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Genetic diversity in the orange subfamily Aurantioideae. II. Genetic relationships among genera and species

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Abstract Genetic relationships were studied by means of ten isoenzymatic systems, at the genus and species level, using two distances and four methods of aggregation in a germplasm collection of 198 cultivars and accessions of 54 species belonging to *Citrus* and 13 related genera. The most consistent results were obtained by the chord distance and the neighbor-joining clustering method. *Citrus* species were distributed in two main groups: the orange-mandarin group and the lime-lemon-citron-pummelo group. The species *C. halimii* and *C. tachibana* are not included in these groups. Mandarin species fall into three main subgroups: one includes *C. sinensis*; the second, *C. aurantium*, the third, small-fruit species. The citron, the pummelo and the ancient lemon subgroups form a cluster to which the species belonging to subgenus Papeda and the cultivated limes, lemons and bergamots are related. *Microcitrus* spp, to which *Severinia buxifolia* and *Atalantia ceylanica* seem to be related, cluster with the lime-lemon-citron-pummelo group while *Fortunella* is close to the orange-mandarin group. *Poncirus trifoliata*, the most important species for citrus rootstock improvement is located far from *Citrus* but connected to it through *Fortunella* spp. A broad distribution of species has been found that should be taken into account to sample new genotypes in the search of desired characters in order to fully and efficiently use genetic resources for citrus improvement.

Key words *Citrus* · *Poncirus* · *Fortunella* · *Microcitrus* · Isozymes · Phylogeny · Taxonomy · Germplasm bank

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Introduction

The protection of global biodiversity is currently a significant topic in international policy and has resulted in expanding efforts to collect and preserve wild germplasm with potential for contributing to crop improvement. The road to a continuous and sustainable increase in the prduction and quality of food is based on the protection and efficient utilization of plant genetic resources. Genetic uniformity that currently exists in many important crops like oranges make them potentially vulnerable to pathogen- or insect-caused catastrophes. New varieties with a wider germplasm base offer a more stable resistance to such phenomena, however the continuous growth of germplasm collections is challenging our ability to manage, evaluate and utilize these resources.

Citrus are one of the major fruit crops in the world and, with banana, they both are the only fruit species represented in the IPGRI's (International Plant Genetic Resources Institute) global network of field collections. Citrus, with seven collections in different countries, presents an example of regional dispersion that is closely related to the problem of their seed conservation (Frankel 1990). In fact, there are many more collections of *Citrus* dispersed all over the world: IPGRI (1986) list 66 collections on all of the continents. The challenge now is to make these resources more accesible to plant breeders. Data on the distribution of genetic variability and relationships among species could be used to design sampling strategies or core collections in which agronomically important genes would be searched for and investigated. Unfortunately, *Citrus* taxonomy is one of the most complex due to hybridization, apomixis and many centuries of cultivation. Several examples of phylogeny studies in *Citrus* on the basis of isozymes are listed in the literature (Esen and Scora 1977; Torres et al. 1978; Hirai et al. 1986). However, several limitations are commonly found: hardly any use of allele frequencies at enzymatic loci; variation in zymograms likely caused by environmental or physiological factors could be erroneously assigned to genotypic differences (Asíns et al. 1995); attention has been focused mainly on the cultivated species of *Citrus*; and intraspecies variability has generally not been taken into account.

The aim of this paper is to contribute to a better understanding of genetic relationships in *Citrus* and related genera by taking into account their pattern of variation as shown by allozyme electrophoresis (Herrero et al. 1996). This information will be used for the future planning of sampling strategies to make the use of the orange subfamily genetic resources more efficient.

Materials and methods

The plant material studied belongs to the Citrus Germ plasm Bank at IVIA (Valencia, Spain) and is shown in Table 1. It consists of 198 cultivars and accessions of 54 species of *Citrus* and 13 related genera of the Aurantioideae subfamily. They are mature, virus- and virus-like-free plants grown in containers kept in a screenhouse (Navarro et al. 1988). Ten isoenzymatic systems were assayed following methods described in Asíns et al. (1995). These systems are: isocitrate dehydrogenase (IDH), phosphoglucoisomerase (PGI), leucine aminopeptidase (LAP), phosphoglucomutase (PGM), glutamate-oxaloacetate transaminase (GOT), superoxide dismutase (SOD), 6-phosphogluconate dehydrogenase (6PG), aconitase (ACO), malate dehydrogenase (MDH) and peroxidase (PRX).

