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Carotenoid Diversity in Cultivated Citrus Is Highly Influenced by Genetic Factors

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Citrus fruits are complex sources of carotenoids with more than 100 kinds of pigments reported in this genus. To understand the origin of the diversity of carotenoid compositions of citrus fruit, 25 genotypes that belong to the 8 cultivated *Citrus* species were analyzed. Juice extracts of mature fruit were analyzed by high-performance liquid chromatography using a C_{30} column. The 25 citrus genotypes presented different carotenoid profiles with 25 distinct compounds isolated. Statistical analyses revealed a strong impact of genotype on carotenoid compositions. Two kinds of classifications of genotypes were performed: on qualitative data and on quantitative data, respectively. The results showed that variability in carotenoid compositions was more interspecific than intraspecific. Two carotenoids, *cis*-violaxanthin and the β-cryptoxanthin, strongly determined the classification on qualitative data, which was also in agreement with previous citrus variety classifications. These findings provide evidence that, as for other phenotypical traits, the general evolution of cultivated *Citrus* is the main factor of the organization of carotenoid diversity among citrus varieties. To the authors' knowledge this is the first study that links the diversity of carotenoid composition to the citrus genetic diversity. These results lead to the proposed major biosynthetic steps involved in the differential carotenoid accumulation. Possible regulation mechanisms are also discussed.

KEYWORDS: *Citrus*; carotenoids; HPLC analysis; juice composition; biosynthesis regulation; citrus phylogeny

INTRODUCTION

Carotenoids are major components in *Citrus* juice quality. External and juice colors are mainly due to the presence of these pigments. Carotenoids of *Citrus* juices are also involved in the prevention of chronic diseases such as certain cancers (*1*), probably because of their antioxidant properties (*2*−*4*). In addition, with more than 100 different kinds of carotenoids isolated in *Citrus*, citrus fruits are complex sources of carotenoids (*5*).

The carotenoid composition of citrus juices has been widely investigated. It has been demonstrated that the carotenoid composition of citrus juices was influenced by several factors such as the growing conditions (*5*), the geographical origin (*6*), the fruit maturity (*7*), and particularly the citrus variety (*8*−*12*). Concerning this last factor, Kato et al. (*11*) showed that mandarin and orange juices accumulated high contents of several carotenoids (violaxanthin, lutein, zeaxanthin, and β-cryptoxanthin), whereas lemon juice was poor in these components. Goodner et al. (*10*) demonstrated that mandarins, oranges, and their hybrids were quite distinct because of their β-cryptoxanthin contents. Juices of red grapefruits contained two major carotenoids: lycopene and β-carotene (*12*). Despite the carotenoid composition of some citrus varieties today being well characterized, the origin of this diversity is still not well understood. Only a few studies have compared the carotenoid composition of citrus varieties that belong to different citrus species. Consequently, very little is known about relationships between carotenoid biosynthesis and citrus genetic diversity. Further investigations dealing with genetic determinism of carotenoid content diversity in the *Citrus* genus are required to be able to manage theses traits in citrus-breeding programs.

At the biochemistry level, carotenoid biosynthesis is now well established (*13*, *14*). Carotenoids are synthesized in plastids by enzymes that are nuclear encoded (*14*). The C_{40}-carotenoid skeleton is formed by the condensation of two molecules of the C_{20}-precursor geranylgeranyl diphosphate (GGDP) to pro-

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duce the colorless 15-*cis* phytoene under the action of the phytoene synthase (PSY) enzyme. Then, in plants, two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), catalyze four consecutive desaturation steps to convert phytoene into the red lycopene. Recently, Isaacson et al. (*15*) and Park et al. (*16*) have isolated the gene that encodes the carotenoid isomerase (CRTISO), which catalyzes the isomerization of poly-*cis*-carotenoids to *all-trans*-carotenoids. Cyclization of lycopene is a branching point: one branch leads to β-carotene and the other to β-carotene. The lycopene β-cyclase (LCY-b) then converts lycopene into β-carotene in two steps, whereas the formation of β-carotene requires the action of two enzymes, lycopene ε-cyclase (LCY-e) and LCY-b (*17*). β-Carotene is converted into lutein by hydroxylations catalyzed by ε-carotene hydroxylase (HY-e) and β-carotene hydroxylase (HY-b). Other xanthophylls are produced from β-carotene with reactions of hydroxylations catalyzed by HY-b and epoxidation catalyzed by zeaxanthin epoxidase (ZEP). The carotenoid biosynthetic pathway has been known for a long time, but just a few studies have investigated the regulation of carotenoid biosynthesis in *Citrus* (*18*−*21*). It is worth noting that these works have been focused on the regulation of carotenoid biosynthesis during citrus fruit maturation. Thus, we should improve our understanding of regulation mechanisms involved in the variability of carotenoid composition of mature citrus fruits. It is important to identify which part of the diversity is related to the allelic diversity of the genes involved in biosynthetic pathway and which one is due to a diversification of the regulation.

