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Genetic diversity in the orange subfamily Aurantioideae. I. Intraspecies and intragenus genetic variability

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Abstract Despite the great economic importance of citrus, its phylogeny and taxonomy remain a matter of controversy. Moreover pathogens of increased virulence and dramatic environmental changes are currently spreading or emerging. The objectives of the present paper, measuring genetic variability and studying its pattern of distribution, are crucial steps to optimize sampling strategies in the search of genotypes that tolerate or resist these threatening factors within the huge array of *Citrus* and *Citrus* related species. Their intraspecific and intrageneric variability was studied comparatively by means of ten enzymatic systems using eight different measures. The analysis of ten enzymatic systems allowed us to distinguish all the species and all but one artificial hybrid. The species with the lowest genotypic variability are *C. myrtifolia*, *C. deliciosa* (willow leaf mandarin), *C. paradisi* (grapefruit), *C. limon* (lemon) and *C. sinensis* (sweet orange), while *Severinia buxifolia* shows the highest value. A broad spectrum of heterozygosity values was found in the collection. Lemons (*C. limon, C. meyeri, C. karna, C. volkameriana*), limes (*C. aurantifolia, C. limettioides, C. lattifolia*) and *C. bergamia* show a very high percentage of heterozygosity which indicates an origin through interspecific hybridization. Two main factors limit genetic intraspecific variability: apomictic reproduction, where nucellar embryos are much more vigorous than the zygotic ones, and nurserymen selecting against variability in the seedling stage of the rootstocks or in propagating the scion cultivars vegetatively. Additionally, self-pollination appears in some species mainly used as rootstocks which would explain their low heterozygosity values. Genetic differences between species and genera are in general high, which suggests that adaptation might have played an important role during the evolution of the orange subfamily.

Key words *Citrus* · *Fortunella* · *Poncirus* · Isozymes · Genetic diversity · Germ plasm bank · Heterozygosity

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Introduction

World production of citrus fruits in 1993 was estimated to be 83.2 million tonnes, largely exceeding all other important fruits including bananas and plantains (*Musa*), grapes (*Vitis*) and apples (*Malus*) (FAO, 1994). Sizable capital investment in horticultural enterprises requires that nurserymen, growers and breeders have confidence in the identification of their material. Furthermore, unambiguous identification is a fundamental step in the certification and registration of new cultivars as well as in the protection of breeders rights.

The genus *Citrus* belongs to the subtribe Citrinae, tribe Citreae, subfamily Aurantioideae of the family Rutaceae. All Aurantioideae species are trees or shrubs with persistent leaves except for the three monotypic genera *Poncirus, Aegle* and *Feronia*, three species of *Clausena* and one of *Murraya*. Their fruit is a hesperidium and seeds are without endosperm, usually with one or more nucellar embryos. The great majority of the species of *Citrus, Fortunella* and *Poncirus* are diploid, having 18 somatic chromosomes and a small genome, 1C=0.62 pg (Guerra 1984). Hybridization, apomixis and many centuries of cultivation have complicated *Citrus* taxonomy with the result that very different systems have been proposed. These systems diverge mainly in the number of species recognised: from 159 (Tanaka 1969) to 16 (Swingle 1943) and 3 (Barret and Rhodes 1976).

Measuring genetic variability and studying its pattern of distribution are critical steps to establish genetic relationships. They are also essential tasks in germ plasm characterization and conservation to effectively control genetic erosion, to design sampling strategies or core collections, and to establish breeding programs. Because a large number of samples need to be screened, markers should be in-

expensive and easily scored. To-date, allozyme electrophoresis has been the genetic technique most widely used to study the genetic structure of populations (Asíns and Carbonell 1987; Pérez de la Vega 1993).

As yet there is no available genetic variability study that covers the huge array of *Citrus* and *Citrus*-related species. The objective of the present paper is to examine the intraspecific and intrageneric genetic variability of these species by means of isozyme markers, as a first step in the discussion of their genetic relationships, taking into account previous results on the reliability of these characters (Asíns et al. 1995).

Materials and methods

Species were named following the nomenclature described by Ortiz (1986) as a modification of that of Carpenter and Reece (1969). All plants analyzed (see Table 1) belong to the *Citrus* germplasm bank at the IVIA. They are mature plants of similar age, growing in containers in a screenhouse and free of virus and virus-like pathogens (Navarro et al. 1988).

Mature leaves from circular twigs were used to obtain crude extracts for electrophoresis following the methodology described by Asíns et al. (1995). Ten enzymatic systems were examined: phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), aconitase (ACO), malic acid dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), peroxidases (PRX), isocitric acid dehydrogenase (IDH) and leucine aminopeptidase (LAP). The differentiation of the Mn, Fe and Cu/Zn forms of SOD was performed following Almansa et al. (1989). The Pineapple cultivar of sweet orange was used as a control in every electrophoresis.

The variability analysis was based on two different data sets, one named L (standing for loci) used variables defined as the presence-absence of each allele, coded as 1 or 0 (i.e. including only those enzymatic systems or zones whose variability was genetically interpretable). The other, named LP (standing for loci and patterns), contained both types of characters, alleles and patterns (see Results) also coded as 1 or 0 according to the presence or absence of a particular allele or pattern. The cultivar named "Gigante", supposedly from *C. limon*, was not considered for the variability analysis because of its uncertain origin.

Genetic variability was studied in a hierarchical fashion: (1) globally within a genus, (2) between species per genus and (3) within species for those represented by more than one cultivar or variety.

The following measures of variability were calculated: the number of polymorphic loci (P); the mean frequency of the number of alleles per locus (A); the coefficient of variation of the mean gene frequency (CV); mean chi-square distance (d2) of Benzecri (1970) or the distance of the chord (dC) of Cavalli-Sforza and Edwards (1967) between varieties or cultivars from a species (or genus) and between species within a genus; the mean number of genotypes per locus (NGL) and the mean number of patterns per enzymatic system (NPS). P, A, CV, d2, dC and NGL were calculated from the L file and d2 was also computed from the LP file. The mean percent of heterozygous loci (H) was also estimated for all species (from the L file). The between-species variability in *Citrus* was obtained by subtracting the mean within-*Citrus* species variability from the total *Citrus* variability calculated as the mean of distances between all pairs of cultivars belonging the genus *Citrus* (Asíns and Carbonell 1989; Bretó et al. 1993).

In order to study the agreement and redundancy of information contained in the different measures of genetic variability, the Spearman's rank correlation was calculated between all pairs of measures for the species.