The genetic relationships were studied at the genus and species level. Two measures of distance were used: the chi-square distance (Benzecri 1970), abbreviated d2, and the chord distance (Cavalli-Sforza and Edwards 1967), designated as dC. d2 was calculated taking into account not only the alleles of each entry for the seven loci *Idh*, *Pgi-2*, *Lap*, *Pgm-1*, *Got-1*, *Got-2* and *MnSod* for which the genetic control is known, but also the isozyme patterns for the ACO-2, 6PG, MDH and PRXa systems (see Herrero et al. 1996 for alleles and patterns descriptions). dC only uses gene frequencies; so it was calculated using the information from the seven aforementioned loci. Two types of graphic representations of relationships were used: minimum spanning trees (MST) and dendrograms. MSTs (Prim 1957) were constructed for genera and species using distances d2 or dC. Three types of dendrograms were obtained: one using the chi-square distance and the Ward's method of aggregation (Ward 1963); the other two dendrograms were based on the chord distance but used two methods of aggregation: UPGMA (unweighted pair-group method with arithmetic average; Sneath and Sokal 1973) and Neighbor-joining (Saitou and Nei, 1987). To evaluate how well the inherent structure in the data is preserved by the classification procedure; i.e. how well the dendrograms and the original distance matrices match, we calculated the cophenetic correlation coefficient (Lapointe and Legendre, 1992).

Results

The highest cophenetic correlation coefficient among the three combinations "type of distance-aggregation method" was obtained by the Chord distance and the neighbor-joining clustering method (0.95). Furthermore, when different samples of observations (accessions, species or genera) were used, the least affected representations of relationships by the sampling were the Neighbor-joining dendrograms based on the Chord distance.

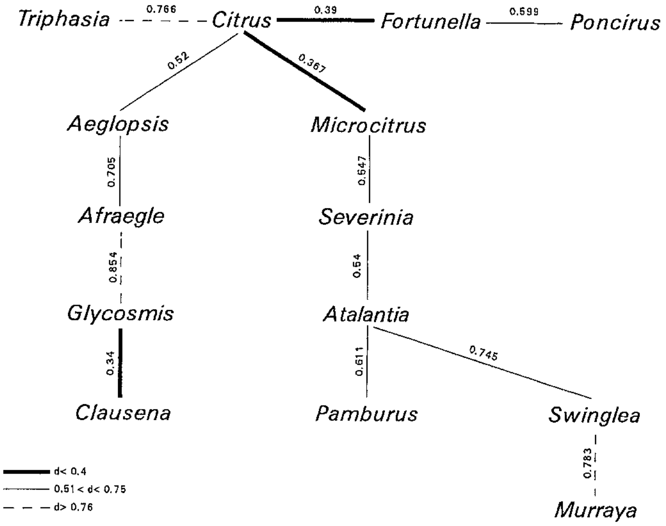
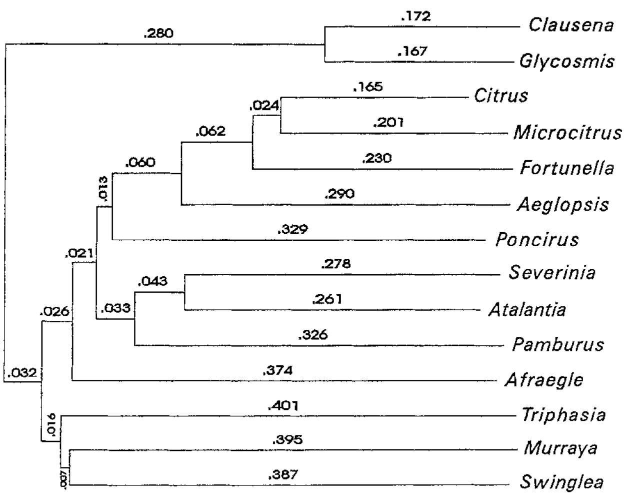


Fig. 1 Minimum spanning tree of genera based on the chord distance

The minimum spanning tree of genera based on dC distance is represented in Fig. 1. When MSTs are based on the chi-square distance (data not shown) all genera are related to *Citrus* (except *Clausena*), with *Microcitrus* and *Fortunella* being the closest genera to *Citrus* while *Afraegle*, *Glycosmis* (related to *Clausena*) and *Triphasia* are the most distant ones. The dendrogram of genera based on dC distance and the neighbor-joining clustering method (Fig. 2) depicts similar genetic relationships as the MST based on dC distance.