The origin and evolution of cultivated *Citrus* have been widely investigated by numerical taxonomy based on morphological characters (*22*, *23*) and molecular marker analyses (*24*−*27*). Strong correlations have been found between phenotypical and molecular organization diversity at the interspecific level. Generalized linkage disequilibrium has resulted from the evolutive story of cultivated *Citrus* (*26*). All authors have agreed to the existence of three major basic taxa from which originated all of the cultivated forms: *Citrus medica* (citrons), *Citrus reticulata* (mandarins), and *Citrus maxima* (pummelos). The global linkage disequilibrium may be the result of an initial allopatric evolution of these three taxa and a further limitation of sexual recombination, probably due to the predominant apomixy of most varieties. *C. medica* is originated from an area covering northeastern India, Burma, and western China; *C. reticulata* from Vietnam, southern China, and Japan; and *C. maxima* from the tropical region of Malaysia and Indonesia (*28*, *29*). At the intraspecific level phenotypic diversity may have resulted from mutational events combined with sexual recombinations for the basic taxa (*C. medica* and *C. maxima* particularly). Only mutational events have been involved in the diversification of secondary species such as *C. sinensis* or *C. paradisi* (*26*). Combined with human selection and clonal multiplication, mutational events may have produced a high level of phenotypic diversity, whereas global genetic diversity analyzed with molecular markers has remained very low or negative. This was particularly true for *C. sinensis*. With regard to the carotenoid contents, which are clearly associated with the visual attractiveness of fruit, one may suppose that human selection has been an important factor in diversification process.

The objective of this paper is to analyze the organization of the diversity of carotenoid content in the *Citrus* genus in order (i) to evaluate how this organization is related, on the one hand, to the global structuration resulting from the origin of cultivated forms and, on the other hand, to more recent mutation/selection processes; (ii) to try to identify the key steps of the biosynthetic pathway involved in this organization by a stop of the pathway or by a differential level of activity; and (iii) at the end to establish potential links between the evolution of cultivated *Citrus* and the functionality of key steps of the biosynthetic pathway.

Table 1. Genotypes Used for Juice Preparation

no.	common name	Tanaka system	ICVN no.[a]
1	Willowleaf mandarin	*C. deliciosa* Ten.	0100133
2	Wase Satsuma	*C. unshiu* Marc.	0100230
3	Hansen mandarin	*C. reticulata* Blanco	0100357
4	seedless pummelo	*C. maxima* (Burm.) Merr.	0100710
5	Deep Red pummelo	*C. maxima* (Burm.) Merr.	0100757
6	Chandler pummelo	*C. maxima* (Burm.) Merr.	0100608
7	Etrog citron	*C. limonimedica* L.	0100130
8	Diamante citron	*C. medica* L.	0100540
9	Marsh grapefruit	*C. paradisi* Macf.	0100188
10	Star Ruby grapefruit	*C. paradisi* Macf.	0100293
11	Ray Ruby grapefruit	*C. paradisi* Macf.	0100604
12	Shamouti orange	*C. sinensis* (L.) Osb.	0100299
13	Sanguinelli orange	*C. sinensis* (L.) Osb.	0100243
14	Cara Cara navel orange	*C. sinensis* (L.) Osb.	0100666
15	Huang pi Chen orange	*C. sinensis* (L.) Osb.	0100567
16	Maroc sour orange	*C. aurantium* L.	0110033
17	Bouquetier de Nice	*C. aurantium* L.	0100688
18	Myrtle-leaf orange	*C. myrtifolia* Raf.	0100708
19	Eureka Frost lemon	*C. limon* (L.) Burm. f.	0100004
20	Volkamer lemon	*C. limonia* Osbeck	0100729
21	Meyer lemon	*C. meyeri* Yu. Tan.	0100549
22	Rangpur lime	*C. limonia* Osbeck	0110050
23	Mexican lime	*C. aurantiifolia* (Christm.) Swing.	0100140
24	Palestine sweet lime	*C. limettioides* Tan.	0100802
25	Clementine	*C. clementina* Hort. ex Tan.	0100092

[a] International Citrus variety numbering.

MATERIALS AND METHODS

Plant Materials and Juice Preparation. Fruitd of 25 genotypes belonging to yjr *Citrus* genus (**Table 1**) were harvested from adult trees at the last stage of fruit development [stage III (*30*)] during the 2004−2005 season. Fruit maturity depends on cultivar and climate. Fruit maturity was estimated using commercial maturity indicators (*31*, *32*). Therefore, the development stage was characterized by determining juice content, soluble solids content (SSC), titratable acidity (TA), and maturity index (SSC/TA ratio) (**Table 2**).

Fruits were provided by the germplasm collection of the Station de Recherches Agronomiques INRA-CIRAD of San Giuliano. All trees were subjected to standard cultural practices. For each of the 25 varieties, 3 individual plants, growing in the same field, were used and 15 pieces of fruit were collected from each plant on the same day as in Dhuique-Mayer et al. (*8*). Thus, 3 samples of 15 fruits were separately analyzed for each variety.

Fruit samples were immediately hand-squeezed and filtered through a stainless steel sieve with 1 mm pore size. Juice content was expressed as percentage of fruit weight. Juices were placed in sealed amber vials (15 mL) under nitrogen and kept frozen at −20 °C before analysis; storage time did not exceed 1 month. Maturity index determination was carried out on an aliquot of each fruit juice sample (15 mL). The TA of juices was determined by titration to pH 8.2 with 0.1 mol L^{-1} NaOH and expressed as percentage of anhydrous citric acid, and SSC was determined with a refractometer (Atago model, 0−32%). Maturity index was evaluated as the SSC/TA ratio.

