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

O. Gulsen¹ and M.L. Roose²

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124

Additional Index Words. Citrus limon, inter-simple sequence repeat, isozyme, simple sequence repeat, microsatellite markers, genetic diversity

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 (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 Citrullus. 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), anchored at the 3’ or 5’ end by two to four arbitrary, often...
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. limettoides*), and seven other Citrus species. The accessions 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. halimi* 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    | Iraqi 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.) Burn. f.    | 3496    | 'Allen Newman Eureka' lemon | X | – | X |
| *C. limon* (L.) Burn. f.    | 3007    | 'Allen Variegated' lemon | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 2429    | 'Amber' lemon           | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3387    | 'Arancino' lemon        | X       | –   | X    |
| *C. limon* (L.) Burn. f.    | 3506    | 'Bergamotto' lemon      | X       | –   | X    |
| *C. limon* (L.) Burn. f.    | 3590    | 'Berna' lemon           | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3265    | 'Bitroumi' o.p. seedling lemon | X | X | X |
| *C. limon* (L.) Burn. f.    | 3499    | 'Blanchard' Eureka lemon | X     | –  | X    |
| *C. limon* (L.) Burn. f.    | 0710    | Chinese lemon           | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3498    | 'Cascade Eureka' lemon  | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3837    | 'Cook Eureka' lemon     | X       | –   | X    |
| *C. limon* (L.) Burn. f.    | 3043    | 'Corona Old Line Eureka' lemon | X | – | X |
| *C. limon* (L.) Burn. f.    | 3591    | 'Coraci' lemon          | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3500    | 'Femininello Lisbon' lemon | X | X | X |
| *C. limon* (L.) Burn. f.    | 3388    | 'Femininello Ovale' lemon | X | X | X |
| *C. limon* (L.) Burn. f.    | 3389    | 'Femininello Sfusato' lemon | X | X | X |
| *C. limon* (L.) Burn. f.    | 3836    | 'Foothill Lisbon' lemon | X       | –   | X    |
| *C. limon* (L.) Burn. f.    | 3005    | 'Frost Eureka' lemon    | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3176    | 'Frost Lisbon' lemon    | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3835    | 'Galilgal Lisbon' lemon | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 0565    | 'Genoa' lemon           | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3737    | 'Improved Meyer' lemon  | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 2323    | 'India' lemon           | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3593    | 'Interdonato' lemon     | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3885    | Iran local lemon        | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 2899    | 'Italian Pink Fleshe'd lemon | X | X | X |
| *C. limon* (L.) Burn. f.    | 3010    | 'Kaweah Lisbon' lemon   | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3045    | 'Kulu' lemon            | X       | X   | X    |
| *C. limon* (L.) Burn. f.    | 3194    | 'Kusner' lemon          | X       | –   | X    |
| *C. limon* (L.) Burn. f.    | 4005    | 'Lapithiostik' lemon    | –       | X   | X    |
| *C. limon* (L.) Burn. 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. | 3000 | ‘Lunario’ 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 | ‘Limoj Sangu’ lemon                             | X       | X   | X    |
| C. limon (L.) Burm. f. | 3892 | ‘Monachello’ lemon                              | X       | X   | X    |
| C. limon (L.) Burm. f. | 3392 | ‘Monacchello’ 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 | ‘Primofiori’ lemon                              | X       | X   | X    |
| C. limon (L.) Burm. f. | 3893 | ‘Ricote’ lemon                                  | X       | X   | X    |
| C. limon (L.) Burm. f. | 3840 | ‘Rosenberger Lisbon’ lemon                      | X       | 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 Synteg’ 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. limon (L.) Burn. | 3932 | ‘Hangleson’ Rangpur lime                        | X       | X   | X    |
| C. limon (L.) Burn. | 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. medicu L. | 3819 | Unnamed citron                                  | X       | X   | X    |
| C. medicu L. | 3527 | ‘Hiawassie’ citron                              | X       | X   | X    |
| C. medicu L. | 3532 | ‘Papuan’ citron                                 | X       | X   | X    |
| C. medicu L. | 3768 | ‘Buddha’s Hand’ citron                          | X       | X   | X    |
| C. medicu L. | 3891 | ‘Ethrog’ citron                                 | X       | X   | X    |
| C. medicu 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. | 3462 | 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 | Fragments (no.) | Polymorphic fragments |
|--------|----------------|-----------------------|
| BDB(CA) | 50 | 15 |
| DBDA(CA) | 55 | 14 |
| (GT)YA | 30 | 4 |
| HVH(CA) | 55 | 18 |
| VHV(TG) | 50 | 16 |
| HVH(TCC) | 37 | 13 |
| (GA)YG | 62 | 12 |
| (TCC)RY | 35 | 11 |
| Total | 374 | 103 |