At the species level, Fig. 3 shows the minimum spanning tree using the chi-square distance (d2). Similar to what was observed at the genus level, the species cluster around the most represented one, *C. sinensis* (see Table 1 for species abbreviations). When dC is used the *Citrus* species appear to be distributed along

Fig. 2 Dendrogram of genera based on the chord distance and the neighbor-joining clustering method



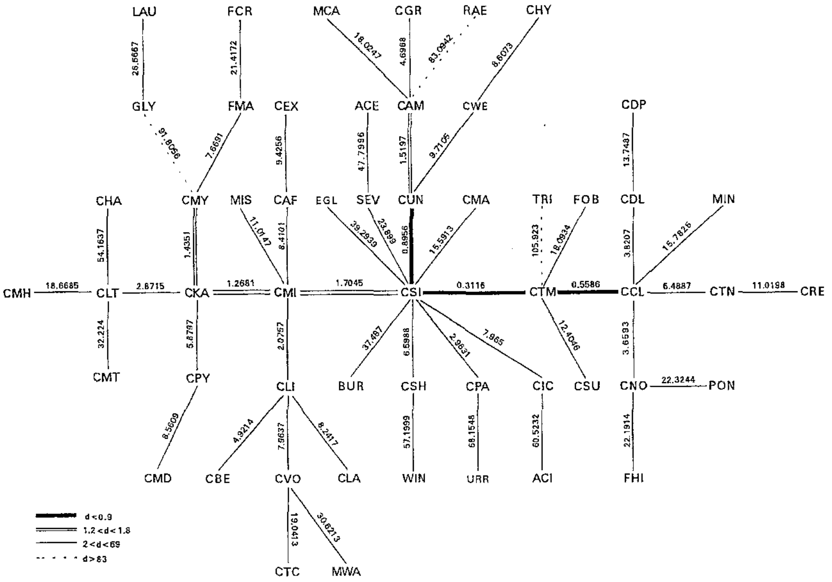


Fig. 3 Minimum spanning tree of species using the chi-square distance

three zones (Fig. 4). First, from *C. sinensis* to the right of the diagram, where an association among *C. sinesis*, *C. aurantium*, *C. myrtifolia* and mandarins can be observed. Secondly, the lemons, limes, bergamotes and citron at the left part of the figure and, thirdly, from *C. sinensis* downwards, the pummelos and grapefruits. Every *Citrus* species is directly connected to each other, except for *C. halimii*, which is joined to a *Fortunella* species. *C. tachibana* is separated by a long distance from the rest of *Citrus* species. With regard to species not belonging to *Citrus*, *Fortunella* species are related to the orange-mandarin group, *Aeglopsis chevalieri* is near to the pummelo group and *Severinia buxifolia* together with the *Microcitrus* and *Atalantia* species is connected with the citron-lime group.

The relationships shown by the neighbor-joining dendrogram based on dC distance (Fig. 5) were similar to those shown by the minimum spanning tree. Two principal groups of *Citrus* species are clearly defined: the orange-mandarin group and the lime-citron-pummelo group in which the pummelo and the lime-lemon-citron group cluster. *C. tachibana* is located outside of these two citrus groups, and *C. halimii* is closer to *Fortunella* species than to the other *Citrus* spp.

The relationships among species were also studied considering only those species with more than 1 cultivar (data not shown). Differences were observed only with respect to the relative position of the subgroups. The main difference was the location of the pummelo subgroup, that then is placed apart from the other subgroups.

Discussion

Distances and dendrograms

Two kinds of distances were measured in this work, each with a different set of data. Chi-square distances (d2) were calculated using information obtained from the ten isoenzymatic systems, i.e. alleles and patterns. Chi-square distance is very sensitive to differences in rare alleles or patterns, and in such cases d2 will not produce clear groupings due to the high genetic diversity of the plant material studied (Herrero et al. 1996). On the other hand, when the distances among hybrids of known origin and their parental genotypes were studied, d2 gave good results for those genotypes that are very closely related because this distance uses more information. An important advantage of dC is that it is not heavily influenced by high levels of within-taxon heterozygosity or variability (Swofford and Olsen 1990), as shown in the Aurantioideae subfamily (Herrero et al. 1996). Another advantage of dC is that it incorporates a number of realistic assumptions about the nature of evolutionary change in gene frequencies without assimilating the undesirable properties of other measures (Swofford and Olsen 1990).

The neighbor-joining clustering method was the best way to construct the dendrograms given that their distances correlated most with the original matrix of distances. The UPGMA aggregation method has been previously used for phylogeny inference in Aurantioide (Yamamoto et al. 1993). While the UPGMA method is considered to be one of the most powerful methods of clustering, the neighbor-joining method reproduces better the original matrix of distances (Nei 1991). Moreover, the neighbor-joining method assumes that evolution rates can vary among taxa, and this is an important consideration to bear in mind in *Citrus* and related genera because important differences regarding evolution between wild and cultivated species or sexual and apomictic species do exist.

