

Citrus

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Citrus fruits are the most extensively produced fruits in the world. About 90.9 million tonnes were produced in 1999/2000, of which 59.5 million tonnes were sweet oranges (FAO, 2000). The volume of fruit processed is increasing: concentrated and frozen orange juice for a large part of the processed fruit products in the United States and Brazil.

Citrus fruits were domesticated in Southeast Asia several thousand years ago and then spread throughout the world (Fig. 1). Citron (*C. medica* L.) was the first species cultivated in the Mediterranean basin, some centuries before the common era, while other species were introduced only during the second millennium. Citrus crops conquered America following the discovery of the New World during the 15th century. The area of citrus cultivation is today very wide, and it is located approximately between 40°N and 40°S latitude.

The cultivation of citrus faces increasing biotic and abiotic constraints in the major regions of production. Tristeza, a degenerating disease caused by the citrus tristeza virus, *Phytophthora* sp., and nematodes are found today throughout the cultivation areas. Other constraints are regional: cold and blight—which is a degenerating disease of still indeterminate origin—in the United States, citrus variegated chlorosis due to *Xilela fastidiosa* in Brazil, cercosporiosis caused by *Phaeoramularia angolensis* in Africa, and greening or citrus huanglongbing in Asia. Among the abiotic constraints, salinity and calcareous soils are major problems of the Mediterranean basin. The widespread use of grafted plants allows farmers to overcome soil-related constraints (calcareous soils, salinity, telluric parasites) to some extent, as well as tristeza. Scions are selected on the basis of qualitative aspects and, in some countries, characters of tolerance to citrus variegated chlorosis, to mal secco or to cercosporiosis (Ollitrault and Luro, 1997).

BOTANY AND GENETIC RESOURCES

Botany and Taxonomy

Partial apomixis by nucellar embryogenesis, associated with a wide sexual compatibility, has led to the production of clonal populations of interspecific

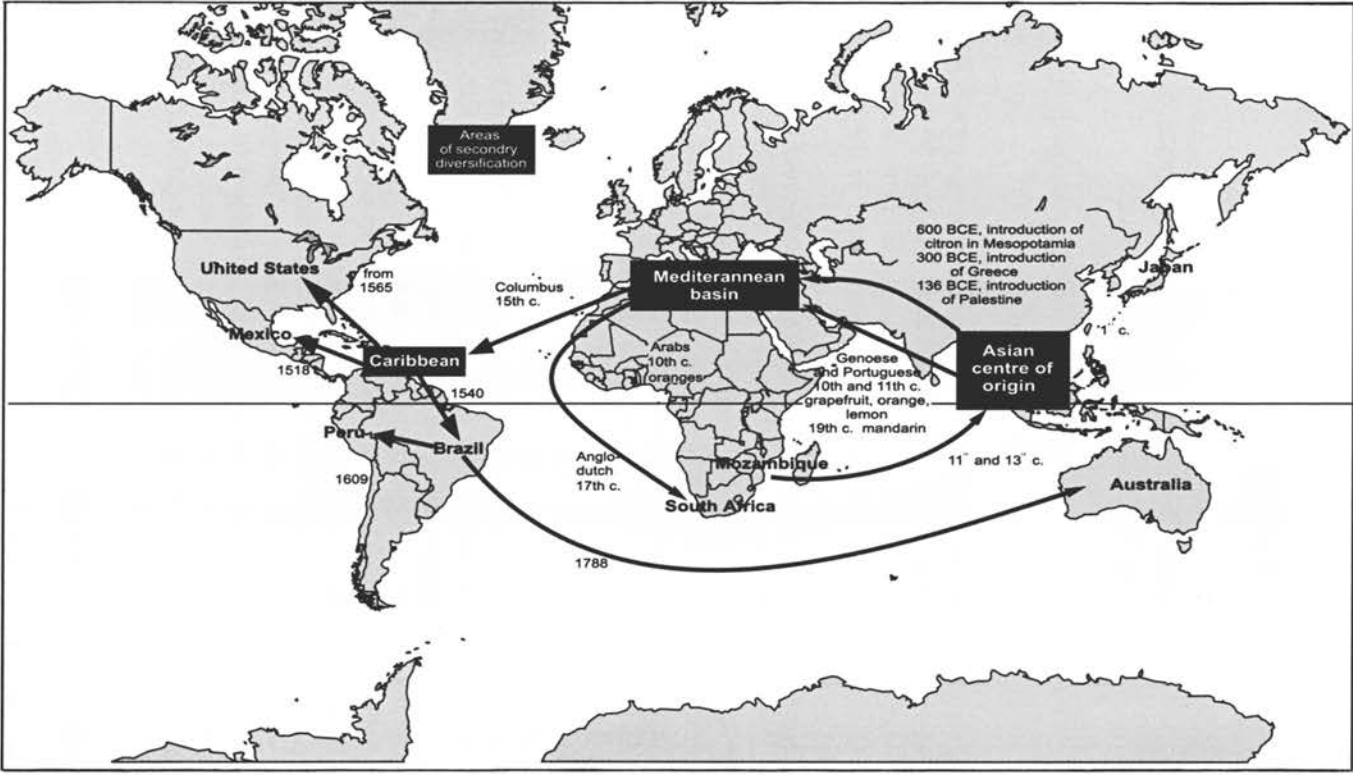


Fig. 1. Regions of origin, dispersal, and diversification of cultivated citrus.

hybrids, which have often been assimilated into new species by taxonomists. Botanic classifications are thus generally complicated. Tanaka (1961) identified 156 species, while Single and Reece (1967) distinguished only 16. The correspondence between these two classifications and the common names is given in Table 1 for the taxa studied in this chapter. In all the *Citrus* species and related genera, the base number of chromosomes (n) is equal to 9 (Krug, 1943). Almost all the *Citrus* are diploid and only a few natural polyploids have been identified, such as *Fortunella hindsii* or the Tahiti lime.

Genetic Resources

There are several collections of citrus throughout the world. They have two objectives, often divergent as to choice of plant material to be conserved: first, to preserve the diversity of *Citrus* and related genera over the long term, and second, to create orchards to provide grafts of valuable varieties. The collection of the Okitsu Branch (Fruit Tree Research Station) in Japan is the most important for cultivated material from the zones of origin, while the conservatory of the University of Malaysia is remarkable for its collection of Aurantioideae of Southeast Asia. The collections of the USDA (United States Department of Agriculture), IVIA (Instituto Valenciano de Investigaciones Agrarias) in Spain, and the University of Adana in Turkey contain certain Rutaceae related to the citrus but are regularly supplied for the most part by new varieties created throughout the world. The INRA and CIRAD station of San Giuliano, in France, has a unique status because of the favourable phytosanitary conditions of Corsica. It shelters a significant collection of healthy plant material, which includes numerous accessions of Southeast Asia and can be evaluated in the field. The Egid database management software, developed by CIRAD and INRA (Cottin et al., 1995) from the descriptors of the IPGRI (International Plant Genetic Resources Institute), has been adopted by the FAO to set up a global network to manage citrus genetic resources.

ORGANIZATION OF DIVERSITY

Agromorphological Variability

The agromorphological variability of citrus is considerable. It involves pomological and organoleptic characters as well as resistance to biotic and abiotic factors. The *Citrus* genus includes several sources of tolerance of biotic and abiotic stresses, which opens up interesting perspectives for the use of genetic resources in plant improvement.