Results

Following previous studies (Torres et al. 1978, 1982, 1985) not all the isoenzymatic variability found in this survey (Figure 1) could be interpreted genetically. Since the variation for isozymatic patterns for ACO-2, 6PG, MDH and PRXa could not be interpreted genetically, each pattern was considered as a variable in the analysis (within the LP file). These patterns are presented in Fig. 2. For the other enzymatic systems, a determination of loci and corresponding alleles was possible because, except for SOD, the variation observed mostly agreed with bibliographic data (Torres et al. 1978, 1982, 1985). Mn-SOD is one of the least variable isozymes (Table 1) and its zymograms observed in *Atalantia* spp. and *Severinia buxifolia* (see Fig. 1) suggest a tetrameric nature and not a monomeric one as reported by Torres et al. (1985) for SOD-1. The relative mobility (Rf) of all allozymes observed for IDH, PGI-2, LAP, PGM-1, GOT-1, GOT-2 and Mn-SOD is shown in Fig. 3.

The isozymatic analysis has allowed us to distinguish all the species and all but one artificial hybrid (the seventh mandarin, "Nova", was indistinguishable from *C. temple*). The resulting classification is presented in Table 1. Each entry for most species and artificial hybrids showed a unique characteristic genotype (*C. bergamia, C. grandis, C. latifolia, C. limettioides, C. madurensis, C. medica*, tangors, tangelos, mandarins). Even species traditionally improved by clonal selection were polymorphic for some enzymatic systems which has allowed us to establish groups within these economically important species. Thus, four genotypes (groups) were found in sour orange, *C. aurantium* (three of them had only one cultivar), seven genotypes were found within *C. clementina* (two of them had only one cultivar), three in *C. limon*, two in *C. paradisi*, two in *C. sinensis* and three in *C. unshiu*.

Rank correlation analysis between all genetic variability estimates and H (Table 2) revealed that measures based on d2, dC and CV resulted in a very similar ordering of species. The ranking given by NGL is also very similar to that obtained by using CV. The most similar ranking to that obtained using NPS is that of NGL. Rankings using A and

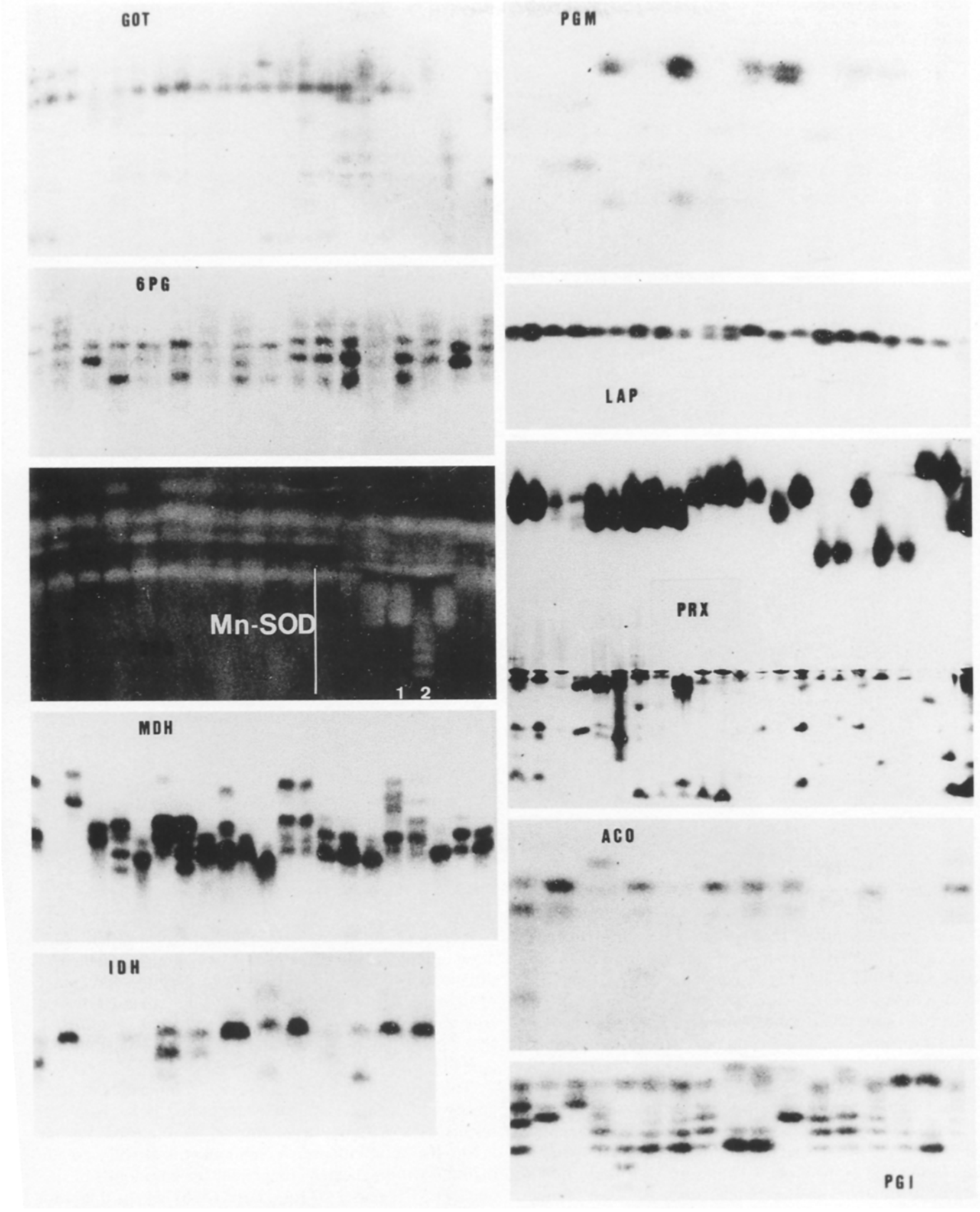

Fig. 1 Profiles of the enzymatic systems employed. *Lanes 1 and 2* of the SOD profiles correspond to *Atalantia ceylanica* and *A. citroides,* respectively, showing the tetrameric nature of Mn-SOD