3R = 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 MgCl2, 0.2 mM 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 varied among primer pairs: primers AGG9, CAC23, and CAC33 were amplified in reactions containing 2 mM MgCl2, 35 cycles with 45 °C annealing temperature. Primer TAA41 was amplified in reactions containing 4 mM MgCl2, 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 MgCl2, 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 phylegetic 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). (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.

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

<sup>3</sup>Range of band numbers shown for taxa with multiple cultivars.

<sup>4</sup>In taxa with multiple accessions, one locus may have missing data for one or more accessions. Value shown is maximum observed.

<sup>5</sup>One band missing due to amplification or staining errors.
Fig. 1. UPGMA dendrogram of 83 accessions of Citrus from ISSR data. Similarity values are shown on top of the dendrogram. 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 'Limoneiro 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.
between the number of ISSR bands and the average number of ranked taxa similarly by heterozygosity and the correlation between heterozygosity index based on the isozyme-SSR data would not differ between taxa due to differential expansion or loss of 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 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)$_8$YG 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$^{10}$, Mdh-1$^{30}$, Mdh-2$^{30}$, Idh$^{10}$, and Got1F$^{30}$). 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. limettoides accessions not included in this study. It had a 0.64 similarity value with ‘Mitha-Tulia’ sweet lime, the only other three C. limetta accessions included in this study. The main group of C. limetta accessions and the C. limettoides 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, ‘Limou Sangui’, ‘Peretta’, ‘Lumia’ (C. Lumia Risso and Poit), ‘Improved Meyer’, ‘Soh Long’, ‘Soh Synteng’, ‘Kulu’, ‘Bergamotto’ and ‘Bitroni’. 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.

‘Hangleston’ 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 citruses 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 Nicaragua 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 citrus 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 citrus. These accessions should be classified as citrus, or perhaps citrus 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. tenuifolia 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 mandarins 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.].
Iwamasa, M., N. Nito, and J.T. Ling. 1988. Intra- and intergeneric
Hodgson, R. W. 1967. Horticultural varieties of citrus, p. 431–591. In:
Herrero, R., M.J. Asins, E.A. Carbonell, and L. Navarro. 1996. Genetic
Handa, T., Y. Ishizawa, and C. Oogaki. 1986. Phylogenic study of
Gulsen, O and M. Roose. 2001. Chloroplast and nuclear genome analysis
Barrett, H.C. and A.M. Rhodes. 1976. A numerical taxonomic study of
Albanese, G., M. Renis, and G. Reforgiato Recupero. 1992. RFLP
Deng, Z.N., A. Gentile, E. Nicolosi, A. Vardi, and E. Tribulato. 1995.
Federici, C.T., D.Q. Fang, R.W. Scora, and M.L. Roose. 1998. Phyloge-
Fang, D.Q., Z. Wen, and X. Shun-Yuan. 1994. Isozymes and classifica-
hybridization in the orange subfamily, Aurantioideae. Proc. Intl. Soc.
Kijas, J.M.H., M.R. Thomas, J.C.S. Fowler, and M.L. Roose. 1997.
Integration of trinucleotide microsatellites into a linkage map of Citrus.
Lucinda, A.M. 1997. Hybrids and phylogenetic systematics III. Com-
Malik, M.N., R.W. Scora, and R.K. Soost. 1974. Studies on the origin of
Nei, M. and W.H. Li. 1979. A mathematical model for studying genetic
variation in terms of restriction endonuclease. 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.
Rohlf, F.J. 1993. NTYSYS-PC, numerical taxonomy and multivariate
analysis system. Version 1.80. Exeter Software, Setauket, N.Y.
Roose, M.L., R.K. Soost, and J.W. Cameron. 1995. Citrus, p. 443–448.
In: J. Smartt and N.W. Simmonds (eds.). Evolution of crop plants.
Longman, Harlow, United Kingdom.
Staub, J.E., F.C. Serquen, and M. Gupta. 1996. Genetic markers, map
construction, and their application in plant breeding. HortScience
31:729–741.
Swingle, W.T. and P.C. Reece. 1967. The botany of Citrus and its wild
relatives, p. 190–430. In: W. Reuther, H.J. Webber, and L.D. Batchelor
(eds.). The citrus industry. vol. 1. Univ. of Calif., Berkeley.
Tanaka, T. 1977. Fundamental discussion of citrus classification. Studia
Citrologica 14:1–6.
Torres, A.M., R.K. Soost, and U. Diedenhofen. 1978. Leaf isozymes as
markers in Citrus. Amer. J. Bot. 65:869–881.
Torres, A.M., R.K. Soost, and T. Mau-Lastovicka. 1982. Citrus isozymes:
Genetics and distinguishing nucellar from zygotic seedlings. J. Hered.
73:335–339.
Webb, D.M. and S.J. Knapp. 1990. DNA extraction from a previously
recalcitrant plant genus. Plant Mol. Biol. Rptr. 8:180–185.
Whitkus, R., J. Doebley, and J.F. Wendel. 1994. Nuclear DNA markers
in systematics and evolution, p. 116–141. In: R.L. Philips and I.K.
Vasil (eds.). DNA based markers in plants. Kluwer, Dordrecht, The
Netherlands.
Xiang, C. and M.L. Roose. 1988. Frequency and characteristics of
nucellar and zygotic seedlings in 12 Citrus rootstocks. Scientia Hort.
37:47–59.
Zietkiewicz, E., A. Rafalski, and D. Labuda. 1994. Genome fingerprint-
ing by simple sequence repeat (SSR)-anchored polymerase chain
reaction amplification. Genomics 20:176–183.