Genetic relationships among non-*Citrus* species

When our results were compared with the known taxonomy of Aurantioideae (Swingle and Reece 1967), a

Table 1 Plant material

Genus	Species	Common name	Species code	Number of cultivars
Citrus	*C. aurantifolia* (Christm.) Swing.	Mexican lime	CAF	1
	C. aurantium L.	Sour orange	CAM	5
	C. bergamia Risso & Poit.	Bergamot	CBE	4
	C. clementina Hort. ex Tan.	Clementine mandarin	CCL	33
	C deliciosa Ten.	Mediterranean mandarin	CDL	3
	C. depressa Hay.	Shekwasha	CDP	1
	C. excelsa Wester	Royal lemon	CEX	1
	C. grandis (L.) Osb.	Bummelo	CGR	3
	C. halimii B.C. Stone		CHA	1
	C. hystrix D.C.	Mauritius Papeda	CHY	1
	C. ichangensis Swing.	Ichang papeda	CIC	1
	C. karna Raf.	Kharna	CKA	1
	C. latifolia Tan.	Tahiti lime	CLA	3
	C. limettioides Tan.	Palestina sweet lime	CLT	2
	C. limon (L.) Burm. f.	Lemon	CLI	19
	C. macrophylla Wester	Alemow	CMH	1
	C. macroptera Montr.	Melanesian papeda	CMT	1
	C. madurensis Lour.	Calamondin	CMA	2
	C. medica L.	Citron	CMD	4
	C. meyeri Y. Tan.	Meyer lemon	CMI	1
	C. myrtifolia Raf.	Chinotto	CMY	2
	C. nobills Lour.		CNO	1
	C. paradisi Macf.	Grapefruit	CPA	7
	C. pyriformis Hassk.	Bonderosa lemon	CPY	1
	C. reshni Hort. ex Tan.	Cleopatra mandarin	CRE	1
	C. shunkokan Hort. ex Tan.	Shunkokan	CSH	1
	C. sinensis (L) Osb.	Sweet orange	CSI	59
	C. sunki Hort. ex Tan.	Sunki mandarin	CSU	1
	C. tachibana (Mak.) Tan.	Tachibana	CTC	1
	C. tangerina Hort. ex Tan.		CTN	1
	C. temple Hort. ex Y. Tan.	Temple mandarin	CTM	1
	C. unshiu (Mak.) Marc.	Satsuma mandarin	CUN	9
	C. volkameriana Ten.	Volkamerian lemon	CVO	1
	C. webberi Wester	Kalpi	CWE	1
Aeglopsis	*Aeglopsis chevalieri* Swing		EGL	1
Afraegle	*Afraegle paniculate* (Schum.) Engl.		RAE	1
Atalantia	*Atalantia ceylanica* (Am. Oliv.	Ceylon atalantia	ACE	1
	Atalantia citroides Pierre ex Guill.	Cochinchine atalantia	ACI	1
Clausena	*Clausena excavata* Burm. f.		LAU	1
	Fortunella crassifolia Swing.	Meiwa kumkat	FCR	1
Fortunella	*Fortunella hindsii* (Champ.) Swing.	Hong Kong wild kumquat	FHI	1
	Fortunella margarita (Lour.) Swing.	Oval kumquat	FMA	1
	Fortunella obovata Tan.	Changshou kumquat	FOB	1
Glycosmis	*Glycosmis pentaphylla* (Retz.) Corr.		GLY	1
	Microcitrus australasica (F. Muell.) Swing.		MCA	1
Microcitrus	*Microcitrus australis* (Planch.) Swing.		MIS	1
	Microcitrus inodora (F.M. Bail.) Swing.		MIN	1
	Microcitrus warburgiana (F.M. Bail.) Tan.		MWA	1
Murraya	*Murraya paniculata* (L.) Jack.	Orange jessamine	URR	2
Pamburus	*Pamburus missionis* (Wight) Swing.	Pamburus	BUR	1
Poncirus	*Poncirus trifoliate* (L.) Raf.	Trifoliate orange	PON	2
Severinia	*Severinia buxifolia* (Poir.) Tenore		SEV	2
Swinglea	*Swinglea glutinosa* (Bl.) Merr.	Tabog	WIN	1
Triphasia	*Tripphasia trifolia* (Burm. f.) P. Wils.		TRI	1

clear association was found among all genera belonging to the "true citrus fruit group" of subtribe Citrinae (*Citrus*, *Microcitrus*, *Fortunella*, *Poncirus*). Sexual compatibility exists among all these genera (Grosser and Gmitter 1990), and abundant evidence of their relationship can be found in the literature with respect to morphology (Barrett and Rhodes 1976), genome organization (Iwamasa and Nito 1988), crossing aptitude (Iwamasa et al. 1988) and isozymes (Rahman et al. 1994). *Microcitrus* is closer to *Citrus* than *Fortunella*, and its proximity is also found using the d2 distance. However, *Microcitrus* species have their area of distribution in northern Australia and in the south of New Guinea, thereby differing from the rest of the true citrus fruit trees studied, which are located in South-east Asia. *Microcitrus* is believed to have been isolated from the rest of genera within the true citrus group for 20 or 30 million years. A more realistic situation is found when