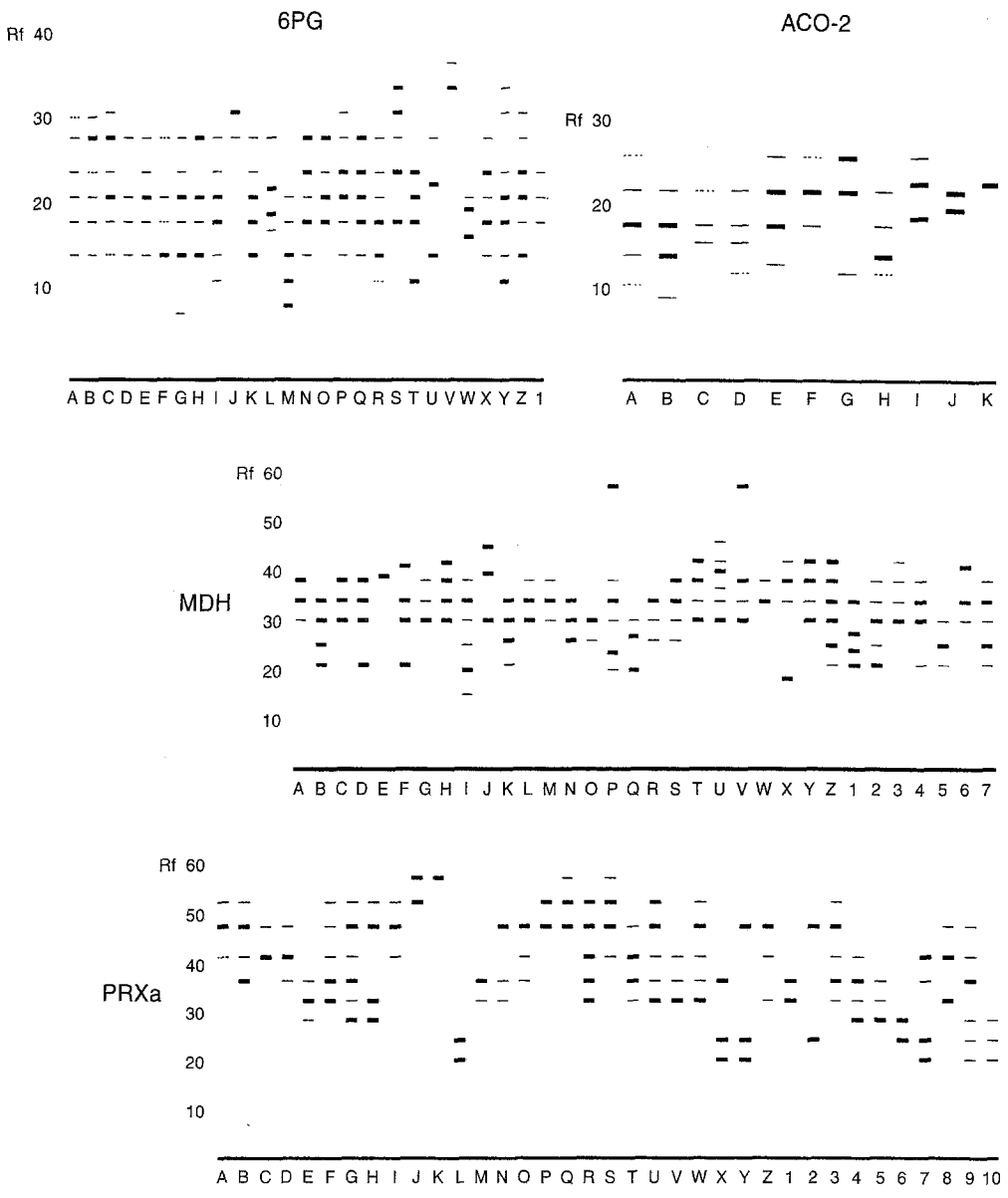

Fig. 2 Patterns of the four enzymatic systems where no hypothesis of genetic control could be fitted

P are almost identical. That based on H is mostly related to that based on P. A graphical representation of the genetic variability estimates based on d2, dC, NGL and P is shown in Fig. 4. The species with less genotypic variability are *C. myrtyfolia*, *C. deliciosa*, *C. paradisi*, *C. limon* and *C. sinensis* while *Severinia buxifolia* shows the highest values.

Figure 5 shows the variability found among species within the subfamily Aurantioideae for the estimated percentage of heterozygosity throughout the genome. *C. limon*, one of the species with less genotypic variability, shows one of the highest values for H. It is worth noting the differences in H between sour and sweet oranges and among mandarins; for instance, among Cleopatra mandarin (*C. reshni*), satsumas (*C. unshiu*) and clementines.

Atalantia and *Microcitrus* had the highest genetic variation among all the genera. NGL was highly affected by the number of species studied. Regarding the percentage of heterozygosity, *Atalantia* shows high values, *Microcitrus* low values (except for *M. australis*), and *Fortunella*, like *Citrus*, low or high values depending on the species (see Fig. 5).

The within- and between-species components of variability within *Citrus* using d2 were quite similar. However results were different depending on the file used. For the L file, the between-species component is slightly greater than the within-species component for both kinds of distance (1.56 versus 1.27 for d2 and 0.267 versus 0.065 for dC). On the other hand, for the LP file the between-species component was smaller (2.46) than the within-species component (2.65) for the d2 distance.

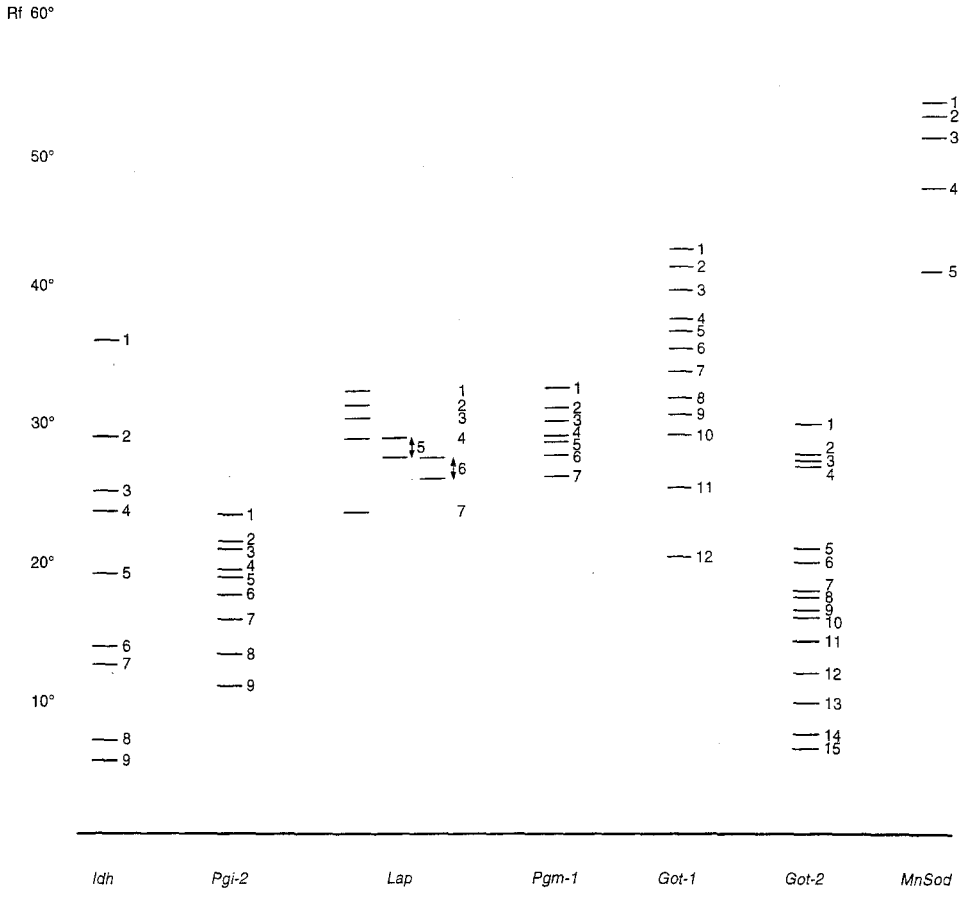

Fig. 3 Relative migration of the allozymes that could be interpreted genetically. *Idh 10, Pgi-2.10, Lap 8, Pgm-1.8, Got-1.13, Got-2.16* and *Mn-Sod 6* are null alleles

Discussion

Some important differences were found in the MDH patterns compared to those reported by Torres et al. (1982). In general, we have found more bands, and their hypothesis of genetic control does not fit with the whole variation observed. Possible causes are differences in electrophoretic conditions and/or in the age of the plant, tissue or type of leaf (Asíns et al. 1995). Similarly, differences from the genetic interpretation given by Torres et al. (1985) for the SOD variation in young seedlings could be additionally explained by the type of SOD under study, Fe, Cu/Zn or Mn SOD (Asíns et al. 1995).