Among the abiotic factors we can cite: cold tolerance in Satsuma mandarin trees; salinity tolerance in Rangpur lime trees and Cleopatra mandarins;

Table 1. Analysed Citrus accessions and genetic characteristics

| Code | Cultivar | Name of species | | Marker morpho. iso. | Genome size (pg/2C) | Enzymatic genotype | | | | | | | | | | | |
|-----------------|------------------|-----------------------------|---------------------|------------------------|---------------------------|--------------------|------------|----------------|--------------|--------------|------|----|----|----|----|----|----|
| | | Swingle and Reece (1967) | Tanaka (1961) | | | ADH-1 AAT | IDH LAP | MDH-1 MDH-2 | PGI PGM-1 | PGM-2 PER | SKDH | | | | | | |
| ● Mandarins (M) | | | | | | | | | | | | | | | | | |
| Mks | King of Siam | <i>reticulata</i> hybrid | <i>nobilis</i> | 1 | 1 | 0.760 | 22 | 11 | 33 | 44 | 33 | 22 | 33 | 34 | 12 | 12 | 33 |
| MSW* | Satsuma Wase | <i>reticulata</i> | <i>unshiu</i> | 1 | 1 | 0.737 | | | | | | | | | | | |
| Mso | Satsuma Qwari | <i>reticulata</i> | <i>unshiu</i> | 1 | 1 | - | 22 | 11 | 33 | 34 | 33 | 22 | 23 | 33 | 22 | 22 | 33 |
| Mda | Dancy | <i>reticulata</i> | <i>tangerina</i> | 1 | 1 | 0.736 | 22 | 11 | 33 | 44 | 33 | 22 | 33 | 33 | 22 | 12 | 33 |
| Mte | Temple | <i>reticulata</i> hybrid | <i>temple</i> | 1 | 1 | 0.748 | 22 | 11 | 33 | 24 | 33 | 22 | 23 | 23 | 22 | 22 | 33 |
| Mcl | Cleopatra | <i>reticulata</i> | <i>reshni</i> | 1 | 1 | 0.733 | 22 | 11 | 33 | 44 | 33 | 22 | 33 | 22 | 22 | 22 | 33 |
| Mpo | Ponkan | <i>reticulata</i> | <i>reticulata</i> | 1 | 1 | 0.744 | 22 | 11 | 33 | 44 | 33 | 22 | 33 | 33 | 22 | 11 | 22 |
| Mco | Common | <i>reticulata</i> | <i>deliciosa</i> | 1 | 1 | 0.730 | 22 | 11 | 33 | 45 | 33 | 22 | 34 | 23 | 22 | 12 | 23 |
| M63 | Clementine SRA63 | <i>reticulata</i> | <i>clementina</i> | 1 | 1 | 0.750 | 22 | 11 | 23 | 24 | 33 | 22 | 24 | 33 | 22 | 22 | 33 |
| Mmu | Murcott | <i>reticulata</i> hybrid | | 1 | 1 | 0.746 | 22 | 11 | 33 | 44 | 33 | 22 | 33 | 33 | 22 | 22 | 33 |
| ● Pummelos (P) | | | | | | | | | | | | | | | | | |
| Pme | Menara | <i>grandis</i> | sp. | 1 | 1 | 0.751 | 22 | 12 | 33 | 22 | 33 | 22 | 22 | 13 | 11 | 12 | 23 |
| Prk | Reinking | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.774 | 22 | 22 | 33 | 45 | 33 | 22 | 22 | 44 | 11 | 11 | 12 |
| Pkp | Kao Pan | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.767 | 22 | 12 | 23 | 35 | 33 | 22 | 22 | 13 | 11 | 11 | 12 |
| Psn | Sunshine | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.794 | 22 | 22 | 23 | 25 | 33 | 12 | 22 | 33 | 11 | 11 | 11 |
| Ppi | Pink | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.779 | 22 | 11 | 33 | 55 | 33 | 22 | 22 | 13 | 11 | 11 | 11 |
| Psp | Seedless | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.787 | 22 | 12 | 33 | 25 | 33 | 22 | 23 | 11 | 11 | 11 | 12 |
| Pin | India | <i>grandis</i> | <i>maxima</i> | 1 | 1 | 0.787 | 22 | 22 | 22 | 55 | 33 | 22 | 22 | 33 | 11 | 11 | 12 |
| Pah | Tahiti | <i>grandis</i> | <i>maxima</i> | 0 | 1 | - | 22 | 12 | 33 | 25 | 33 | 22 | 22 | 11 | 11 | 11 | 11 |
| Pph | Philippines | <i>grandis</i> | <i>maxima</i> | 0 | 1 | - | 22 | 22 | 33 | 55 | 33 | 22 | 22 | 11 | 11 | 11 | 11 |
| Psu | Surinam | <i>grandis</i> | <i>maxima</i> | 0 | 1 | - | 22 | 12 | 23 | 35 | 33 | 22 | 22 | 13 | 11 | 11 | 11 |
| Pei | Eingedi | <i>grandis</i> | <i>maxima</i> | 1 | 0 | 0.763 | - | - | - | - | - | - | - | - | - | - | - |
| Pch | Chandler | <i>grandis</i> | <i>maxima</i> | 1 | 0 | 0.764 | - | - | - | - | - | - | - | - | - | - | - |
| ● Limes (L) | | | | | | | | | | | | | | | | | |
| Lbs | Brazil Sweet | <i>aurantifolia</i> | <i>limettioides</i> | 1 | 1 | 0.756 | 22 | 12 | 33 | 36 | 13 | 22 | 23 | 22 | 12 | 11 | 12 |

(Contd.)

(Table 1. Contd.)

