and 23% respectively, Table 4). RAPD and SCAR marker data suggest that Rangpur lime derives from citron and mandarin (Nicolosi et al., 2000).

Subgroup 1–2 includes four citron accessions and a few suspected lemon hybrids; Chinese lemon, Wild lemon, and Nicaraguan lemon. Chinese lemon showed the same banding patterns as some citron accessions for all five isozyme loci. Wild lemon was the same as the other citron types for *Mdh-1* and *Mdh-2*. These three accessions also had relatively few total bands (Table 3), indicating low heterozygosity similar to that of citron. These accessions should be classified as citrons, or perhaps citron backcrosses, instead of lemon hybrids.

The number of ISSR bands observed in the six citrons tested varied from 36 to 44, and 40 of these bands were also found among the 70 bands observed in the main group of lemons (Tables 3 and 4). These numbers suggest that citrons have contributed approximately half of the lemon genome and that citrons are quite homozygous, a characteristic also suggested by their low total band numbers (Table 3). A similar conclusion was reached in a study of RFLP markers (Federici et al., 1998).

Although the term shaddock is a synonym for pummelo, 'Cuban' shaddock clustered with citrons. Its internal fruit characters and some other phenotypes are similar to those of citron or lemon (Hodgson, 1967). Fifteen and five bands unique to citron and pummelo, respectively, were found in 'Cuban' shaddock. This suggests that it has both citron and pummelo parentage. An RFLP study of this accession (Federici et al., 1998) also clustered it with citrons. Clustering with distance methods such as UPGMA and Neighbor Joining tends to insert a hybrid close to one of the parents (Lucinda, 1997).

Group 2 includes two subgroups having similarity values of 0.55. Subgroup 2–1 includes four *C. maxima* accessions; Unnamed, Chinese pummelo seedling, 'Siamese acidless', and 'African' pummelo. The number of common fragments between pummelos and the main lemon group, 25 out of 98, was lower than that between citrons and lemons, 40 out of 103 (Table 4). Clearly, this shows that pummelo contributed less than citron to the lemon genome.

Subgroup 2–2 is composed of a mandarin x pummelo hybrid ('Cocktail' grapefruit), 'Olinda Valencia' sweet orange, 'Algerian Clementine' mandarin (*C. clementina*), and a suspected pummelo x mandarin hybrid (*C. tengu* Hort. ex Tanaka). The number of ISSR bands shared by lemons and 'Algerian Clementine' and 'Olinda Valencia' orange was 30 and 28 out of 103, respectively (Table 4). 'Olinda Valencia' shared 35% of its bands with mandarins, 20% with citrons, and 25% with pummelos. This does not necessarily indicate that all three of these taxa contributed to sweet orange. Only one mandarin, four pummelos, and six citrons were studied and therefore it is likely that some bands observed only in citrons or pummelos occur in other mandarins. This problem can only be addressed by identifying species-specific bands, which would require studying a much larger sample from each of the ancestral taxa.

Citrons were found to have the highest number of unique ISSR fragments among the three ancestral species, which was 24 out of 103 (data not presented). In other words, 24 bands were found only in citrons, but not in the mandarin and pummelos tested. Pummelos and mandarin each had nine unique fragments. Only six fragments were shared by all 83 samples tested. Citrons and pummelos shared five bands, citrons and mandarin eight bands, and pummelos and mandarins shared 15 bands. Twenty-six of the 103 ISSR fragments scored were not found in any of the three basic species, but were observed in one or more of the 71 other samples. Possibly, these unique fragments were accumulated during earlier hybridization involving some other *Citrus* taxa or related genera, but it is also likely that our samples of the ancestral taxa were not adequate to detect all alleles present in them. Of these 26, six bands were found only in the main lemon group. Determination of the origin of these six bands is necessary to determine the parentage of the main lemon group. In a separate study, we found that the chloroplast genome of lemon is identical to that of sour orange, and that all ISSR fragments of lemon occur in either sour orange or citron (Gulsen and Roose, in press). The hypothesis that lemon is a sour orange x citron hybrid is also supported by additional chloroplast and nuclear genome marker data (Nicolosi et al., 2000).

The overall picture of diversity and evolution in the lemon group that emerges from this study is that most lemon cultivars derive from a single hybrid between citron and another genotype that includes genes from both mandarin and pummelo. Divergence among these cultivars has occurred by mutation only. However, there are additional lemon cultivars with more diverse origins, including possibly selfing of lemon and independent hybridization of citron with other citrus taxa. Finally, some accessions currently classified as lemons are much more closely related to citron than to other lemons. These divergent, but "lemon-like" accessions should provide a useful resource for breeders interested in improving lemon for traits such as resistance to mal secco [*Phoma tracheiphila* (Petri) Kantsch and Gik.].

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