The success in classifying the cultivars or entries by means of the isoenzymatic systems employed has been very high. There are two clones at the germ plasm bank of unknown origin (marked # in Table 1). One of them is the accession named "Gigante" lemon. From its genotype it should be included not in *C. limon* but rather in *C. bergamia* given that it is most similar to *C. bergamia* cv "Burjasot". The other accession is a promising rootstock, "Gou-Tou-Cheng", that has been included in *C. aurantium* (sour orange). In commercial citriculture, sour orange has been a universal rootstock that is well-known for many attributes related especially to yield, fruit and juice quality, and tolerance to cold temperatures and various soil conditions (Castle 1987). However, it has one major weakness, it is highly susceptible to decline by isolates of the citrus tristeza virus. Gou-Tou-Cheng has been reported tolerant to CTV in China (Chao et al. 1979), South Africa (Van Vuuren et al. 1991), Australia (Broadbent, cited by Castle et al. 1992), and even to severe CTV strains in Florida (Castle et al. 1992). Regarding its origin, some noticeable differences from sour orange are evident: the presence of the allele *Pgi-2.4* and, mainly, the homozygosity of the allele *Got-2.14*. *Pgi-2.4* is a common allele scattered throughout the citrus germ plasm; however *Got-2.14* has a much more restricted distribution. We have observed it only in *C. bergamia, C. grandis, C. limettioides* and *C. hyxtris* but not in *C. auriantium*. Therefore, any of these species may have been involved in the origin of Gou-Tou-Cheng. From its wide distribution and its sexual reproduction, *C. grandis* is a very likely candidate. Concerning morphological characters it has been considered as a putative hybrid, most likely involving pummelo (*C. grandis*), mandarin (*C. reticulata*), and sour orange (Castle et al. 1992). Therefore, isozymatic characters can be used efficiently for pedigree ascertainment and germ plasm bank management in *Citrus*.

It is very difficult to concentrate all the information on genetic variability in terms of only one measure, therefore

Table 1 Alleles and isozymatic patterns of entries. # stands for entries of unknown origin: cultivars "Gou-Tou-Cheng" of *C. aurantium* and "Gigante" of *C. limon*

Species or hybrid	Number of cultivars	*Idh*	*Pgi-2*	*Lap*	*Pgm-1*	*Got-1*	*Got-2*	*MnSod*	6PG	ACO-2	MDH	PRXa
C. aurantifolia (Christm.) Swing.	1	5 9	4 7	3 3	1 6	5 9	6 6	1 1	E	A	7	D
C. aurantium L.	2	5 5	3 7	3 3	1 7	5 5	15 15	1 1	K	B	A	B
	1	5 5	3 7	3 3	1 7	5 5	15 15	1 1	K	A	A	B
C. aurantium #	1	5 7	3 7	3 3	1 7	5 5	15 15	1 1	I	B	A	Z
	1	5 5	4 7	3 3	1 1	5 5	14 14	1 1	H	A	A	D
C. bergamia Risso & Poit.	1	5 7	3 7	3 3	1 1	1 9	6 14	1 1	G	B	D	B
	1	5 9	4 7	3 3	1 7	4 9	6 14	1 1	G	A	D	F
	1	5 9	3 3	3 3	1 7	4 9	14 14	1 1	G	A	A	N
	1	5 9	3 7	3 3	1 7	4 9	6 14	1 1	G	A	D	F
C. clementina Hort. ex Tan.	1	5 7	3 7	3 3	1 2	5 5	15 15	1 1	P	A	A	A
	16	5 7	3 7	3 3	1 2	5 5	15 15	1 1	C	A	A	A
	3	5 7	3 7	3 3	1 2	5 5	15 15	1 1	C	A	E	A
	8	5 7	3 7	3 3	1 2	5 5	15 15	1 1	B	A	A	A
	2	5 7	3 7	3 3	1 2	5 5	15 15	1 1	A	A	A	A
	2	5 7	3 7	3 3	1 1	5 5	15 15	1 1	C	A	A	A
	1	5 7	3 7	3 3	1 2	5 5	15 15	1 1	J	A	E	A
C. deliciosa Ten.	3	5 5	2 4	3 3	1 2	5 5	15 15	1 1	C	A	A	B
C. depressa Hay.	1	5 5	2 4	3 3	1 1	2 5	15 15	1 1	K	A	M	R
C. excelsa Wester	1	5 7	7 9	3 3	1 1	5 9	6 6	1 1	G	D	7	D
C. grandis (L.) Osb.	1	5 7	7 7	3 3	7 7	5 5	14 14	1 1	G	A	A	4
	1	5 5	7 7	3 3	7 7	5 5	6 14	1 1	G	A	A	4
	1	5 5	4 7	3 4	7 7	5 5	6 14	1 1	G	B	A	V
C. halimii B.C. Stone	1	6 6	7 9	3 3	2 2	5 7	7 7	1 1	W	A	T	4
C. hystrix D.C.	1	5 5	7 7	3 4	6 7	5 5	6 14	1 1	U	B	N	D
C. ichangensis Swing.	1	5 7	7 7	3 3	6 7	5 9	15 15	1 1	U	A	L	E
C. karna Raf.	1	5 7	7 7	3 3	1 7	5 9	6 15	1 1	K	A	D	3
C. latifolia Tan.	1	5 9	7 7	3 3	1 5	5 9	6 6	1 1	G	A	B	C
	1	5 9	4 7	3 3	1 5	5 9	6 15	1 1	E	A	B	3
	1	5 9	7 7	3 3	1 5	5 9	6 6	1 1	F	B	B	C
C. limettioides Tan.	1	5 7	3 7	3 3	1 2	1 9	6 14	1 1	G	B	D	R
	1	5 5	4 7	3 3	1 7	5 5	7 15	1 1	K	A	D	T
C. limon L. Burm. f.	14	5 9	3 7	3 3	1 7	5 9	6 15	1 1	G	A	D	B
	4	5 9	3 7	3 3	1 7	5 9	6 15	1 1	F	A	D	B
	1	5 9	3 7	3 3	1 7	5 9	6 15	1 1	E	A	D	B
C. limon #	1	5 7	7 7	3 3	1 1	1 9	6 14	1 1	D	B	F	B
C. macrophylla Wester	1	5 7	7 9	3 3	1 5	5 9	6 6	1 1	G	A	1	T
C. macroptera Montr.	1	5 5	7 9	3 5	6 6	5 9	6 6	1 1	W	H	5	T
C. madurensis Lour.	1	5 5	4 4	3 3	1 1	5 5	7 15	1 1	L	A	H	H
	1	5 5	3 6	3 3	1 1	5 5	7 15	1 1	Y	A	H	H
C. medica L. var. ethrog	1	7 7	7 7	8 8	1 1	9 9	6 6	1 1	G	E	I	D
	1	7 7	7 7	7 7	1 1	9 9	6 6	1 1	G	A	I	D
C. medica L.	1	5 7	3 7	1 3	7 7	5 9	6 15	1 1	G	A	I	R
C. medica L. var. sarcodactylis	1	7 9	7 7	8 8	1 2	9 9	6 6	1 1	G	E	I	C
C. meyeri Y. Tan.	1	5 7	4 7	3 3	1 7	5 9	6 15	1 1	K	A	D	A
C. myrtifolia Raf.	2	5 5	3 7	3 3	1 1	5 5	15 15	1 1	K	A	A	R
C. nobilis Lour.	1	5 5	3 7	3 3	1 2	5 5	15 15	1 1	H	E	A	A
C. paradisi Macf.	6	5 5	7 7	3 4	7 7	5 5	6 15	1 1	A	A	A	G
	1	5 5	7 7	3 4	7 7	5 5	6 15	1 1	A	B	A	G
C. pyriformis Hassk.	1	7 7	7 7	4 4	7 7	5 9	6 6	1 1	G	A	I	F
C. reshni Hort. ex Tan.	1	5 5	4 4	2 2	1 1	5 5	15 15	1 1	R	A	A	S
C. shunkokan Hort. ex Tan.	1	5 5	4 7	3 4	7 7	5 5	15 15	1 1	I	H	A	I
C. sinensis (L.) Osb.	58	5 7	4 7	3 3	1 7	5 5	15 15	1 1	A	A	A	A
	1	5 7	4 7	3 3	1 1	5 5	15 15	1 1	A	A	A	A
C. sunki Hort. ex Tan.	1	5 5	4 4	3 3	1 2	5 5	15 15	1 1	Y	A	S	D
C. tachibana (Mak.) Tan.	1	5 7	4 4	4 4	2 3	1 1	15 15	1 1	Z	A	M	M
C. tangerina Hort. ex Tan.	1	5 7	3 7	3 3	1 2	5 5	15 15	1 1	Q	A	A	P
C. temple Hort. ex Y. Tan.	1	5 7	4 7	3 3	1 2	5 5	15 15	1 1	A	A	A	A