| | | | | | | | | | | | | | | | | | |
|---------------------|------------------|---------------------|---------------------|---|---|-------|----|----|----|----|----|----|----|----|----|----|----|
| Lga | Gallet | <i>aurantifolia</i> | <i>aurantifolia</i> | 1 | 1 | 0.787 | 12 | 12 | 13 | 36 | 13 | 12 | 22 | 22 | 22 | 11 | 12 |
| Lta | Tahiti | <i>aurantifolia</i> | <i>latifolia</i> | 1 | 1 | 1.170 | | | | | | | | | | | |
| Lme | Mexican | <i>aurantifolia</i> | <i>aurantifolia</i> | 1 | 1 | 0.779 | | | | | | | | | | | |
| Lel | Elkseur | <i>aurantifolia</i> | <i>latifolia</i> | 1 | 1 | 1.170 | | | | | | | | | | | |
| Lbe | Bears | <i>aurantifolia</i> | <i>latifolia</i> | 1 | 1 | 1.170 | 22 | 22 | 13 | 36 | 13 | 12 | 22 | 23 | 12 | 11 | 22 |
| Lca | Calédonie | <i>aurantifolia</i> | <i>aurantifolia</i> | 1 | 1 | 0.784 | | | | | | | | | | | |
| Lki | Kirk | <i>aurantifolia</i> | <i>aurantifolia</i> | 1 | 1 | 0.779 | | | | | | | | | | | |
| Lra | Rangpur | <i>aurantifolia</i> | <i>limonia</i> | 1 | 1 | 0.772 | 22 | 12 | 13 | 36 | 13 | 22 | 23 | 22 | 22 | 11 | 13 |
| Lka | Kanghzi | <i>aurantifolia</i> | <i>aurantifolia</i> | 0 | 1 | - | 22 | 22 | 12 | 36 | 13 | 22 | 22 | 22 | 12 | 11 | 22 |
| Lsr | IAC SRA618 | <i>aurantifolia</i> | <i>aurantifolia</i> | 1 | 0 | 1.170 | - | - | - | - | - | - | - | - | - | - | - |
| ● Lemons (C) | | | | | | | | | | | | | | | | | |
| Cme | Meyer | <i>limon</i> | <i>meyeri</i> | 1 | 1 | 0.772 | 22 | 12 | 23 | 46 | 13 | 22 | 23 | 23 | 12 | 12 | 12 |
| Cfi | Fino | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.784 | | | | | | | | | | | |
| Cve | Verna | <i>limon</i> | <i>limon</i> | 1 | 1 | - | | | | | | | | | | | |
| Cad | Adamapoulos | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.769 | | | | | | | | | | | |
| Cdx | Doux | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.778 | | | | | | | | | | | |
| Cli | Lisbon | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.786 | 22 | 12 | 13 | 46 | 13 | 22 | 24 | 23 | 12 | 12 | 12 |
| Cvi | Villafranca | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.776 | | | | | | | | | | | |
| Cmo | Monachello | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.787 | | | | | | | | | | | |
| Ceu | Euréka | <i>limon</i> | <i>limon</i> | 1 | 1 | 0.777 | | | | | | | | | | | |
| Clu | Lunari | <i>limon</i> | <i>limon</i> | 0 | 1 | - | | | | | | | | | | | |
| Cst | Santa Teresa | <i>limon</i> | <i>limon</i> | 1 | 0 | 0.786 | - | - | - | - | - | - | - | - | - | - | - |
| ● Sweet oranges (O) | | | | | | | | | | | | | | | | | |
| Owa | Washington Navel | <i>sinensis</i> | <i>sinensis</i> | 1 | 1 | 0.757 | | | | | | | | | | | |
| Odf | Double Fine | <i>sinensis</i> | <i>sinensis</i> | 1 | 1 | 0.778 | | | | | | | | | | | |
| Ota | Tarocco | <i>sinensis</i> | <i>sinensis</i> | 1 | 1 | 0.772 | | | | | | | | | | | |
| Onh | New Hall | <i>sinensis</i> | <i>sinensis</i> | 1 | 1 | 0.778 | | | | | | | | | | | |
| Ona | Navelina | <i>sinensis</i> | <i>sinensis</i> | 1 | 1 | 0.755 | 22 | 11 | 23 | 24 | 33 | 22 | 23 | 33 | 12 | 22 | 12 |

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| | | | | | | | | | | | | | | | | | |
|----------------|--------------------------|--------------------------|------------------------|---|---|-------|----|----|----|----|----|----|----|----|----|----|----|
| Gce | Cecily | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.778 | | | | | | | | | | | |
| Gal | Alanoek | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.759 | | | | | | | | | | | |
| Gre | Reed | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.772 | | | | | | | | | | | |
| Gsr | Star Ruby | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.772 | 22 | 12 | 33 | 25 | 33 | 22 | 22 | 13 | 11 | 12 | 23 |
| Grb | Red Blush | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.788 | | | | | | | | | | | |
| Glr | Little River | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.784 | | | | | | | | | | | |
| Gth | Thomson | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.784 | | | | | | | | | | | |
| Gma | Marsh | <i>paradisi</i> | <i>paradisi</i> | 1 | 1 | 0.783 | | | | | | | | | | | |
| Gru | Ruby | <i>paradisi</i> | <i>paradisi</i> | 1 | 0 | 0.781 | - | - | - | - | - | - | - | - | - | - | - |
| ● Other Citrus | | | | | | | | | | | | | | | | | |
| ROL | rough lemon | <i>limon</i> | <i>jambhiri</i> | 1 | 1 | 0.777 | 12 | 12 | 23 | 46 | 13 | 22 | 23 | 22 | 22 | 11 | 22 |
| PEC | <i>pectinifera</i> | <i>reticulata</i> hybrid | <i>depressa</i> | 1 | 1 | 0.751 | 22 | 11 | 33 | 24 | 23 | 22 | 33 | 33 | 22 | 12 | 33 |
| JUN | - | <i>ichang austera</i> | <i>junos</i> | 1 | 1 | 0.810 | 22 | 12 | 33 | 24 | 23 | 22 | 33 | 13 | 12 | 11 | 22 |
| GUL | - | <i>maxima</i> | <i>pseudogulgul</i> | 1 | 1 | 0.745 | 22 | 12 | 33 | 24 | 33 | 22 | 23 | 11 | 11 | 11 | 22 |
| ICH | <i>ichangensis</i> lemon | <i>ichangensis</i> | <i>ichangensis</i> | 1 | 1 | 0.774 | 22 | 11 | 22 | 44 | 23 | 22 | 23 | 34 | 12 | 11 | 22 |
| BGM | bergamot | <i>aurantifolia</i> | <i>bergamia</i> | 1 | 1 | 0.771 | 22 | 12 | 13 | 44 | 13 | 22 | 24 | 13 | 12 | 11 | 12 |
| PDC | commander pear | <i>limon</i> | <i>lumia</i> | 1 | 1 | - | 22 | 12 | 33 | 24 | 33 | 22 | 22 | 34 | 12 | 12 | 22 |
| COM | <i>combava</i> | <i>hystrix</i> | <i>hystrix</i> | 1 | 1 | 0.803 | 22 | 12 | 33 | 14 | 12 | 22 | 22 | 33 | 11 | 11 | 12 |
| INT | - | <i>paradisi</i> | <i>intermedia</i> | 1 | 1 | 0.764 | 22 | 12 | 33 | 23 | 33 | 22 | 23 | 23 | 11 | 12 | 22 |
| MAC | - | <i>aurantifolia</i> | <i>macrophylla</i> | 1 | 1 | 0.798 | 22 | 22 | 23 | 44 | 12 | 22 | 12 | 23 | 12 | 11 | 22 |
| PEN | - | <i>aurantifolia</i> | <i>pennivesiculata</i> | 1 | 1 | 0.813 | 22 | 22 | 13 | 22 | 11 | 12 | 22 | 23 | 12 | 11 | 12 |
| EXE | - | <i>aurantifolia</i> | <i>excelsa</i> | 1 | 1 | 0.793 | 22 | 22 | 23 | 44 | 33 | 12 | 12 | 23 | 22 | 11 | 22 |
| SIA | siamelo | hybrid | hybrid | 1 | 1 | 0.745 | 22 | 11 | 33 | 24 | 33 | 22 | 23 | 33 | 12 | 11 | 12 |
| KPA | <i>khasi papeda</i> | <i>latipes</i> | <i>latipes</i> | 1 | 1 | 0.780 | 22 | 12 | 34 | 44 | 23 | 23 | 12 | 34 | 12 | 11 | 12 |
| HAL | - | <i>halimii</i> | <i>halimii</i> | 1 | 1 | 0.778 | 22 | 22 | 22 | 44 | 22 | 23 | 12 | 34 | 22 | 11 | 22 |
| VOL | - | <i>limon</i> | <i>limonia</i> | 1 | 1 | 0.764 | 12 | 12 | 13 | 46 | 13 | 22 | 23 | 22 | 22 | 11 | 12 |
| NAS | <i>nasnaran</i> | <i>reticulata</i> hybrid | <i>amblycarpa</i> | 0 | 1 | - | 12 | 12 | 33 | 44 | 33 | 22 | 13 | 33 | 11 | 11 | 12 |

*The codes in bold face represent the common enzymatic type in the analyses. All the individuals in a set of rows shaded in grey have the same enzymatic profile.

calcareous soil tolerance in *C. jambhiri*, *C. macrophylla*, *C. volkameriana*, *C. amblycarpa*, and sour oranges; and drought tolerance in Rangpur lime. Tolerance of the major pests and diseases has also been identified: tolerance of *Phytophthora* sp. in some pummelos, sour orange, *C. volkameriana*, and *C. amblycarpa*; African cercosporiosis tolerance in grapefruit, lemon, and Satsuma and Beauty mandarin; tristeza tolerance in Cleopatra mandarin, *C. amblycarpa*, Rangpur lime, *C. jambhiri*, and *C. volkameriana*; blight tolerance in orange; tolerance of citric canker due to *Xanthomonas campestris* in *C. junos* and some mandarins (Satsuma and Dancy, for example); and resistance to phytophagous acarids of Marsh pomelo and mandarins (Satsuma and Dancy). In view of these examples, there seems to be no link between the distributions of sources of resistance to biotic factors and the specific structure of the genus *Citrus*.