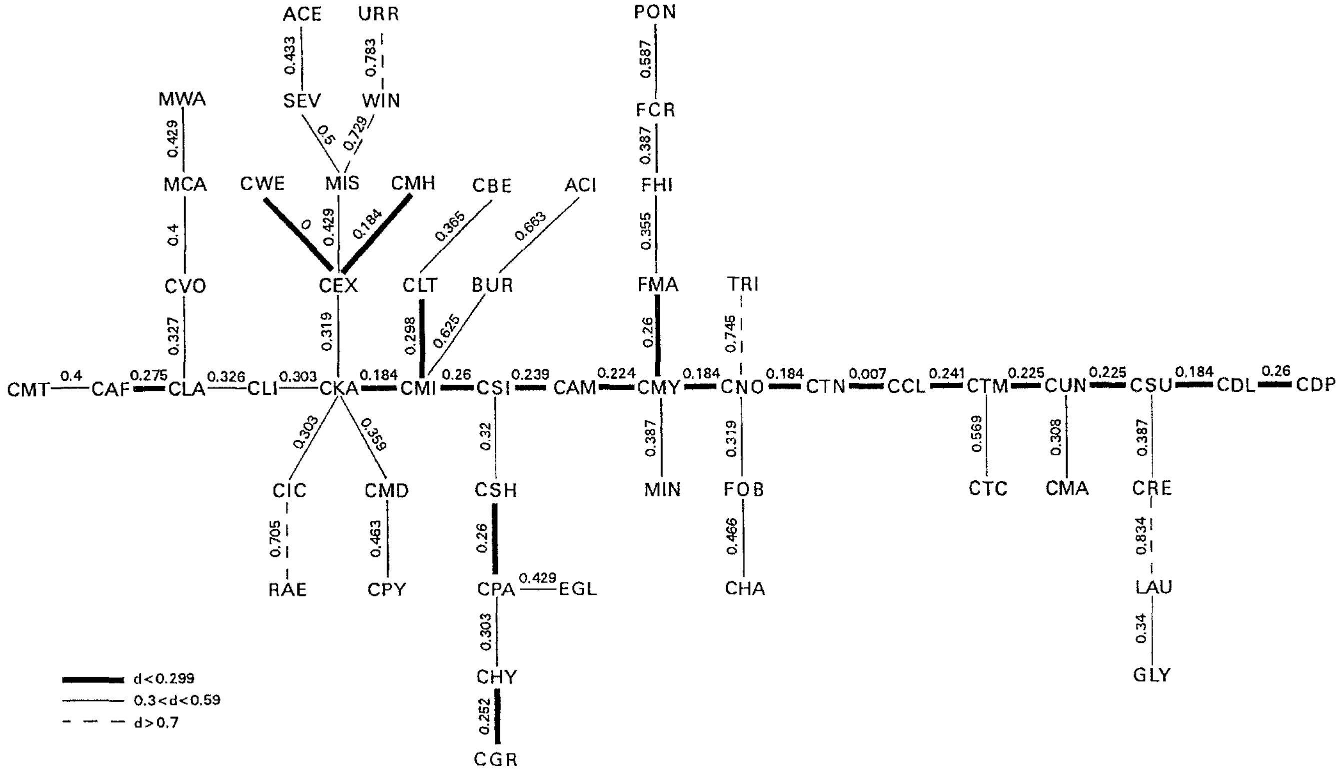


Fig. 4 Minimum spanning tree of species based on the chord distance

the species are considered separately given that the *Citrus* genus is very wide from a genetics point of view. Thus, *Microcitrus* spp are related to the citron-lime group, while *Fortunella* spp are related to the orange-mandarin group. Kozaki and Hirai (1981) also observed this connection of *Microcitrus* when examining pollen morphology. *M. australasica* and *M. warburgiana* are the closest to the lime-lemon-citron group. Other authors have also reported a relationship between these 2 species and the citron (Swingle and Reece 1967; Barrett and Rhodes 1976) and between *Fortunella* and mandarin species (Barrett and Rhodes 1976; Handa and Oogaki 1985; Scora 1988).

Poncirus, although included in the true citrus group, is located a large distance from the rest of genera of this group according our results, as was also observed for isozymes (He et al. 1988) and other characters such as restriction patterns of organular DNA (Green et al. 1986; Yamamoto et al. 1993) and morphological characters (Barrett and Rhodes 1976). Swingle and Reece (1967) provide more evidence about the separation of this genus: like the existence of deciduousness, absent in Citrinae; its area of distribution in northern China; and its flowering period not being coincident with that of *Citrus*. This geographical and sexual isolation would explain the separation of *Poncirus* from the true citrus group. *Poncirus* has been traditionally used in citrus breeding due to such important characters as disease resistances, dwarfing induction and cold resistance. The connection found between *Poncirus* and *Fortunella* suggest the study of *Fortunella* spp (much closer to citrus) to search for genes controlling those important characters.