Table 1 (Continued)

Species or hybrid	Number of cultivars	*Idh*	*Pgi-2*	*Lap*	*Pgm-1*	*Got-1*	*Got-2*	*MnSod*	6PG	ACO-2	MDH	PRXa
C. unshiu (Mak.) Marc.	7	5 5	4 7	3 3	1 1	5 5	15 15	1 1	A	B	A	A
	1	5 5	4 7	3 3	1 1	5 5	15 15	1 1	A	D	A	A
	1	5 5	4 7	3 3	1 2	5 5	15 15	1 1	A	B	A	A
C. volkameriana Ten.	1	5 9	4 7	3 3	1 1	1 9	6 15	1 1	R	A	D	M
C. webberi Wester	1	5 7	7 9	3 3	1 1	5 9	6 6	1 1	G	B	R	D
Tangor	1	5 7	4 7	3 3	1 7	5 5	15 15	1 1	C	A	A	Q
	1	5 7	2 4	3 3	1 2	5 5	15 15	1 1	K	A	A	A
	1	5 5	2 4	3 3	1 1	5 5	15 15	1 1	I	A	A	A
	1	5 5	4 7	3 3	2 7	5 5	15 15	1 1	G	A	A	A
	1	5 7	4 7	3 3	1 2	5 5	15 15	1 1	I	A	A	S
Tangelo	1	5 5	4 7	3 3	1 7	5 5	6 15	1 1	O	B	A	A
	1	5 5	4 7	3 3	1 7	5 5	15 15	1 1	A	B	A	A
	1	5 5	4 7	3 3	1 7	5 5	15 15	1 1	A	A	A	A
Mandarin hybrids	1	5 5	2 4	3 3	1 2	5 5	15 15	1 1	A	C	A	B
	1	5 5	3 7	3 3	2 7	5 5	15 15	1 1	E	B	A	A
	1	5 5	3 7	3 3	2 7	5 5	15 15	1 1	C	B	A	A
	1	5 5	2 4	3 3	1 7	5 5	15 15	1 1	C	A	A	C
	1	5 5	4 4	3 3	1 7	5 5	15 15	1 1	I	A	A	B
	1	5 5	4 7	3 3	1 2	5 5	15 15	1 1	A	B	C	A
	1	5 7	4 7	3 3	1 2	5 5	15 15	1 1	A	A	A	A
	1	5 7	4 7	3 3	1 2	5 5	15 15	1 1	E	A	A	A
	1	5 7	4 7	3 3	1 7	5 5	15 15	1 1	I	B	A	A
	1	5 5	4 4	3 3	1 2	5 5	15 15	1 1	I	A	A	O
	1	5 7	2 4	3 3	1 7	5 5	15 15	1 1	I	A	A	A
	1	5 5	4 7	3 3	1 7	5 5	15 15	1 1	A	B	A	A
	1	5 5	4 4	3 3	2 7	5 5	15 15	1 1	A	A	A	D
Citrumelo	1	3 5	4 7	3 4	6 6	5 5	7 15	1 1	Q	A	2	C
Citrangequat	1	7 7	4 7	3 3	1 7	5 5	6 6	1 1	C	A	M	W
Limequat	1	5 9	4 7	3 3	1 1	5 9	6 6	1 1	K	A	Z	X
	1	5 5	7 7	3 3	1 1	5 9	6 15	1 1	O	A	K	7
Orangequat	1	5 5	4 7	3 3	1 1	5 5	2 15	1 1	T	E	M	Y
Pummelo	1	7 7	7 7	3 3	7 7	5 5	6 6	1 1	G	B	A	C
	1	5 7	7 7	3 3	7 7	5 5	6 6	1 1	A	A	A	R
	1	5 7	7 7	3 3	7 7	5 5	6 15	1 1	R	A	A	U
Citrange	2	3 7	7 7	1 3	4 7	5 5	7 15	1 1	K	A	2	Q
Aeglopsis chevalieri Swing.	1	5 5	7 9	3 4	7 7	5 9	5 11	1 1	I	I	X	5
Alfraegle panicuiata (Schum.) Engl.	1	2 3	6 8	1 3	6 7	6 6	1 5	1 1	K	G	S	B
Atalantia ceylanica (Arn.) Oliv.	1	5 8	7 7	1 3	4 4	5 9	6 11	2 4	M	F	J	C
Atalantia citroides Pierre ex Guill.	1	1 1	4 4	3 3	4 7	1 5	5 5	4 5	U	A	O	E
Clausenia excavata Burm. f.	1	10 10	10 10	2 2	8 8	13 13	16 16	6 6	*	*	*	J
Fortunella crassifolia Swing.	1	5 5	4 7	3 3	1 2	5 7	3 8	1 1	I	A	3	L
Fortunella hindsiil (Champ.) Swing.	1	5 5	7 7	3 3	1 2	1 5	2 8	1 1	A	E	4	L
Fortunella margarita (Lour.) Swing.	1	5 5	7 7	3 3	1 1	5 5	8 15	1 1	N	A	G	E
Fortunella obovata Tan.	1	5 5	7 7	3 3	2 2	5 5	7 15	1 1	I	A	Y	2
Gylcosmis pentaphylla (Retz) Corr.	1	10 10	10 10	1 1	8 8	13 13	16 16	6 6	*	*	*	F
Microcitrus australasica (F. Muell.) Swing.	1	5 5	7 9	3 3	1 1	9 9	15 15	1 1	S	A	L	8
Microcitrus australis (Planch.) Swing.	1	5 7	7 9	3 3	1 1	5 5	6 15	2 2	X	A	L	V
Microcitrus inodora (F. M. Bail.) Swing.	1	5 5	3 3	3 3	1 1	5 5	15 15	2 2	O	A	L	D
Microcitrus warburgiana (F. M. Bail.) Tan.	1	5 5	7 7	5 5	1 1	9 11	15 15	1 1	G	A	L	1
Murraya paniculata (L.) Jack.	1	4 4	1 1	4 4	6 6	3 3	13 13	2 2	V	I	P	10
	1	4 4	1 1	4 4	4 7	3 3	13 13	2 2	V	I	V	9
Pamburus missionis (Wight) Swing.	1	3 3	4 4	3 3	7 7	1 9	6 6	2 2	B	F	6	6
Poncirus trifoliata (L.) Raf.	1	3 3	4 7	2 2	4 6	5 7	3 10	1 1	C	E	I	A
	1	3 3	4 7	3 3	4 6	5 5	4 7	1 1	C	E	I	D
Severinia buxifolia (Poir.) Tenore	1	3 5	7 7	3 3	4 4	5 9	6 6	2 4	G	F	L	A
	1	5 7	7 7	1 3	1 1	8 8	9 9	2 4	M	J	U	B
Swinglea glutinosa (Bl.) Merr.	1	7 7	4 4	6 6	6 6	5 8	5 12	2 2	X	H	W	L
Triphasia trifolia (Burm. f.) P. Wils.	1	4 4	5 6	3 6	2 6	10 12	15 15	3 3	F	K	Q	K