On the other hand, the morphophysiological variability is strongly marked between the species, even though certain characters selected by humans have a strong intraspecific diversity (precocity, calibre, colour of fruits). For example, within the genus *Citrus*, the diameter of fruits varies from a few centimetres for certain mandarins and limes to more than 30 cm for some grapefruits. Albedo is nearly non-existent in mandarins but is the essential characteristic of the fruit in the citron. The fruit pulp is green, orange, yellow, or red. Its acidity is nil in some sweet oranges and very high in limes and lemons. Although the leaves of all the species of *Citrus* are monofoliolate, their size and shape as well as the shapes of the trees vary considerably according to the species.

A more refined study of the structure of morphological diversity in the genus *Citrus* has been done from 20 descriptors of the vegetative apparatus observed among 74 cultivars. It supports the analysis of relations between morphological diversity and molecular diversity presented in this chapter.

Biochemical and Molecular Variability

Essential oils and polyphenols were the first markers used to characterize varieties (Tatum et al., 1974) and to study the phylogenesis of citrus (Scora, 1988). Isozymes were used routinely to identify the zygotic or nucellar origin of seedlings (Soost et al., 1980; Khan and Roose, 1988; Ollitrault et al., 1992). They also make it possible to specify phylogenetic relations between species (Torres et al., 1982; Hirai et al., 1986; Ollitrault and Faure, 1992; Herrero et al., 1996, 1997). The techniques of direct analysis of DNA polymorphism—DNA, RFLP, RAPD, variable number of tandem repeats (VNTR)—were mainly applied in genome mapping programmes (Durham et al., 1992; Jarrel et al., 1992; Luro et al., 1994b; Fang et al., 1998; Moore et al., 2000) or programmes of varietal characterization and taxonomy (Luro et al., 1994a, 1995; Fang and Roose, 1996; Federici et al., 1998; Nicolosi et al., 2000). Nevertheless, the allelic determinism of these markers is sometimes difficult to clarify, so they have limited use in genetic studies of populations concerning heterozygosity and index of fixation or index of gametic inequilibrium.

Cytogenetic studies and flow cytometry analyses have demonstrated the existence of great variations between species as to chromosome size (Nair and Randhawa, 1969; Ollitrault et al., 1994). They also have proved many cases of structural heterozygosity (Raghuvanshi, 1969; Gmitter et al., 1992; Guerra, 1993; Miranda et al., 1997). These elements on the structure of genomes of different taxa are determinants for analysis of the organization of allelic diversity in evolutionary terms.

In order to study the parameters of population structure, the analysis of allelic diversity presented in this chapter relies on the polymorphism of 9 isozymic systems. The nuclear structural diversity is also examined by evaluation of genome size using flow cytometry. The varietal sampling for the cultivated forms is the same as for the study of morphological diversity. Seventeen non-cultivated *Citrus* spp. complete the analysis.

ISOZYMIC DIVERSITY

Thirty-five alleles were identified for 11 polymorphous loci. Only 5 of these alleles were not observed in cultivars. The null allele of the locus *LAP* (*LAP-6*), identified at the homozygous state in the citrons, was detected in the heterozygous state in a certain number of acid citrus (lemons, limes) when controlled hybrids were examined. Several cultivars of a single species presented identical profiles. This was particularly the case for orange, sour orange, pomelo, and lemon. The 74 cultivars were thus grouped into 30 isozymic genotypes (Table 1).

There appears to be widely varying intraspecific diversity among the edible species (Table 2). The citrons have nil allelic diversity due to a high homozygosity and the absence of polymorphism between the cultivars analysed. The grapefruit, sweet orange, and sour orange have similar intraspecific structures. The allelic diversity and heterozygosity in them are moderate and the intercultivar polymorphism is nonexistent. Lemons are

Table 2. Structure of intraspecific allelic diversity observed for 11 loci coding for isozymes

| | No. | Mean no. of alleles per locus | Total diversity | Intercultivar diversity | Observed heterozygosity | Deviation of panmixia |
|--------------|-----|-------------------------------|-----------------|-------------------------|-------------------------|-----------------------|
| Citron | 4 | 1.00 | 0.00 | 0.00 | 0.00 | — |
| Grapefruit | 10 | 1.45 | 0.23 | 0.00 | 0.45 | ***(5 loci) |
| Sour orange | 10 | 1.36 | 0.18 | 0.00 | 0.36 | ***(4 loci) |
| Sweet orange | 10 | 1.45 | 0.23 | 0.00 | 0.45 | ***(5 loci) |
| Lemon | 10 | 1.00 | 0.42 | 0.02 | 0.82 | ***(9 loci) |
| Lime | 10 | 2.09 | 0.34 | 0.08 | 0.54 | ** (2 loci) |
| Pummelo | 10 | 2.09 | 0.25 | 0.13 | 0.24 | ns |
| Mandarin | 10 | 2.00 | 0.19 | 0.10 | 0.17 | ns |

ns: non-significant at threshold of 5%.

**significant at threshold of 1%.

***significant at threshold of 1‰.

highly heterozygous but have very little intervarietal polymorphism. Indeed, only the cultivar "Meyer" can be differentiated from the other ones. The limes are also highly heterozygous and manifest a stronger intervarietal polymorphism than the lemons. The pummelos and mandarins have a very high allelic richness, mainly due to significant intervarietal polymorphism. The two species that have great intercultural diversity—mandarins and pummelos—do not display a significant deviation to panmixis, which undoubtedly demonstrates an important genetic exchange within these taxa. All the other species, with the exception of citrons, which are totally fixed, have an excess of heterozygotes.

The total diversity of the sample of cultivated citruses, in the sense of Nei (1973), is 0.45. It is broken down in a balanced manner in terms of intraspecific diversity (0.23), and interspecific diversity (0.22), with a high value of the G_{ST} coefficient (0.49). This value indicates a marked allelic differentiation between the cultivated taxa. Indeed, it is significant for 10 of the 11 loci analysed. This differentiation between taxa, observed for nearly all the loci, is also found in the multilocus structure evaluated from the 30 genotypes of cultivated *Citrus*. The linkage disequilibrium thus involves 23 locus pairs out of 55 and 9 loci out of 11.

This strong structuration observed within the cultivars is confirmed when one looks at 47 enzymatic genotypes identified, which relates the 30 genotypes of cultivars to 17 other *Citrus* spp. Nine loci out of 11 present significant deviation to panmixis and a shortage of heterozygotes. This type of deviation is classically linked to structures in sub-populations (Walhund effect) and to systems of reproduction that limit gene flow.