The location of *Severinia* and *Atlantia* in the dendrogram is consistent with the taxonomic classification (Swingle and Reece 1967). As has been previously found (Herrero et al. 1996), *Atalantia* is one of the most variable genera, even for heterozygosity. Both *Atalantia* species are very distantly placed; thus *Atalantia ceylanica* clusters with *Severinia buxifolia* while *Atalantia citroides* clusters with *Pamburus missionis* (Fig. 5). The complexity of *Atalantia* was previously shown by Swingle and Reece (1967) who separated its species into two subgenera: Euatalantia, to which *A. citroides* belongs, and Rissoa, which includes *A. ceylanica*. On the other hand, *Severinia* species were considered to be species of *Atalantia* for many years; this fact and the high heterozygosity of *A. ceylanica* make us hypothesize a hybrid origin for the latter. Swingle and Reece (1967) did not see clearly the relationship between *Pamburus* and the other genera of its subtribe (Triphasinae). Its species *P. missionis* (some time ago named *Atalantia missionis* (Wt.) Oliv.) is related to subtribe *Citrinae* according to our results, given its proximity to *Atalantia citroides*. *P. missionis* grows commonly in the dry region of Ceylon and in the flat sandy coastal lands of Madras State in India, showing xerophytic structures. *Severinia buxifolia*, which presents graft compatibility with *Citrus*, also shows important adaptative traits such as resistance to boron and injurious saline solutes (Swingle and Reece 1967) in addition to being resistant to citrus tristeza virus. Therefore, we consider these 3 somehow related genera, *Atalantia*, *Severinia* and *Pamburus*, to be impor-tant genetic resources for *Citrus* rootstock breed-