Reagents and Standards. Extraction solvents were RPE grade hexane, ethanol, and dichloromethane from Carlo-Erba (Val de Reuil, France). Analytic solvents were HPLC grade methanol from Carlo-Erba and methyl *tert*-butyl ether (MTBE) from Sigma-Aldrich (Steinheim, Germany). Reagents for analyses were pure grade sodium chloride, sodium sulfate, magnesium hydroxide carbonate, and 0.1 N sodium hydroxide from Carlo-Erba. Standards used were purchased from Extrasynthese (Genay, France): β-carotene, β-cryptoxanthin,

Table 2. Characterization of Maturity Stage of the Genotypes Used

no.	common name	juice content (% ± SD[a])	SSC[b] ± SD	TA[c] (% ± SD)	maturity index[d] ± SD
1	Willowleaf mandarin	35.0 ± 3.2	9.9 ± 0.7	1.0 ± 0.1	10.0 ± 1.6
2	Wase Satsuma	33.2 ± 1.6	10.0 ± 0.4	1.3 ± 0.1	7.5 ± 0.3
3	Hansen mandarin	46.9 ± 2.4	9.4 ± 0.6	1.5 ± 0.1	6.2 ± 0.9
4	seedless pummelo	5.2 ± 2.0	13.1 ± 0.2	2.2 ± 0.3	6.1 ± 1.2
5	Deep Red pummelo	21.5 ± 3.7	10.0 ± 0.4	1.2 ± 0.1	8.1 ± 0.8
6	Chandler pummelo	19.4 ± 0.9	11.5 ± 0.5	1.1 ± 0.1	10.6 ± 0.3
7	Etrog citron			4.8 ± 0.5	
8	Diamante citron			5.3 ± 0.2	
9	Marsh grapefruit	31.6 ± 1.4	10.8 ± 0.2	1.6 ± 0.0	6.6 ± 0.2
10	Star Ruby grapefruit	31.9 ± 0.1	10.2 ± 1.1	1.7 ± 0.2	6.0 ± 0.0
11	Ray Ruby grapefruit	36.3 ± 0.5	8.9 ± 0.1	1.6 ± 0.1	5.7 ± 0.1
12	Shamouti orange	31.2 ± 2.5	11.3 ± 0.5	1.2 ± 0.0	9.9 ± 0.6
13	Sanguinelli orange	41.0 ± 1.5	10.2 ± 0.2	1.2 ± 0.1	8.5 ± 0.6
14	Cara Cara navel orange	44.3 ± 3.3	10.1 ± 0.1	0.9 ± 0.0	10.8 ± 0.7
15	Huang pi Chen orange	39.9 ± 3.9	7.4 ± 0.3	1.2 ± 0.1	6.4 ± 0.6
16	Maroc sour orange	24.1 ± 3.5		4.1 ± 0.3	
17	Bouquetier de Nice	18.6 ± 2.3		3.3 ± 0.2	
18	Myrtle-leaf orange	32.4 ± 1.4		3.6 ± 0.0	
19	Eureka Frost lemon	39.1 ± 0.9		4.9 ± 0.1	
20	Volkamer lemon	37.3 ± 1.5		4.5 ± 0.3	
21	Meyer lemon	41.1 ± 2.3		4.1 ± 0.1	
22	Rangpur lime	42.4 ± 1.0		5.0 ± 0.2	
23	Mexican lime	32.7 ± 1.8		7.5 ± 0.1	
24	Palestine sweet lime	33.5 ± 2.0	7.4 ± 0.3	0.0 ± 0.0	
25	Clementine	32.6 ± 2.2	10.8 ± 0.3	0.7 ± 0.0	16.3 ± 1.7

[a] SD, standard deviation. [b] SSC, soluble solid content. [c] TA, titratable acidity expressed as percentage of anhydrous citric acid. [d] SSC/TA ratio.

Table 3. Spectral Characteristics of Carotenoids Found in Juices of 25 Genotypes

			λ_{max} (nm) observed					λ_{max} (nm) literature					
no.	RT (min ± SD[a])	tentative identification		peakI	peak II	peak III	% III/II		peak I	peak II	peak III	% III/II	ref
1	15.76 ± 0.09	*cis*-apocarotenoid	cis328	408	430	458			405	430	460	10	*33*
2	16.33 ± 0.04	*cis*-neoxanthin	cis328	416	439	468	76		418	441	470	81.6	*9*
3	17.35 ± 0.04	neochrome		399	422	448	75		399.7	420.0	446.0		*10*
4	17.96 ± 0.05			400	422	448							
5	18.71 ± 0.07	*cis*-violaxanthin	cis328	412	436	464	81	cis328	414	438	466	95.1	*9*
6	19.59 ± 0.04	luteoxanthin		396	418	443	75		397	419	445	92.4	*9*
7	19.92 ± 0.04	mutatoxanthin		404	426	448	31		406.5	427.5	451.5		*36*
8	20.57 ± 0.09	lutein[b]		422	444	472	48		421	445	474	60	*33*
9	20.78 ± 0.06			407	428	451							
10	21.89 ± 0.04	zeaxanthin[b]		426	450	476	17		428	450	478	26	*33*
11	22.55 ± 0.04	*cis*-isolutein	cis330	417	440	468	47		418.7	440.5	466.8		*10*
12	24.25 ± 0.08			422	446	473	44						
13	25.40 ± 0.09		cis338	420	444	470							
14	25.89			406	428	452	40						
15	26.10 ± 0.04	α-cryptoxanthin		422	445	473	47		421	445	475	60	*33*
16	26.96 ± 0.06	phytoene		276	286	298			276	286	297	10	*33*
17	28.16 ± 0.08	*β*-cryptoxanthin[b]		427	450	477	20		428	450	478	27	*33*
18	28.59 ± 0.03	phytofluene		331	348	368	68		331	348	367	90	*33*
19	32.25 ± 0.09	*ζ*-carotene		379	400	424	90		379	400	424	85.9	*9*
20	33.35 ± 0.09	α-carotene		422	444	470	34		422	445	473	55	*33*
21	35.51 ± 0.09	*β*-carotene[b]			452	477	12		425	450	477	25	*33*
22	36.84 ± 0.09	*cis*-*β*-carotene	cis342	425	449	475		cis340	422	446	473	34	*19*
23	38.82 ± 0.04			433	456	488							
24	45.95	lycopene *cis*-isomer	cis355	441	466	490		cis358	438	465	493	45	*9*
25	55.57±0.06	lycopene[b]		446	472	502	71		448	474	506	73.7	*9*