The high level of genetic organization observed using genetic parameters of populations is found in the principal coordinates analysis (PCoA) done on the genotypes of cultivars, where 50.4% of the total variance is represented on the 1-2 plane (Fig. 2). The diversity of cultivated *Citrus* is structured around three gene pools: the first contains the mandarins, the second contains the grapefruit and pummelo, and the third is made up of the citrons, which show a marked relationship to the limes. The oranges and sour orange are close to the mandarins, with a probable introgression of pummelo. The lemons, highly heterozygous, may have evolved from a hybridization between the citron/lime group and the group made up of the mandarins, sweet oranges, and sour oranges. Factorial analysis allows us to identify the hybrid forms and their potential parents for this highly organized population.

This organization of cultivated forms around three pools is not called into question by the introduction of non-cultivated forms, as shown by the diversity tree that is constructed by NJ analysis of Dice dissimilarity (Fig. 3). Certain non-cultivated *Citrus* are associated with the groups formed by the cultivars: *C. pectinifera* with the mandarins; siamelos with the oranges; *C. pseudogulgul* and *C. intermedia* with the group of grapefruits and pummelos;

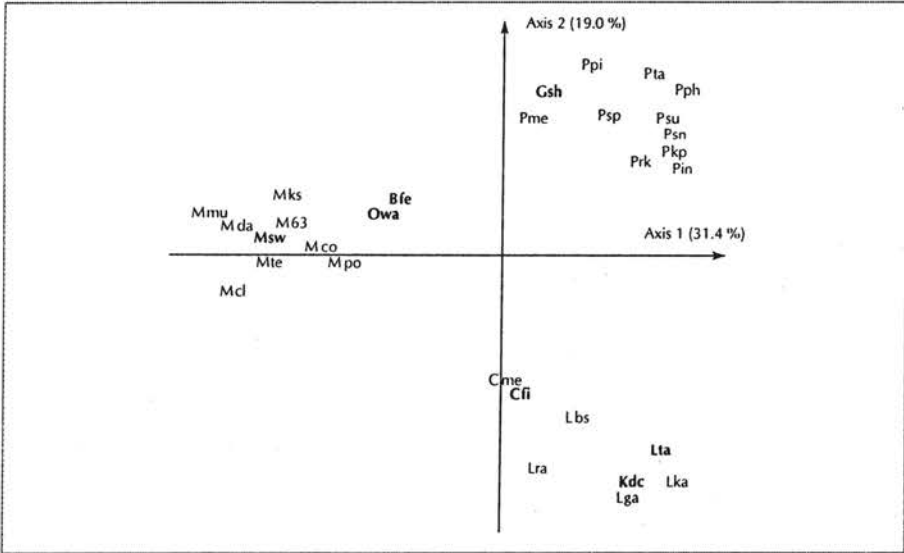


Fig. 2. Isozymic diversity of cultivated citrus on the basis of 11 loci: representation of the first factorial plane of PCoA done on a Dice matrix of dissimilarity between 30 different genotypes identified among 74 cultivars. The codes are the same as those used in Table 1.

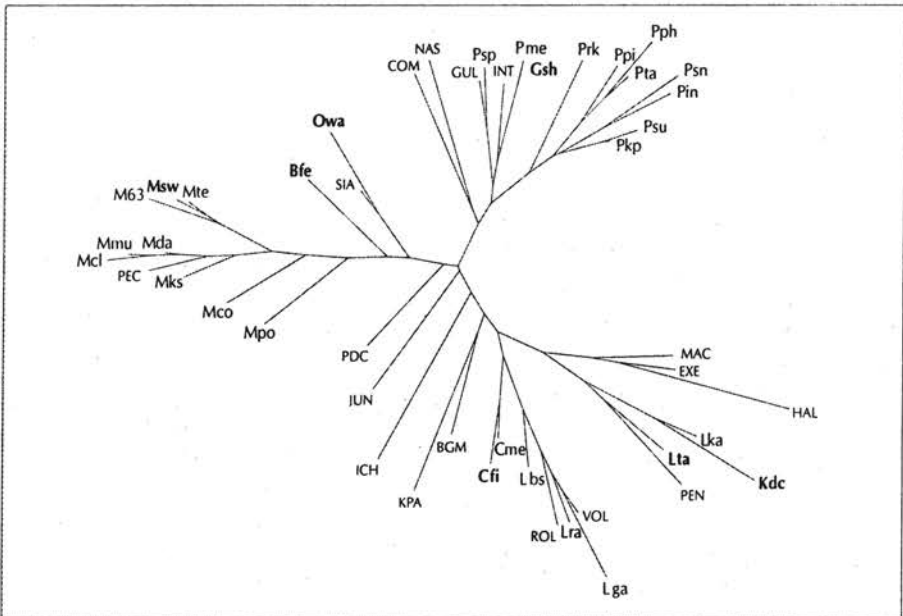


Fig. 3. Isozymic diversity of the genus *Citrus* on the basis of 11 loci: tree representation according to the NJ method, done on a Dice matrix of dissimilarity between 47 genotypes (30 cultivated genotypes and 17 other *Citrus*). The codes are the same as those used in Table 1.

C. pennivesiculata, *C. volkameriana* and *C. jhambivi* with the group of limes. The others are distinguished from these groups either because they carry alleles that are not observed in the cultivars—as with *C. macrophylla*, *C. excelsa*, *C. junos*, *C. ichangensis*, *C. latipes*, *C. hystrix*, and *C. amblycarpa*—or because they have original recombined allelic structures, such as *C. bergamia* or *C. lumia*.

GENOME SIZE

The size of nuclear genomes of individuals is given in Table 1. The diploid genotypes have relatively small genomes, between 0.73 and 0.82 pg of DNA per diploid genome (Fig. 4). The values of 1.17 pg correspond to triploid genotypes; they were observed for four cultivars of lime, Tahiti, Bears, Elkseur, and IAC SRA618. Among the edible species, the interspecific differences are