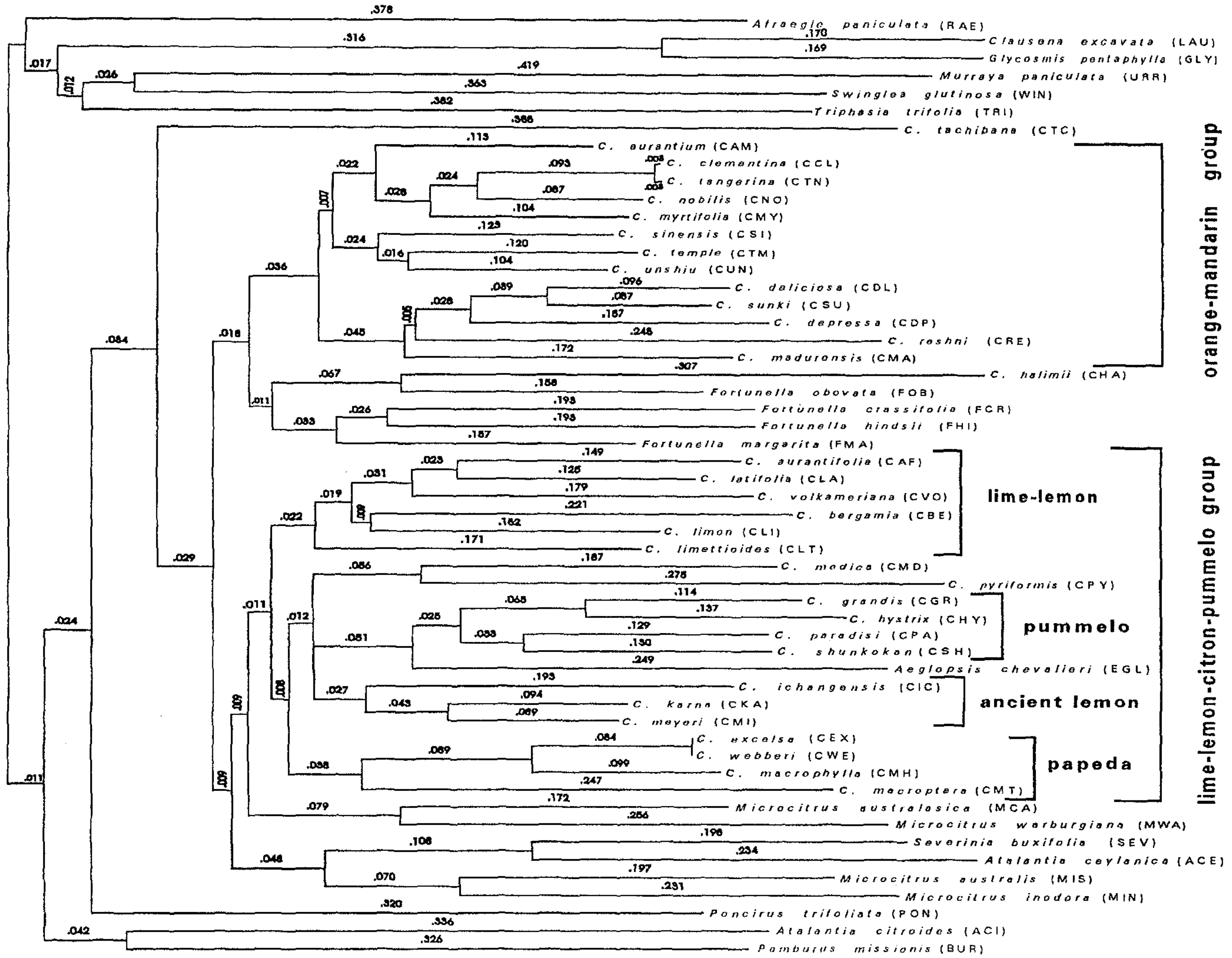


Fig. 5 Dendrogram of species based on the chord distance and the neighbor-joining clustering method

ing. Their sexual isolation could be overcome by the use of protoplast fusion methodologies or by trying to use *Microcitrus* spp (*M. australis* for instance) as a bridge species towards *Citrus*.

Microcitrus may have an interest in citrus breeding given the wide range of ecological adaptations of this genus (Swingle and Reece 1967) and its genetic proximity to *Citrus* explains its easy hybridization with *Citrus*. More studies are needed to clarify the genetic relationships of *Aeglopsis*. If the proximity of this genus to *Citrus* is confirmed, it could be a very useful genetic resource for *Citrus* improvement. Therefore, a new view of the taxonomy of subtribe Citrinae is reported here with respect to the location of *Poncirus* in the true citrus fruit trees group and the relatedness of *Aeglopsis* to this group.

Genetic relationships among *Citrus* species

Barrett and Rhodes (1976) established three groups of *Citrus* species defined by three basic species, namely *C. grandis* (pummelo), *C. medica* (citron) and *C. reticulata* (that comprises the mandarins). Many other authors have also reported differences among these three principal groups of *Citrus* species with respect to morphological characters (Handa and Oogaki 1985), isozymes (Ollitrault 1990), mitochondrial or chloroplast DNA (Green et al. 1986; Yamamoto et al. 1993), Restriction fragment length polymorphisms (RFLPs) (Roose 1988) and random amplified polymorphic DNAs (Luro et al. 1992). We have found that these three groups are not equally separated from each other, with the citron and pummelo groups being more closely connected. Consequently, *Citrus* species can be distributed among two main groups: the orange-mandarin group and the lime-lemon-citron-pummelo group, with the citron and pummelo groups being connected. *C. halimii* and *C. tachibana* are not included in these groups. The geographic distribution of *C. halimii* in Thailand and Malaysia (Stone et al. 1974) is not coincident with the distribution of the other species of *Citrus* studied, and this species has been proposed as the fourth basic species of *Citrus* (Scora and Kumamoto 1983; Luro et al. 1992). A relationship of *C. halimii* with the species of *Fortunella* has been reported in the literature (Roose 1988; Scora

1988), and our results confirm this hypothesis (see Fig. 5). Moreover, *C. halimii*, *F. hindsii*, *F. margarita* and *C. madurensis* (closely related to *Fortunella*) show the same sensitivity reaction at the bud union when they are grafted on "Rough Lemon" after several years. The distribution of *C. tachibana* in Japan would account for its differentiation from other *Citrus* species. Swingle and Reece (1967) and Hirai et al. (1990) propose an early separation of *C. tachibana* from the *Citrus* species distributed in South-east Asia, and the unique RFLPs found in this species (Roose 1988) supports this theory. However, Swingle and Reece (1967) describe *C. tachibana* as being very similar in many of its characters to the mandarin and consider it as a satellite species of *C. reticulata*. It is noteworthy that its closest species using dC is *C. temple* (a type of mandarin) (Fig. 4).