Table 2 Spearman's rank correlation values among all the measures of variability used at the species level

	d2LP	d2L	dC	CV	A	P	NGL	NPS
d2L	0.9599 0.0001[a]							
dC	0.9296 0.0002	0.982 0.0001						
CV	0.8803 0.0004	0.9547 0.0001	0.9702 0.0001					
A	0.5869 0.0189	0.677 0.0068	0.7246 0.0038	0.7854 0.0017				
P	0.4359 0.0812	0.522 0.0368	0.5705 0.0225	0.6592 0.0084	0.8907 0.0004			
NGL	0.6841 0.0062	0.7843 0.0017	0.8361 0.0008	0.9187 0.0002	0.855 0.0006	0.7121 0.0044		
NPS	0.481 0.0544	0.4547 0.0689	0.489 0.0505	0.5697 0.0227	0.6716 0.0072	0.5505 0.0277	0.6762 0.0068	
H	0.0418 0.8672	0.0977 0.6961	0.188 0.452	0.1954 0.4344	0.6189 0.0133	0.6993 0.0052	0.2829 0.2578	0.4129 0.0986

[a] Level of significance

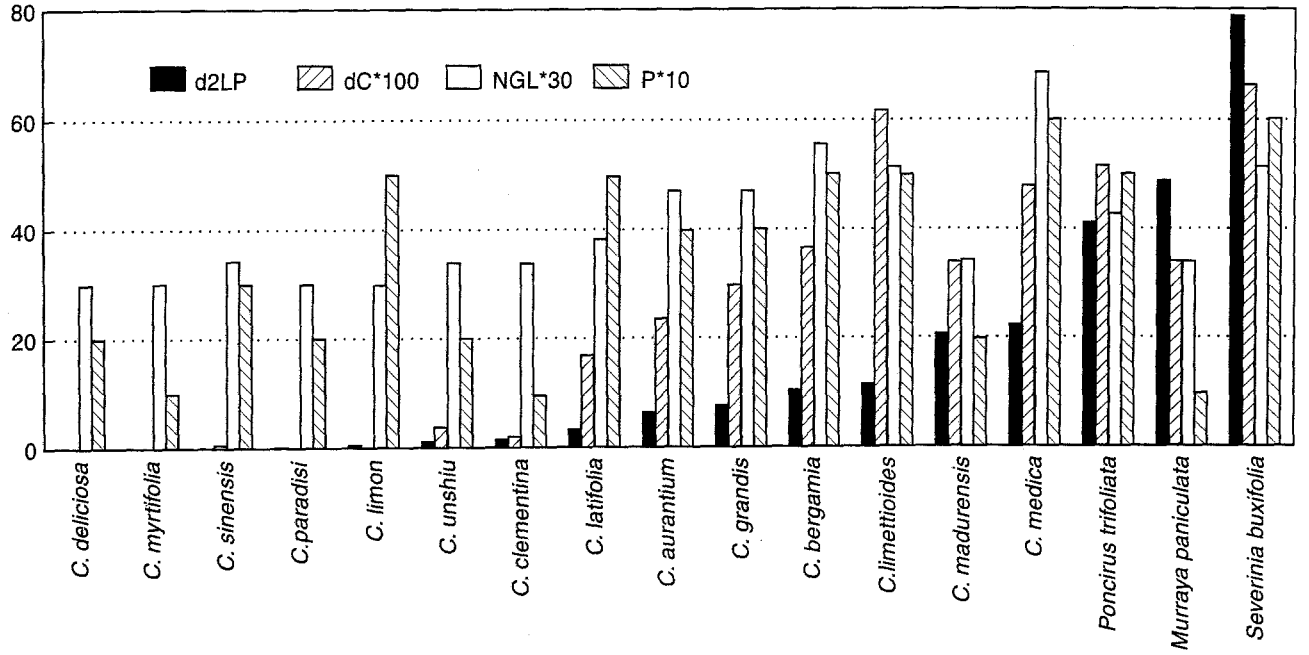

Fig. 4 Comparison of total variability among species regarding the most complementary measures