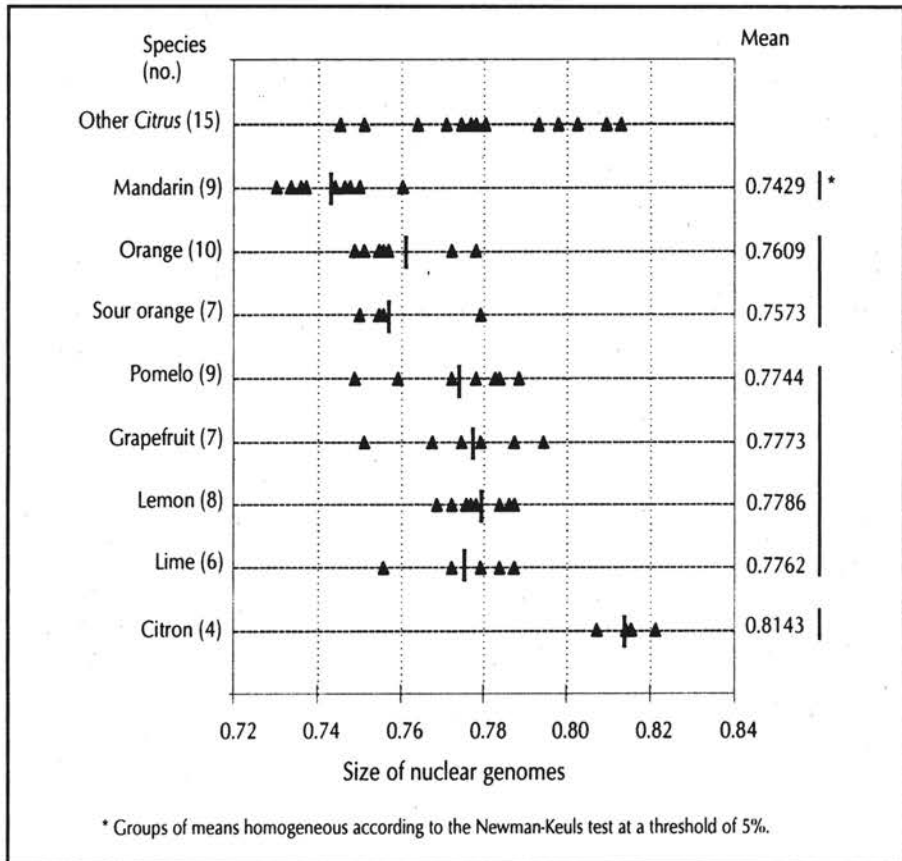


Fig. 4. Size of nuclear genomes of 75 individuals, of which 60 are edible cultivars grouped by species (mean of 3 measurements in picograms of DNA per diploid genome).

statistically significant and represent a deviation of 10% between the mandarins and the citrons (Fig. 5). The other species are divided into two groups of intermediate sizes. One comprises oranges and sour oranges, the other, corresponding to the larger sizes, comprises lemons, limes, grapefruits, and pummelos. The inedible types also have genome sizes between those of mandarins and citrons. Thus, two out of three taxa that structure the diversity, mandarin and citron, have genome sizes that are at the extremes observed in the genus *Citrus*. The other taxa have genome sizes that agree with the genetic affinities determined by isozymic analyses.

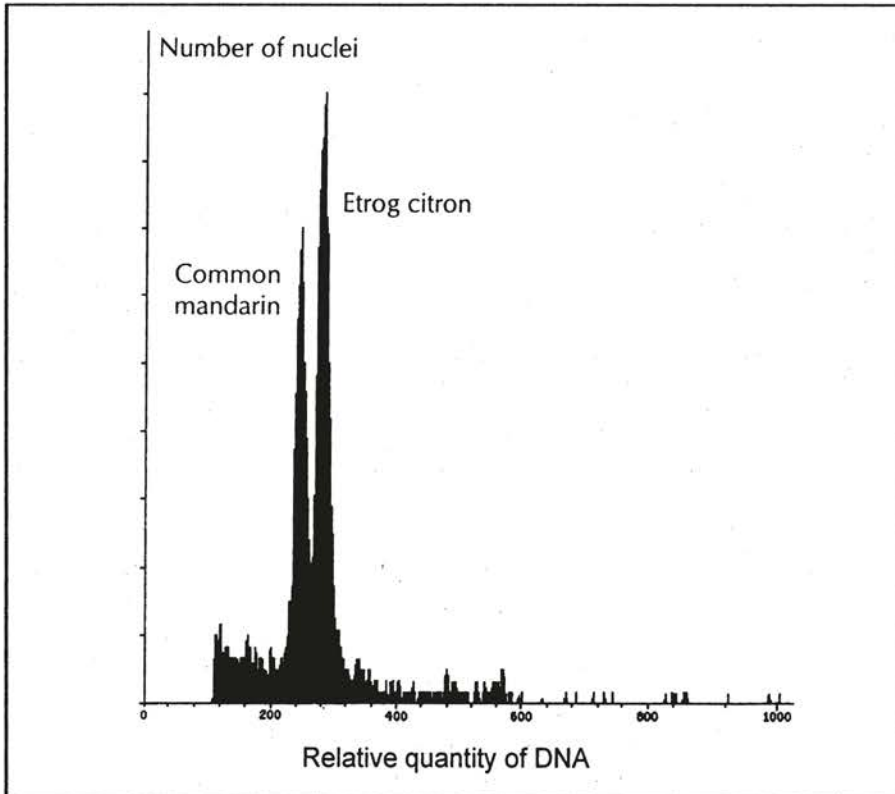


Fig. 5. Relative sizes of nuclear genomes of Etrog citron and common mandarin: flow cytometry of a mixture of nuclei stained with propidium iodide.

RELATIONS BETWEEN THE DIFFERENT LEVELS OF VARIABILITY

Analysis of morphological diversity from 20 vegetative descriptors allows us to find the overall organization around three gene pools previously

identified according to the isozymic data (Fig. 6). The relative positions of cultivated species around these three axes are in the conserved set. On the other hand, the monomorphic species in the enzymatic sense present a morphological dispersal equivalent to that of polymorphic species in the molecular sense (Fig. 7). Two levels thus coexist in the organization of morphological diversity: one major level, which responds to the constraints affecting the evolution of the genome as a whole, and a secondary level, dissociated from the molecular evolution visualized by the isozymes.

Interspecific Organization

Except for the system of gametophytic self-incompatibility, there is no sexual incompatibility within the genus *Citrus*: hybrids are obtained easily for all the interspecific combinations. The notion of specific differentiation could thus be called into question. Nevertheless, this genus seems to be very highly organized to the extent that generalized gametic disequilibrium has been identified for the isozymes and to the extent that the major axes of molecular and morphological structuration appear similar. This indicates an organization into sub-populations between which the gene flows are limited, as confirmed by the deviations from the panmixia observed for almost all loci.

The organization of *Citrus* diversity around three taxa (*C. reticulata*, *C. medica*, and *C. maxima*) confirms the results of numerical taxonomy of Barret and Rhodes (1976), which have suggested that these taxa were the origin of the cultivated *Citrus* group. It is also in agreement with total protein analysis (Handa et al., 1986), isozyme analysis (Herrero et al., 1996, 1997), RFLP and RAPD analysis (Luro et al., 1994a; Federici et al., 1998; Nicolosi et al., 2000) and STMS analysis (Luro et al., in press). The differentiation between these sexually compatible taxa can be explained by foundation effect in three geographic zones and by an allopatric evolution. The pummelos originated in the Malay Archipelago and Indonesia, the citrons evolved in northeastern India and the nearby regions of Burma and China, and the mandarins were diversified over a region including Vietnam, southern China, and Japan (Webber, 1967; Scora, 1975).

The other cultivated species—sweet orange, sour orange, lemon, grapefruit, lime—appeared subsequently by recombinations among the basic taxa, which came into contact during the course of trade and migrations. The enzymatic data—generally high heterozygosity and absence of intervarietal polymorphism, confirmed recently with STMS (Luro et al., in press)—prove that there are typical cases of false species, in which varietal diversification is produced from an ancestral hybrid by accumulation of mutations without the intervention of sexual recombination. It is to be noted that all the cultivars of these species are polyembryonic, which allows us to fix the heterozygosity and to conserve the morphological and pomological type even without manual methods of vegetative propagation, such as layering, budding, or grafting.