[a] RT, retention time ± standard deviation (SD). [b] Identified using authentic standards. Solvents used were water, MeOH, and MTBE (refs *19*, *10*, and *36*); water, MeCN:MeOH (75:25), and MTBE (ref *9*); and petroleum ether or EtOH or hexane (ref *33*). Whatever the solvent, gradient programs were different. As a result, these data were used only to compare spectral characteristics found with those already described in different solvents.

zeaxanthin, lutein, lycopene, *β*-apo-8′-carotenal (purity of standards was verified by HPLC and photodiode array detection).

Preparation of Standards. Concentrations of standard solutions were calculated by spectrophotometric measurement dissolving standard with the appropriate solvent and using a molar extinction coefficient (ϵ_{mol}) (*33*). To prepare the solution of internal standard, lycopene or *β*-apo-8′-carotenal was diluted in dichloromethane to obtain a final concentration of 120 mg L^{-1} for lycopene and 260 mg L^{-1} for *β*-apo-8′-carotenal.

Carotenoid Extraction. Carotenoid extraction was carried out according to the method of Dhuique-Mayer et al. (*8*). Twenty grams of juice was stirred with 120 mg of $MgCO_3$ and 35 mL of extraction solvent (ethanol/hexane, 4:3 v/v, containing 0.1% of BHT as antioxidant) for 5 min. Lycopene (750 μL of solution, equivalent to 90 μg) or *β*-apo-8′-carotenal (150 μL, equivalent to 40 μg) was added as an internal standard. Residue was separated from the liquid phase by filtration with a filter funnel (porosity no. 2) and re-extracted with 35 mL of ethanol/hexane (4:3, v/v). The residue was washed with 30 mL

Table 4. Carotenoid Contents (Milligrams per Liter) in Juices of 25 Genotypes

		retention time												
carotenoid[a]		15.76	16.33	17.35	17.96	18.71	19.59	19.92	20.57	20.78	21.89	22.55	24.25	25.4
Willowleaf mandarin	mean	0.331	0.770	0.417	–[b]	2.389	–	–	0.670	–	0.682	0.942	0.586	0.338
	SD[c]	0.045	0.080	0.075	–	0.249	–	–	0.067	–	0.060	0.069	0.071	0.072
Wase Satsuma	mean	0.280	1.571	0.443	–	4.327	–	–	0.953	–	0.586	0.981	2.220	0.390
	SD	0.031	0.072	0.042	–	0.314	–	–	0.055	–	0.039	0.167	0.199	0.068
Hansen mandarin	mean	0.558	1.555	0.590	–	7.604	0.487	–	1.266	–	1.194	2.197	0.957	0.327
	SD	0.041	0.245	0.042	–	0.924	0.027	–	0.089	–	0.152	0.208	0.118	0.045
seedless pummelo	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Deep Red pummelo	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Chandler pummelo	mean	–	0.099	0.074	–	0.819	–	–	0.086	–	–	0.150	–	–
	SD	–	0.007	0.017	–	0.009	–	–	0.002	–	–	0.008	–	–
Etrog citron	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Diamante citron	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Marsh grapefruit	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Star Ruby grapefruit	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Ray Ruby grapefruit	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Shamouti orange	mean	0.663	2.904	1.411	0.485	10.581	0.463	0.409	1.155	–	0.853	2.594	0.176	–
	SD	0.046	0.155	0.212	0.035	1.038	0.043	0.055	0.072	–	0.065	0.437	0.036	–
Sanguinelli orange	mean	0.355	2.097	1.547	0.499	13.372	0.591	0.731	2.534	–	1.577	4.402	–	–
	SD	0.070	0.076	0.344	0.072	0.388	0.029	0.185	0.332	–	0.066	0.589	–	–
Cara Cara navel orange	mean	0.365	1.250	0.901	0.226	5.469	0.249	0.383	1.138	–	0.550	1.691	0.074	–
	SD	0.098	0.203	0.115	0.023	0.647	0.020	0.079	0.203	–	0.134	0.239	0.015	–
Huang pi Chen orange	mean	–	0.222	0.126	–	0.315	0.099	–	0.190	–	tr	tr	–	–
	SD	–	0.026	0.019	–	0.053	0.019	–	0.049	–	–	–	–	–
Maroc sour orange	mean	–	–	–	–	0.079	–	0.099	0.131	–	0.050	tr	0.075	0.062
	SD	–	–	–	–	0.023	–	0.018	0.034	–	0.011	–	0.013	0.013
Bouquetier de Nice	mean	–	–	–	–	0.400	0.082	–	–	0.132	0.092	0.255	0.129	0.104
	SD	–	–	–	–	0.050	0.008	–	–	0.027	0.014	0.043	0.037	0.046
Myrtle-leaf orange	mean	–	–	–	–	0.462	0.131	0.157	0.186	–	0.119	0.297	0.180	0.186
	SD	–	–	–	–	0.082	0.034	0.034	0.016	–	0.024	0.052	0.012	0.030
Eureka Frost lemon	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Volkamer lemon	mean	0.291	0.219	0.218	–	0.990	0.349	0.191	–	0.267	0.123	0.416	–	0.161
	SD	0.004	0.008	0.012	–	0.055	0.033	0.020	–	0.024	0.012	0.085	–	0.006
Meyer lemon	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Rangpur lime	mean	–	0.698	0.248	–	0.618	–	0.180	0.306	–	0.293	–	0.606	0.155
	SD	–	0.021	0.038	–	0.090	–	0.047	0.049	–	0.055	–	0.118	0.048
Mexican lime	mean	–	–	–	–	–	–	–	0.057	–	–	–	–	–
	SD	–	–	–	–	–	–	–	0.008	–	–	–	–	–
Palestine sweet lime	mean	–	–	–	–	–	–	–	–	–	–	–	–	–
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–
Clementine	mean	0.351	1.247	0.614	–	3.261	0.256	0.230	0.733	–	0.830	1.160	0.761	0.329
	SD	0.039	0.163	0.073	–	0.321	0.036	0.016	0.080	–	0.050	0.088	0.113	0.046
F value		127.1	304.9	56.6	202.6	257.7	347.9	46.3	141.5	194.1	223.0	99.0	161.2	78.8
P > *F* (%)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
LSD[e]		0.052	0.131	0.168	0.028	0.64	0.028	0.075	0.148	0.012	0.084	0.31	0.113	0.044