it is advisable to use different indexes (Asíns and Carbonell, 1987). The presence of very rare alleles or patterns due to the broad spectrum of species studied and the marked differences in heterozygosity are the main factors responsible for the disagreement between measures. P (and A) is highly affected by the degree of heterozygosity (estimated as H), therefore it is not a good measure of variability within *Citrus* species. As can be clearly seen in Fig. 4, NGL is highly dependent on the number of observations at each level of classification. Considering the germ plasm bank employed (a representative one in terms of the different number of samples of cultivated versus uncultivated species) it is not advisable to use NGL or NPS as a variability measure for comparative purposes among species. Regarding the different distance indexes, the ranking of species is very similar (see Table 2); the only differences are located in the highly variable group (*M. paniculata, P. trifoliata, C. medica* and *C. madurensis*). Similarly to what has been observed in previous studies (Asíns and Carbonell 1987, 1989; Bretó et al. 1993) d2 is very sensitive to differences in rare variables (alleles or patterns) and this could lead to an over-estimation of distance within the species mentioned. The reason for using the chord distance of Cavalli-Sforza and Edwards (1967) is that in addition to the advantages of being a Euclidean measure (Nei's distances are not and therefore frequently violate the triangle

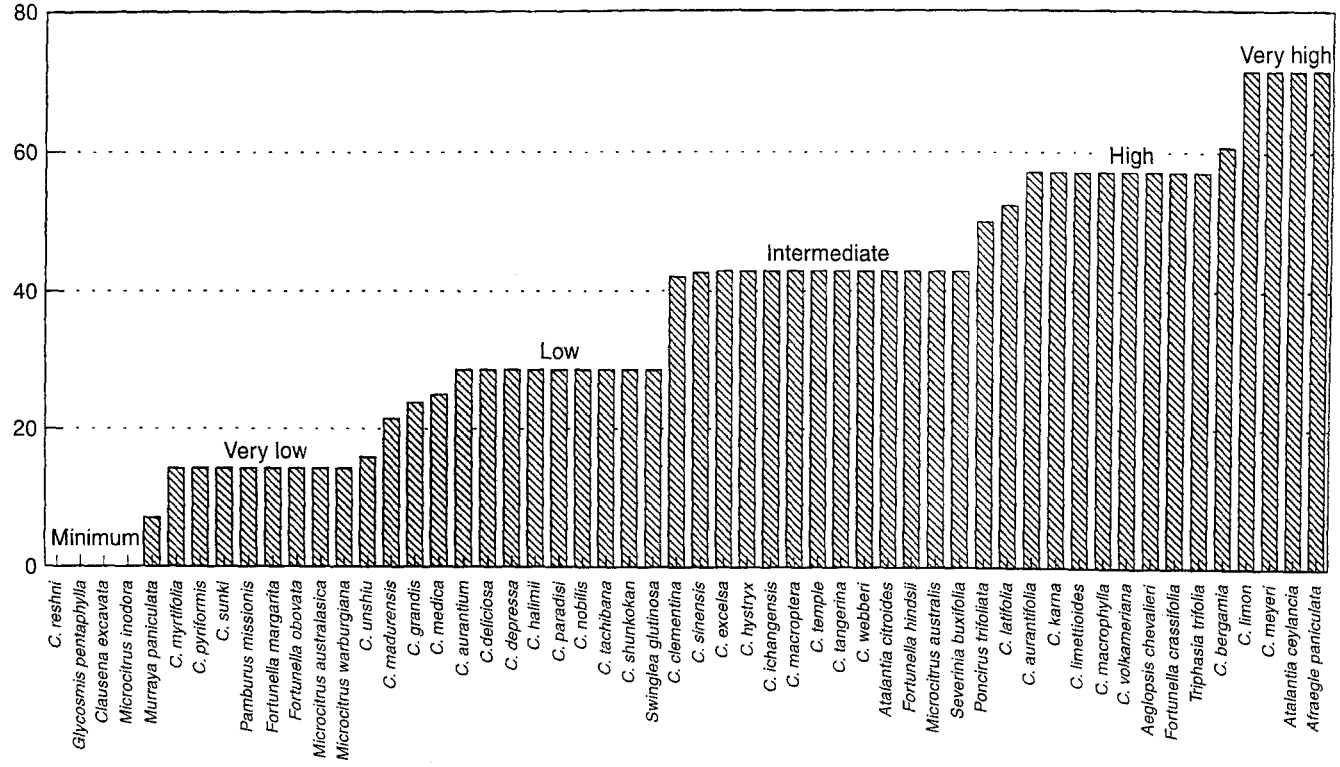

Fig. 5 Comparison of the mean percentage of heterozygosity among species

inequality), it is not heavily influenced by the within-taxon heterozygosity (Wright 1978; Hillis 1984) which has to be taken into account in these species. The angular transformation of gene frequencies that this distance incorporates, makes the variances of the transformed frequencies independent of the ranges in which they fall and then standardizes the distance with respect to random drift (Swofford and Olsen 1990). The disadvantage over d2 is that it can only be calculated for gene frequencies so that the information from enzymatic patterns has to be ignored.

As for the factors affecting genetic variation, mutation, cross hybridization, and especially the type of reproduction, they are very important for explaining genetic variability in the orange subfamily. Most *Citrus* species are apomicts or facultative apomicts. Sexual fusion of gametes results in a single zygotic embryo. In addition, a variable number of vegetative embryos develop by the proliferation of the nucellar tissue adjacent to the embryo sac (Frost 1926). There is competition among the developing embryos and many do not survive to seed maturity. In most cases, the zygotic embryo degenerates, but in a small proportion of seeds the zygotic embryo survives to maturity, along with a variable number of nucellar embryos (Frost and Soost 1968). The frequency of zygotic seedlings depends on both the genotype and the environment (Khan and Roose 1988; Moore and Castle 1988). Thus mono- and poly-embryony can be largely equated to sexual and nucellar embryony respectively, although sexual polyembryony also exists (Bacchi 1943; Cameron and Garber 1968). Within *Citrus*, only *C. medica, C. grandis, C. clementina, C. tangerina, C. temple, C. nobilis* and *C. halimii* are cited as monoembryonic (Barret and Rhodes 1976; Singh and Nath 1969; Scora and Kumamoto 1983). Regarding cytogenetic changes as a source of genetic variability, numerical chromosome changes are rare. Diploidy is the general rule in the orange subfamily, with only a few tetraploid and triploid plants (Iwamasa and Nito 1988). However, structural chromosome changes , such as translocations and inversions, have been detected in some citrus cultivars (Raghuvanshi 1962; Iwamasa and Nito 1988). In an apomictic genus, like *Citrus*, all the structural changes in the chromosomes can survive to some extent, depending on the percentage of nucellar embryony in any particular case. The significance of any kind of mutation becomes clear when we take into consideration the fact that most cultivated *Citrus* species are propagated vegetatively. Bud mutations are very common in *Citrus* (Raghuvanshi 1962) and the improvement of most cultivars of sweet orange, satsumas, clementines, grapefruits, lemons, etc., has come from careful selection of identified bud mutations, which would explain the low within-species genetic variability we have found for them. This low genetic intraspecific polymorphism found in the cultivated species contrasts with their great variability for agronomically important characters such as maturation date, fruit color, fruit size, etc. There has been considerable debate over whether molecular or morphological features are inherently better sources for estimating phylogeny (Patterson 1987). Comparative studies have shown that morphological change and molecular divergence are quite independent, responding to different evolutionary pressures and following different rules (Wilson et al. 1974, 1977). Doubtless, improve-