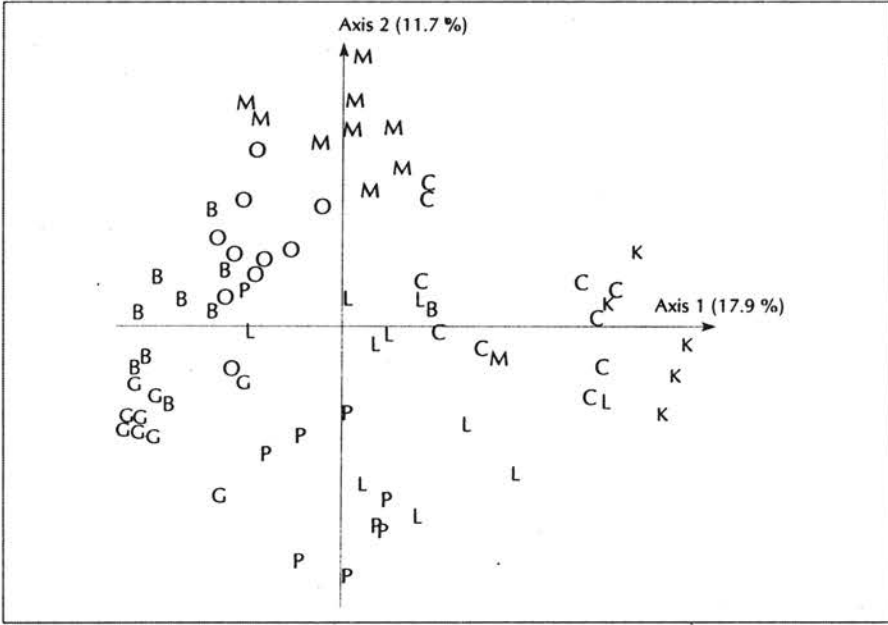


Fig. 6. Morphological diversity: representation of the primary factorial plane of PCoA done on a Sokal and Michener matrix of distance between 74 cultivars on the basis of 20 vegetative descriptors. The codes used are the same as those in Table 1.

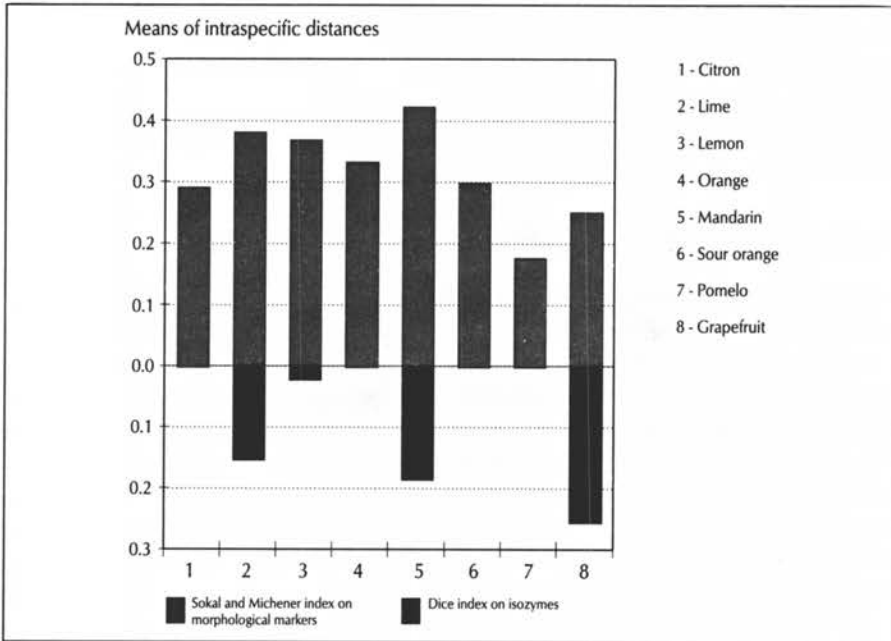


Fig. 7. Simultaneous illustration of the intraspecific dispersal calculated with the isozyme and morphological markers.

Our conclusions are in agreement with the ones obtained by isozyme analysis (Herrero et al., 1996), RFLP (Federici et al., 1998), RAPD and SCAR (Nicolosi et al., 2000), and STMS (Luro et al., in press). Sweet oranges and sour oranges are close to mandarins but have introgressed nuclear genomic fragments of pummelo. The last species also transmits its cytoplasmic genomes to sweet and sour oranges (Nicolosi et al., 2000; Ollitrault et al., 2000). Grapefruit is close to pummelo but includes nuclear genomic fragments of the mandarins/oranges group. It should have resulted from a hybridization between pummelo and sweet oranges introduced in the Caribbean islands after the discovery of the New World by Christopher Columbus. The genetic relationship between citron, limes, and lemons is clearly established by morphological and nuclear molecular markers. Synthesis of nuclear and cytoplasmic data (Ollitrault et al., 2000) indicated that mandarin and pummelo gene pools also contribute to lemon genesis. Nicolosi et al. (2000) suggested that it should result from a hybridization between citron and sour orange. Lime is the only cultivated species for which there is evidence of interspecific origin between cultivated and non-cultivated taxa; it should result from a hybridization between citron and *C. micrantha* (Nicolosi et al., 2000).

The strong organization, still observed today at the molecular rather than morphological scale indicates that the genetic exchanges between the three original groups are limited. The partial apomixis, linked to the polyembryony, has certainly been an essential element in the limitation of gene flows. Other factors, such as the structural differentiation of genomes, have also favoured the maintenance of gametic disequilibrium by limiting recombination on large portions of the genome. This differentiation in genome size is in agreement with the cytogenetic observations of Nair and Randhawa (1969) and of Raghuvanshi (1969). It testifies to the advanced state that the three basic taxa have reached on the way to real speciation.

Intraspecific Diversification

Intervarietal morphological polymorphism, relatively significant within sweet orange, sour orange, grapefruit, lemon, and lime, is explained largely by human selection. This is particularly marked for the pomological and phenological criteria. It can lead to a rapid morphophysiological evolution, independent of the molecular evolution analyses using isozymes. The most obvious example is that of the clementine. Appearing about a century ago in a seedling of common mandarin planted by Father Clement, it has since been considerably diversified. This diversification, result of a simple selection of bud mutations in the orchard, involves precocity—the period of production today extends from October to March—as well as pomological characters such as calibre, colour, and the presence of pips (Bono et al., 1982).

Over a much longer period, sweet oranges have diversified in the same way. This species, for which molecular studies with isozymes, RAPD

(Luro et al., 1994a), and microsatellites (Luro et al., 1995, in press) have not displayed any intervarietal polymorphism, is, however, highly polymorphic for morphological and phenological characters. Even though its introduction in the Mediterranean Basin is relatively recent (around the year 1000), this area constitutes the main centre of diversification, where all the main types of modern sweet oranges have been selected, such as common oranges, blood oranges, and navel oranges (Aubert, in press).

On the other hand, sexual recombination has also played a determining role in the diversification of pummelo, of which the cultivars are all monoembryonic, and of mandarin, certain cultivars of which are monoembryonic. These two have high intervarietal isozymic polymorphism without significant difference in the panmixia.

GENETIC RESOURCE MANAGEMENT

The situation of citrus illustrates the uses and limitations of molecular markers in the construction of core collections. In the evolution of the genus *Citrus* we find factors that, on the global scale, show a good correlation between organization of the phenotypic diversity and organization of the molecular diversity (foundation effect, allopatric evolution, and limitation of gene flow that allow the maintenance of global gametic disequilibrium). For the secondary species, there are also, on the intraspecific scale, evolutionary mechanisms, such as somatic reproduction and strong selection pressures on the mutations affecting morphophysiological characters, which lead to dissociation of the two levels of evolution. In the case of citrus, the chief utility of the marking studies lies in the identification of sequences and evolutionary factors at the origin of taxa and their diversification. Studies on the constitution of a core collection must thus be based more on this general information than on the allelic constitution of individuals.