		retention time												
carotenoids[a]		25.89	26.1	26.96	28.16	28.59	32.25	33.35	35.51	36.84	38.82	45.95	55.57	total
Willowleaf mandarin	mean	–	0.298	1.126	10.287	1.267	0.465	0.133	1.781	–	–	–	–	22.481
	SD[c]	–	0.043	0.262	0.698	0.258	0.047	0.051	0.373	–	–	–	–	1.620
Wase Satsuma	mean	–	0.642	0.504	17.507	1.737	0.991	0.082	0.427	–	–	–	–	33.642
	SD	–	0.044	0.052	0.897	0.166	0.044	0.026	0.030	–	–	–	–	1.919
Hansen mandarin	mean	–	0.543	3.004	11.661	3.633	2.487	0.099	1.230	–	–	–	–	39.392
	SD	–	0.053	0.547	1.714	0.552	0.365	0.009	0.228	–	–	–	–	5.317
seedless pummelo	mean	–	–	0.138	–	0.081	0.065	–	0.329	–	–	–	0.300	0.912
	SD	–	–	0.009	–	0.007	0.007	–	0.100	–	–	–	0.102	0.189
Deep Red pummelo	mean	–	–	0.138	–	0.079	–	–	0.943	–	–	–	1.536	2.697
	SD	–	–	0.025	–	0.014	–	–	0.115	–	–	–	0.076	0.096
Chandler pummelo	mean	–	–	0.083	–	–	–	–	0.360	–	–	–	0.495	2.165
	SD	–	–	0.020	–	–	–	–	0.105	–	–	–	0.122	0.024
Etrog citron	mean	tr[d]	–	–	0.093	–	–	–	0.134	–	–	–	–	0.227
	SD	–	–	–	0.022	–	–	–	0.007	–	–	–	–	0.019
Diamante citron	mean	–	–	–	0.057	–	–	–	0.122	–	–	–	–	0.179
	SD	–	–	–	0.012	–	–	–	0.016	–	–	–	–	0.026
Marsh grapefruit	mean	–	–	tr	–	tr	tr	–	tr	–	–	–	–	tr
	SD	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 4. (Continued)