Literature Cited
Albanese, G., M. Renis, and G. Reforgiato Recupero. 1992. RFLP
analysis of different lemon cultivars. Proc. Intl. Soc. Citricult. 1:208–
209.
Barrett, H.C. and A.M. Rhodes. 1976. A numerical taxonomic study of
affinity relationships in cultivated Citrus and its close relatives. System-
etic Bot. 1:105–136.
Bassam, B.J., G. Caeteno-Anolles, and P.M. Gresshoff. 1991. Fast and
sensitive silver staining of DNA in polyacrylamide gels. Anal. Bio-
chem. 196:80–83.
Dice, L.R. 1945. Measures of the amount of ecologic association
between species. Ecology 26:297–302.
Fang, D.Q. and M.L. Roose. 1999. Inheritance of intersimple sequence
repeat markers in citrus. J. Hered. 90:247–248.
Fang, D.Q., M.L. Roose, R.R. Krueger, and C.T. Federici. 1997. Finger-
printing trifoliate orange germ plasm accessions with isozymes, RFLPs,
and inter-simple sequence repeat markers. Theor. Appl. Genet. 95:211–
219.
Fang, D.Q., Z. Wen, and X. Shun-Yuan. 1994. Isozymes and classifica-
tion of closely related Citrus cultivars with inter-simple sequence repeat markers. Theor. Appl.
Genet. 95:408–417.
Green, R.M., A. Vardi, and E. Galun. 1986. The plastome of Citrus,
physical map, variation among Citrus cultivars and species and compari-
son with related genera. Theor. Appl. Genet. 72:170–177.
Gulsen, O and M. Roose. 2001. Chloroplast and nuclear genome analysis
of the parentage of lemons. J. Amer. Soc. Hort. Sci. 126(2):210–215.
Handa, T., Y. Ishizawa, and C. Oogaki. 1986. Phylogenic study of
Fraction 1 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. Intraspecies and
intragenus genetic variability. Theor. Appl. Genet. 92:599–609.
Hodgson, R. W. 1967. Horticultural varieties of citrus, p. 431–591. In:
W. Reuther, H.J. Webber, and L.D. Batchelor (eds.). The citrus
industry. vol. 1. Univ. of Calif., Berkeley.
Iwamasa, M., N. Nito, and J.T. Ling. 1988. Intra- and intergeneric