Among the three basic species, pummelos and mandarins have significant molecular polymorphism. Intraspecific varietal improvement can be done traditionally by sexual hybridization. The management of intraspecific genetic resources can thus be rationalized conventionally in the form of core collections. The results obtained from a collection of 100 mandarin trees indicate the existence of genetic organization on the intraspecific scale, which could help establish, among other things, a sampling strategy on the basis of molecular data.

The set of characters defining the other cultivated species—sweet orange, sour orange, grapefruit, lemon—relies on genotypes that have a relatively high heterozygosity but are stabilized by vegetative propagation. Conservation of the genetic resources of each of these species must be based on the constitution of genotype collections. This intraspecific diversity is difficult to recombine sexually for improvement of the 'species' because the characters

defining the 'species' are thereby recombined. The genotype collections, which aim to conserve the widest adaptive diversity and morphological diversity within each 'species', help inform citrus farmers about cultivars best adapted to particular regions. Classical molecular markers (isozymes, STMS, RFLP, RAPD) offer no information at this level, given the mechanisms of intraspecific evolution described earlier; the stratification must be based mainly on geographic criteria and agromorphological data.

When we discuss citrus diversity in general, genetic resource management can be rationalized also in terms of gene conservation. The three taxa identified as being the origin of most of the cultivated forms thus constitute an essential reservoir since a large part of the allelic diversity exists at the intercultural level. The mandarins and pummelos seem in this case to be more important in the conservatories. The limes group displaying important genotypic diversity as well as the evidence of the contribution of a fourth taxon (probably *C. micrantha*; Nicolosi et al., 2000) must also be preserved on a priority basis. Moreover, as our study has shown, certain non-cultivated citrus carry a rich allelic diversity. These taxa thus are not particular genotype combinations arising from hybridization between the three basic taxa of the cultivated forms. It seems essential to conserve them, particularly because they may contribute tolerances to biotic or abiotic factors in the process of stock improvement. Finally, the development of biotechnologies, particularly somatic hybridization, considerably enlarges the gene pool that can be used for the breeding (Grosser et al., 2000). It is thus advisable today to conserve the genetic resources of citrus at the level of the tribe Citreae.

APPENDIX

Plant Material

Seventy-four cultivars representing the 8 species cultivated for their fruits (Swingle and Reece, 1967) and 17 non-edible types, some of which are used as stock, served as the basis of the enzymatic study (Table 1). To the extent possible, 10 cultivars were retained for each species cultivated, with the exception of citron, for which we had only 4 genotypes available in the collection. The trees, protected from any viral or viroidal disease, were cultivated at the agronomic research station of INRA and CIRAD of San Giuliano, in Corsica. Ninety of these genotypes were the subject of a morphological description.

Enzymatic Analyses

Nine enzymatic systems were analysed by electrophoresis on starch gel or polyacrylamide gel (Ollitrault et al., 1992): alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH), shikimate dehydrogenase (SKDH), phosphoglucomutase (PGM), phosphoglucoisomerase (PGI), peroxydases (PER), leucine aminopeptidase (LAP), and aspartate aminotransferase (AAT). For the locus *PGM-2*, only two allele positions were retained. For the other systems, the interpretation and allelic nomenclature were the same as those of Ollitrault et al. (1992) and were in accordance with the interpretation given by Torres et al. (1978, 1982) for MDH, IDH, PGI, and LAP.

Flow Cytometry Analysis

The nuclear genome size of each of the diploid genotypes was estimated by the mean of three measurements relative to that of a triploid cultivar (Tahiti lime), used as an internal control. Leaf pieces of the sample and of the control were prepared in mixtures and coloured with propidium iodide according to the protocol described by Ollitrault et al. (1994). Two thousand nuclei were then analysed on a Fascal cytometer. The nuclear genome size of each genotype was estimated in picograms per diploid genome from the mean of relative values multiplied by 1.17 pg, which corresponds to the genome size of Tahiti lime estimated by Ollitrault et al. (1994).

Morphological Studies

Twenty qualitative descriptors of the vegetative parts (Table 3) were studied. The set of data on the morphology of citrus was managed by the computerized database system for the citrus germplasm network EGID (Cottin et al., 1995).

Table 3. The twenty qualitative morphological descriptors

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| <p>A. Shape of tree</p> <ol style="list-style-type: none"> 1. Erect 2. Spheroid 3. Flat ellipsoid <p>B. Position of branches</p> <ol style="list-style-type: none"> 1. Erect 2. Spread out 3. Drooping 4. Weeping <p>C. Density of foliage</p> <ol style="list-style-type: none"> 1. Sparse 2. Dense <p>D. Surface of trunk</p> <ol style="list-style-type: none"> 1. Smooth 2. Rough <p>E. Colour of leaf surface</p> <ol style="list-style-type: none"> 1. Light green 2. Green 3. Dark green <p>F. Colour of underside of leaf in relation to leaf surface</p> <ol style="list-style-type: none"> 1. Identical 2. Lighter <p>G. Nerves on leaf surface</p> <ol style="list-style-type: none"> 1. Prominent 2. Not prominent <p>H. Angle of leaf base</p> <ol style="list-style-type: none"> 1. Acute 2. Obtuse <p>I. Angle of leaf tip</p> <ol style="list-style-type: none"> 1. Acute 2. Obtuse <p>J. Articulation of leaf</p> <ol style="list-style-type: none"> 1. Present 2. Absent <p>K. Attachment of petiole to branch</p> <ol style="list-style-type: none"> 1. Straight 2. Angled <p>L. Density of spines</p> <ol style="list-style-type: none"> 1. Nil 2. Low 3. Moderate 4. High | <p>M. Length of spines</p> <ol style="list-style-type: none"> 1. Nil 2. Very short (0 to 5 mm) 3. Short (5 to 15 mm) 4. Medium (15 to 40 mm) 5. Long (> 40 mm) <p>N. Shape of section of young branches</p> <ol style="list-style-type: none"> 1. Angular 2. Round <p>O. Leaf edge</p> <ol style="list-style-type: none"> 1. Crenellate 2. Dentate 3. Entire 4. Undulate <p>P. Leaf form</p> <ol style="list-style-type: none"> 1. Elliptical 2. Oval 3. Inverse oval 4. Lanceolate 5. Orbiculate <p>Q. Length of petiole</p> <ol style="list-style-type: none"> 1. Nil 2. Short (0 to 10 mm) 3. Medium (10 to 15 mm) 4. Long (15 to 35 mm) 5. Very long (>35 mm) <p>R. Shape of lamina</p> <ol style="list-style-type: none"> 1. Absent 2. Cordiform 3. Deltoid 4. Oval <p>S. Size of lamina</p> <ol style="list-style-type: none"> 1. Insignificant 2. Small 3. Medium 4. Large 5. Very large (equal to the limb) <p>T. Colour of young shoots</p> <ol style="list-style-type: none"> 1. Anthocyanate 2. Green |
|--|--|

Statistical Analyses

The parameters of genetic structuration were studied using Genepop software for analysis of deviations at panmixia, differentiation between cultivated taxa (study of allele distribution in the species by the exact test of Fisher), and gametic disequilibrium. The descriptive parameters of the diversity—total diversity, diversity between taxa, diversity between individuals, G_{ST} —are those proposed by Nei (1973). The tree representations and PCoA were done on the basis of the Dice matrix of distance for the enzymatic data and the Sokal and Michener matrix of distance for the morphological data. The trees were constructed by the neighbour-joining method with the help of Darwin software (Perrier et al., 1999).

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