ments in the broadness of the orange and mandarin harvest calendar has always been a major goal of *Citrus* breeders in searching for new bud mutations.

Hybridization seems to be a major source of variation. *Citrus* species are known to hybridize among themselves without much difficulty (Iwamasa et al. 1988) and numerous instances of naturally occurring hybrids have been reported (Swingle 1943). Recently, the CMA/DAPI chromosome banding patterns of six *Citrus* species revealed that each individual was heterozygous for at least one chromosome pair (Guerra 1993). *Citrus* is usually referred to as a very heterozygous genus (cited by Barret and Rhodes 1976) but we have shown that this is not true for all its species. A high percentage of heterozygosity has been found in the group of lemons (*C. limon, C. meyeri, C. karna, C. volkameriana*), limes (*C. aurantifolia, C. limettioides, C. lattifolia*) and *C. bergamia,* which reveals an origin through interspecific hybridization. This was previously suggested for *C. limon* by several authors (Torres et al. 1978; Green et al. 1986; Roose 1988). However, not all *Citrus* species present such high heterozygosity values. Those species showing very low or minimum values of heterozygosity suggest that self-pollination may have played an important role in their origin or evolution. Some of them are, or were, used as rootstock (*C. reshnii, C. sunki, C. myrtifolia*) and were therefore reproduced from seeds. If zygotic seedlings have arisen from them through self-pollination it would explain our results on low heterozygosity. The data of Moore and Castle (1988), based on the isozyme genotyping at seven loci of seedling populations of 15 rootstocks, support our hypothesis. Roose et al. (1994) have also recently reported that all zygotic seedlings from open-pollinated seed of *P. trifoliata* cv "Flying Dragon" had isoenzymatic genotypes consistent with an origin by self-pollination.

For monoembryonic (sexual) *Citrus* species, the genetic variability found in *C. medica* is much greater than that found in *C. grandis* and we think that this is mainly due to a greater complexity of the former species. *C. grandis* is predominantly cross-pollinating (Soost 1964) although some of its clones have a tendency to self-fertilization (Barret and Rhodes 1976) and yield a relatively vigorous selfed progeny. Much less is known about cross and self fertility from *C. medica* but limited observations suggest it produces vigorous selfed seedlings. Our results imply that variability in *C. medica* has been generally underestimated because a narrow spectrum of varieties has in general been used to characterize this species. A majority of authors have considered only one or two accessions and the cultivar "Poncil" has never been used. The genotype for most enzymatic loci of this cultivar (the third one in Table 1) is quite different from the other cultivars of the species. In fact it is the only *Citrus* species that presents the rare *Lap-1* allele which has otherwise been observed only in *Afraegle, Atalantia, Glycosmis, Severinia* and both citranges (artificial cross between *C. sinensis* and *P. trifoliata*). *C. clementina,* another monoembryonic species, in spite of being self-incompatible shows the lowest genetic variability within this group of species. Two reasons can be suggested for this: one, the method of improvement, involving the selection of favourable bud mutations (no cross breeding), and two, that *C. clementina* is used as scion cultivar and, therefore, is propagated vegetatively to ensure an early yield and to avoid any variation. Moreover, given that it is extremely important to avoid seed formation for commercial purposes, it has led to the necessity of obtaining new seedless cultivars. This is a clear example of how man is restricting natural variation.

Cultivated species are subjected to a strong selection against variability at their propagation. The scion cultivar is always grafted onto a seedling rootstock in order to obtain a more uniform and early yielding tree. Therefore only the rootstock is reproduced from seed. The possible variation raised from genetic segregation in zygotic seedlings (of the rootstock) is removed by nurserymen. Vegetative propagation allows less genetic variability. It would explain the much greater variability, although less heterozygosity, of sour orange (a very common rootstock) compared to its closely related species, sweet orange. Therefore, it is important to recommend germ plasm conservation of these species, as well as those closely related species with sexual reproduction, given that man is commercially interested in eliminating all genetic variation and the latter are a natural and ready-to-use pool of genes within the genus *Citrus*.

Three *Citrus*-related species have been considered for the intraspecific study, which has revealed their genetic richness. Unfortunately, at the time of the analysis of the germ plasm bank no species of *Eremocitrus* were available; however, it is important to point out that efforts to pollinate castrated and bagged *Eremocitrus* flowers from *Citrus* or other genera have not so far succeeded. Among the genera studied, *Poncirus trifoliata* is of especial interest due to its high genetic variability, crossability with *Citrus* and its important characteristics. It is used as a rootstock by itself and to improve cultivated *Citrus* species for resistance to several diseases (CTV decline, phytophthora root rot, nematodes), cold, or to induce a dwarfing habit. Other closely related genera have revealed a great variability involving also differences in the percentage of heterozygosity, which suggests evolution through hybridization. Due to its crossability with *Citrus*, its high genetic variability and its wide range of ecological adaptations (from tropical rain forest to xerophytic habitats of Australia), the genus *Microcitrus* must be also taken into account as an important source of genetic resources for *Citrus* improvement.

It has been shown that the orange subfamily presents a great deal of genetic diversity, even at the level of isozymatic markers. Most species present more than one genotype and therefore any study of phylogenetic relationships must take into account such variability. Part of this variability can be explained through hybridization given the high heterozygosity values found in some species. High heterozygosity and genotypic uniformity are found together in some species which could be explained by both, their origin through interspecific hybridization and their type of reproduction and propagation. In addition to this,

there are some polyembryonic species with very low heterozygosity suggesting that self-pollination has been involved, in their evolution. Three important factors tend to limit the genetic variability in this group; apomixis, facultative apomixis accompanied by self-pollination, and strong selection against variability due to man's action. These factors justify the need for collecting and preserving more *Citrus* genotypes and *Citrus*-related species, specially those with complete or partial sexual reproduction.

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