carotenoids[a]		retention time 25.89	26.1	26.96	28.16	28.59	32.25	33.35	35.51	36.84	38.82	45.95	55.57	total
Star Ruby grapefruit	mean	–	–	2.130	–	1.711	0.369	–	2.826	0.171	0.287	–	10.072	17.566
	SD	–	–	0.098	–	0.068	0.020	–	0.105	0.022	0.045	–	0.652	0.775
Ray Ruby grapefruit	mean	–	–	0.581	–	0.510	0.293	–	1.142	–	–	–	6.855	9.381
	SD	–	–	0.143	–	0.132	0.061	–	0.362	–	–	–	2.758	3.444
Shamouti orange	mean	–	0.288	0.758	2.694	0.703	0.957	0.091	0.587	–	–	–	–	27.770
	SD	–	0.036	0.082	0.362	0.084	0.125	0.024	0.053	–	–	–	–	2.064
Sanguinelli orange	mean	–	1.026	1.123	3.979	1.107	1.241	0.232	0.289	–	–	–	–	36.703
	SD	–	0.111	0.111	0.230	0.100	0.071	0.029	0.020	–	–	–	–	0.573
Cara Cara navel orange	mean	–	0.182	13.059	1.698	5.062	1.213	0.122	1.521	–	0.073	0.254	2.263	37.743
	SD	–	0.030	1.107	0.327	0.298	0.077	0.013	0.106	–	0.027	0.073	0.501	1.385
Huang pi Chen orange	mean	–	–	0.265	tr	0.191	0.255	–	0.079	–	–	–	–	1.743
	SD	–	–	0.088	–	0.053	0.066	–	0.008	–	–	–	–	0.094
Maroc sour orange	mean	–	–	–	1.415	–	0.149	–	0.203	–	–	–	–	2.263
	SD	–	–	–	0.153	–	0.018	–	0.050	–	–	–	–	0.210
Bouquetier de Nice	mean	–	–	–	2.044	–	0.151	–	0.058	–	–	–	–	3.446
	SD	–	–	–	0.266	–	0.041	–	0.008	–	–	–	–	0.497
Myrtle-Leaf orange	mean	–	–	–	3.312	–	0.192	–	–	–	–	–	–	5.224
	SD	–	–	–	0.642	–	0.030	–	–	–	–	–	–	0.918
Eureka Frost lemon	mean	tr	–	–	0.165	tr	0.126	–	tr	–	–	–	–	0.291
	SD	–	–	–	0.007	–	0.026	–	–	–	–	–	–	0.020
Volkamer lemon	mean	–	–	0.151	2.510	0.285	0.241	–	0.105	–	–	–	–	6.517
	SD	–	–	0.010	0.098	0.024	0.028	–	0.016	–	–	–	–	0.181
Meyer lemon	mean	–	–	0.097	0.863	–	0.108	–	0.192	–	–	–	–	1.260
	SD	–	–	0.016	0.046	–	0.012	–	0.032	–	–	–	–	0.067
Rangpur lime	mean	–	–	–	3.996	0.187	0.253	–	0.862	–	–	–	–	8.403
	SD	–	–	–	0.119	0.024	0.021	–	0.237	–	–	–	–	0.157
Mexican lime	mean	–	–	–	–	–	–	0.053	0.239	–	–	–	–	0.349
	SD	–	–	–	–	–	–	0.008	0.031	–	–	–	–	0.046
Palestine sweet lime	mean	–	–	–	0.280	–	–	–	tr	–	–	–	–	0.280
	SD	–	–	–	0.009	–	–	–	–	–	–	–	–	0.007
Clementine	mean	–	0.214	0.946	9.087	1.265	1.098	0.147	2.434	–	–	–	–	24.962
	SD	–	0.041	0.081	0.545	0.069	0.059	0.060	0.201	–	–	–	–	1.169
F value		200.8	247.0	316.5	62.2	212.1	155.5	39.1	86.9	1193.9	67.9	46.9	51.8	
P > *F* (%)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	
LSD[e]		0.002	0.047	0.426	0.806	0.246	0.135	0.029	0.240	0.003	0.019	0.020	0.954	

[a] Carotenoids are designated by their retention time in order of elution. [b] Not detected. [c] SD, standard deviation. [d] tr < 0.05 mg L^{-1}. [e] Least significant difference. For β-cryptoxanthin the limit of detection (LOD) is 0.0046 μg and the limit of quantification (LOQ), 0.0152 μg. Concentrations are the mean of at least three independent determinations. The 25 genotypes are significantly different for all 25 carotenoid pigments ($P < 0.01\%$).

of ethanol and with 30 mL of hexane until it was colorless. Organic phases were transferred in a separatory funnel and successively washed with 2 × 50 mL of 10% sodium chloride and 3 × 50 mL of distilled water. The aqueous layer was removed. The hexanic phase was dried using anhydrous sodium sulfate, filtered, and evaporated at 40 °C in a rotary evaporator. The residue was dissolved in 500 μL of dichloromethane and 500 μL of MTBE/methanol (80:20, v/v). This solution was diluted 6-fold in a MTBE/methanol mixture for varieties with numbers 1, 2, 3, 10−14, and 25 (see **Table 1**) and 3-fold for varieties with numbers 16−18 and 20−22. Samples were placed in amber vials before HPLC analysis.

Saponification. Hexanic extract was evaporated with a rotary evaporator, redissolved with 20 mL of hexane, and placed in a 50 mL amber vial to which was added 20 mL of 10% methanolic KOH. Saponification was performed overnight at room temperature protected from the light. The sample was shaken under nitrogen in the sealed vial. The sample was transferred to a separatory funnel to which 50 mL of distilled water was added to separate the layers. The hexanic layer was washed with distilled water until free of alkali. The methanolic KOH layer was extracted with 3 × 10 mL of dichloromethane. The extracts were pooled and washed to remove alkali. The extracts were dried using anhydrous sodium sulfate, filtered, and evaporated in a rotary evaporator. The residue was dissolved as described above. Analyses were carried out under red light to avoid carotenoid degradation during extraction and saponification.

HPLC Analysis of Carotenoids. Carotenoids were analyzed by HPLC using an Agilent 1100 system (Massy, France) according to the previously published method of Dhuique-Mayer et al. (*8*). Carotenoids were separated along a C_{30} column [250 × 4.6 mm i.d., 5 μm YMC (EUROP GmbH)]; the mobile phases were H_2O as eluent A, methanol as eluent B, and MTBE as eluent C. Flow rate was fixed at 1 mL min^{-1}, column temperature was set at 25 °C, and injection volume was 20 μL. A gradient program was performed: the initial condition was 40% A/60% B; 0−5 min, 20% A/80% B; 5−10 min, 4% A/81% B/15% C; 10−60 min, 4% A/11% B/85% C; 60−71 min, 100% B; 71−72 min, back to the initial condition for reequilibration. Absorbance was followed at 290, 350, 400, 450, and 470 nm using an Agilent 1100 photodiode array detector. Chromatographic data and UV−visible spectra were collected, stored, and integrated using an Agilent Chemstation plus software.