Three subgroups were found in the orange-mandarin group. The sour orange subgroup, which includes *C. clementina*, *C. tangerina* and *C. nobilis* mandarins together with *C. myrtifolia* and *C. aurantium*. This result supports the relationship of *C. aurantium* with *C. myrtifolia* found by Swingle and Reece (1967). *C. clementina* and *C. tangerina* are very closely related, and this can be supported by data based on morphological characters (Barrett and Rhodes 1976). In fact, these mandarins are so closely related that perhaps it would be advisable to include them in the same species. The 3 species of mandarin in this subgroup were also found to be related when the chi-square distance is used (Fig. 3). As to the relationship with *C. aurantium*, *C. clementina* was proposed as a hybrid having *C. aurantium* as one of its parental species (Blondel 1978). The sweet orange (*C. sinensis*) subgroup includes *C. temple* and *C. unshiu* mandarins. These 3 species are also related by the d2 distance. Swingle and Reece (1967) had previously noticed this connection, and suggested that *C. temple* is a tangor having *C. sinensis* as its parental species. The relationship of mandarin species to both orange species could have a justification in the postulated origin of sweet and sour orange through the hybridization of *C. grandis* with mandarins (Torres et al. 178; Potvin et al. 1983); thus, sweet and sour orange would differ in the parental mandarin species (Scora and Kumamoto 1983). The third subgroup includes the rest of mandarin species. We have found no report in the literature supporting the relationship between *C. sunki* and *C. depressa*, which we have included in the third subgroup. These two species of small fruits were included in only a few studies. Another member of this subgroup, *C. reshni*, a species used as rootstock in many alkaline areas because it shows resistance to important diseases and adaptation to alkaline soils, appears to be clearly separated from the other mandarin species. This result agrees with its area of origin, India, differing from the other mandarin species studied that are native to southern China or Japan (Hodgson 1967). *C. madurensis* clusters with the third subgroup of mandarins. An origin of this species by hybridization between *C. sunki* and *Fortunella margarita* has been postualted (Swingle and Reece 1967; Barrett and Rhodes 1976; Handa and Oogaki 1985), which agrees with its inclusion into the orange-mandarin group.

The association of species included in the lime-lemon-citron-pummelo group is not a very common occurrence in the literature (Ye et al. 1981). The relationship of *C. grandis* with *C. paradisi* has also been found by other authors (Handa and Oogaki 1985; Ollitrault 1990). An origin of *C. paradisi* through hybridization of *C. grandis* with *C. sinenis* has been proposed (Swingle and Reece 1967; Esen and Scora 1977). A close relationship between *C. medica* and the ancient lemons (*C. karna* mainly) has also been found (Fig. 4), which agrees with the thesis that postulates *C. medica* as the parental species in a hybrid origin of limes and lemons (Malik et al. 1974; Barrett and Rhodes 1976; Potvin et al. 1983; Score and Kumamoto 1983; Herrero et al. 1996). Our results clearly relate *C. bergamia* with the lime-lemon cluster, which disagrees with its postulated origin from *C. aurantium* (Hodgson 1967; Hirai et al. 1986; Scora 1988).

From our results it can be deduced that the separation between subgenera Papeda and Citrus is not so clear as was assumed in the taxonomy of *Citrus*. *C. webberi*, *C. macroptera* and *C. macrophylla* form a group, while the other species of Papeda, *C. hystrix* and *C. ichangensis* locate apart from them (Fig. 5). A hypothesized hybrid origin of *C. webberi* from *C. macroptera* could acount for their association (Swingle and Reece 1967). *C. hystrix* is near *C. grandis*, and *C. ichangensis* locates apart from the other species of Papeda. Torres et al. (1978) also found important isozymatic differences among *C. hystrix*, *C. ichangensis* and *C. macroptera*.

According to our results *Citrus* spp are distributed over two main groups: the orange-mandarin group and the lime-lemon-citron-pummelo group. If sweet and sour orange are considered to be different species it does not seem logical to put all the mandarins together in a single species, *C. reticulata*, as Swingle and Reece (1967) do. The orange-fruited *Citrus* species form a compact group that we have named the orange-mandarin group, which is connected with *Fortunella* spp (also with orange colored fruits) and *C. halimii*. This situation closely resembles that found in *Lycopersicon* spp where the three red-fruited species form a natural assemblage within the genus (Bretó et al. 1993). *Aeglopsis chevalieri* is closely related to the pummelo subgroup. The citron, the pummelo and the ancient lemon subgroups form a cluster to which the species belonging to subgenus Papeda and the cultivated limes, lemons and bergamots are related. *Microcitrus* spp, to which *Severinia buxifolia* and *Atalantia ceylanica* seem to be related, are closer to the lime-lemon-citron-pummelo group than to the orange-mandarin one. These relationships provide a new view of the orange subfamily and suggest the further prospecting of certain key species such as *C. halimii*, *Aeglopsis chevalieri*, *C. ichangensis*, *C. hystrix*, *Severinia buxifolia* and *C. tachibana*. As far as citrus improvement is concerned a broad distribution of species has been found that should be taken into account when sampling genotypes in the search for new genes controlling characters such as resistance to biotic and abiotic stresses in order to fully and efficiently use citrus genetic resources.

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