Identification and Quantification of Carotenoids. Carotenoids were identified using retention times, absorption spectra, and coinjection with authentic standards. The spectral fine structure value, % III/II, was calculated as the percentage of the quotient between band III and band II (λ_{max}), taking the trough between the two bands as the baseline. The UV−visible spectra and % III/II were compared with those reported in the literature. Quantification of carotenoids was achieved using calibration curves with β-carotene, β-cryptoxanthin, lutein, lycopene, and β-apo-8′-carotenal with five concentrations. Correlation coefficients ranged from 0.994 to 0.998. Other carotenoids were quantified as β-carotene. Each carotenoid was quantified using area collected at 290, 350, 400, 450, or 470 nm depending on its maximum λ. Recoveries were determined by adding internal standard (lycopene or β-apo-8′-carotenal) before the extraction of each sample analyzed and used to correct carotenoid contents after HPLC analysis. The concentration of each carotenoid was expressed as milligrams per liter. Analysis precision was checked from three consecutive extractions−saponifications−injections of one sample (corresponding to one plant), and coefficients of variation were ≤5%. Coefficients of variation obtained from three consecutive extractions−saponifications−injections

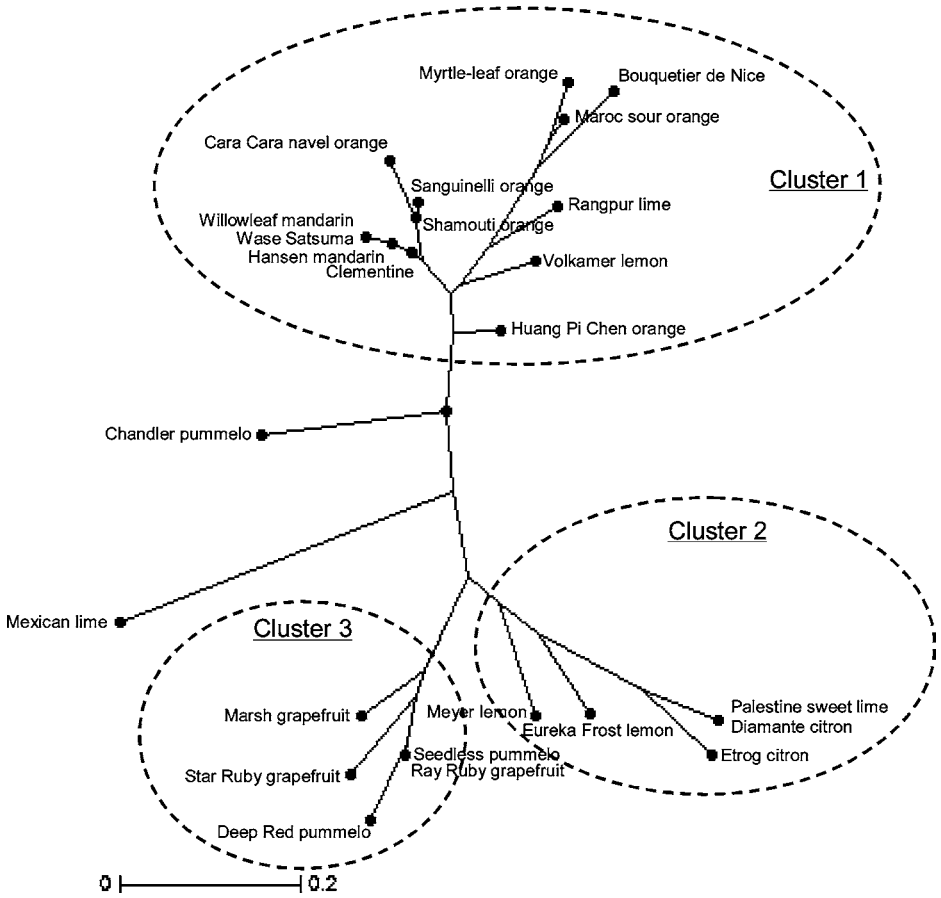

Figure 1. Diversity of carotenoid profiles of the 25 citrus genotypes on the basis of the presence or absence of carotenoids. The tree was constructed according to the neighbor-joining method using a dice matrix of dissimilarity. The three clusters that we have identified are circled.

of three samples belonging to the same variety (corresponding to three plants) were ≤20% for almost all carotenoids and ≤30% for β-carotene and lycopene of grapefruit and pummelo varieties. Concentrations are given as the mean of data from three extractions. The total contents in carotenoid pigments of juices from the 25 genotypes were calculated by summing concentrations of all compounds. Limits of detection (LOD) and quantitation (LOQ) were calculated for β-cryptoxanthin by preparing serial dilutions of this compound in mobile phase (concentrations ranging from 1 to 10 mg L^{-1}). Calibration curves and then LOD and LOQ were determined with LOD = 3 × *S*/*a* and LOQ = 10 × *S*/*a* (where *S* is the standard deviation of the blank signal and *a* the slope of the calibration curve).

Statistical Analyses. The data matrix was composed of the contents (mean of three samples) of 25 carotenoid variables and 25 genotypes. The carotenoid variables were scored as 1 for the presence and 0 for the absence for qualitative analysis. @DARwin 4.0 software (CIRAD, Montpellier, France) was used for dissimilarity analysis and tree construction. Two representations were constructed according to the neighbor-joining method and from the presence or absence of variables and a matrix of Dice's distances for the first one and from carotenoid contents and Euclidian distances for the second one. Connections between clusters and qualitative variables were analyzed according to the chi2 method using the MEANS and FREQ procedures of SAS (SAS Institute Inc., Cary, NC, 1989). The statistical comparison of data was performed by ANOVA using the GLM procedure of SAS (SAS Institute Inc., 1989) to reveal significant differences among the 25 genotypes studied.

RESULTS

Diversity of Carotenoid Composition of *Citrus* Genotypes. For each of the 25 varieties at least 3 juice samples (three plants) were analyzed by HPLC. Twenty-five carotenoids were detected, and their chromatographic and spectral characteristics are reported in **Table 3**. Variations in retention time were ≤6%, and variations in wavelength were ≤7% for all pigments. Five carotenoids were identified by comparison of their retention time and UV−visible spectra with those of standards. Others pigments were tentatively identified using spectral characteristics reported in the literature. Spectral characteristics of peaks match those reported with an average difference of ≤5%. Carotenoid contents were determined and expressed as the average concentration of three data in milligrams per liter (**Table 4**). The comparison of data by ANOVA showed that the 25 genotypes were significantly different for all carotenoid pigments ($P < 0.01\%$). Consequently, a very strong effect of genotype is found to explain the variability of each component.

Mandarins, oranges (apart from Huang pi Chen orange), and Clementines were the species richest in carotenoid contents (total contents ≥ 22.481 mg L^{-1}). Star Ruby and Ray Ruby grapefruits were also among the richest citrus varieties with high total carotenoid contents of 17.566 and 9.381 mg L^{-1}, respectively, whereas Marsh grapefruit presented only traces of phytoene, phytofluene, ζ-carotene, and β-carotene. Sour oranges and pummelos presented total contents between those of mandarins, oranges, and Clementines and those of lemons, limes, and citrons, which were the poorest in pigments (total contents ≤ 1.26 mg L^{-1}). However, Volkamer lemon and Rangpur lime were richer in pigments than sour oranges or pummelos, with 6.517 and 8.403 mg L^{-1}, respectively, as total contents. β-Cryptoxanthin, β-carotene, *cis*-violaxanthin, and lycopene appeared to widely contribute to the total amount of carotenoids, although some of them were absent in fruits of several genotypes. Thus, differences between the 25 genotypes were

qualitative and quantitative. Moreover, the analyses revealed more interspecific differences than intraspecific differences.

Organization of the Carotenoid Diversity Based on the Presence/Absence of Each Compound. The tree of **Figure 1** was constructed on the basis of the presence or absence of each carotenoid. The 25 genotypes were classified in 3 clusters. The first one comprised 13 genotypes (3 mandarins, Clementine, 4 oranges, 3 sour oranges, Rangpur lime, and Volkamer lemon). The second cluster was smaller, with 5 genotypes (2 citrons, 2 lemons, and Palestine sweet lime). The third one also contained 5 genotypes (2 pummelos and 3 grapefruits). Mexican lime and Chandler pummelo were not included in these three clusters. Clusters and carotenoid variables were analyzed according to the chi2 method to determine which pigments were mainly responsible for these clusters. Two carotenoids strongly determined this classification: *cis*-violaxanthin and β-cryptoxanthin. *cis*-Violaxanthin was present in all varieties belonging to cluster 1, whereas this pigment was absent in the varieties of cluster 2. Similarly, β-cryptoxanthin was present in all varieties belonging to clusters 1 and 2 but was absent for varieties of cluster 3. Moreover, other xanthophylls were also important for cluster 1: lutein, isolutein, and zeaxanthin. Conversely, some carotenoids did not contribute to the cluster formation such as peaks 4, 9, 14, and 21−24 (see **Table 3**). These pigments were present in only one or two varieties apart from β-carotene (peak 21), which was present in almost all of them. Thus, for qualitative data it was possible to distribute the 23 citrus genotypes in 3 main clusters. Varieties belonging to the same species were included in the same cluster except for Rangpur lime and Volkamer lemon, which were located in cluster 1 and not in cluster 2 as expected.

Organization of the Carotenoid Diversity Based on the Concentration of Each Compound. The tree of **Figure 2** was obtained on the basis of the average concentration of each carotenoid from juices of three samples. To evaluate the quantitative data impact on the classification previously obtained (**Figure 1**), we have imposed the structure of the tree of **Figure 1** for the construction of the one of **Figure 2**. It is important to note that the distance scales are not the same in **Figures 1** and **2**. Quantitative data revealed more information at the intraspecific level. Concerning the varieties that were included in cluster 1 of **Figure 1**, mandarins and Clementines were separated from oranges in **Figure 2**. Mandarins and Clementines accumulated high amounts of β-cryptoxanthin, whereas oranges accumulated high quantities of *cis*-violaxanthin (the β-cryptoxanthin/*cis*-violaxanthin ratio was $\geq$1.5 for mandarin and Clementine varieties and $\leq$0.3 for orange varieties) (see **Table 4**). Sour oranges, Rangpur lime, and Volkamer lemon were no longer in cluster 1. Huang pi Chen orange was closer to cluster 2, this genotype having a low carotenoid content in juice. β-Cryptoxanthin was the major pigment in sour oranges, Rangpur lime, and Volkamer lemon. *cis*-Violaxanthin was the major pigment in Huang pi Chen orange, but the contents of these pigments were roughly 3 times lower than for oranges, mandarins, and Clementines. It is worth noting that the repartition of the mandarin varieties in **Figures 1** and **2** does not overlap. This was due to differences in β-cryptoxanthin contents. Similarly, the spread of the group of oranges could be explained by contents in *cis*-violaxanthin. Cara Cara orange was far away from the other oranges; the accumulation of carotene such as phytoene, phytofluene, and lycopene was higher.

Concerning cluster 2, all genotypes accumulated low amounts of carotenoids, which explained why all varieties overlapped. In cluster 3, the spread of the group of pummelos and grapefruits was also greater. Two genotypes were distant: Marsh grapefruit contained only traces of pigments, which explained why it overlapped varieties of cluster 2, and Star Ruby grapefruit presented the highest content of lycopene.

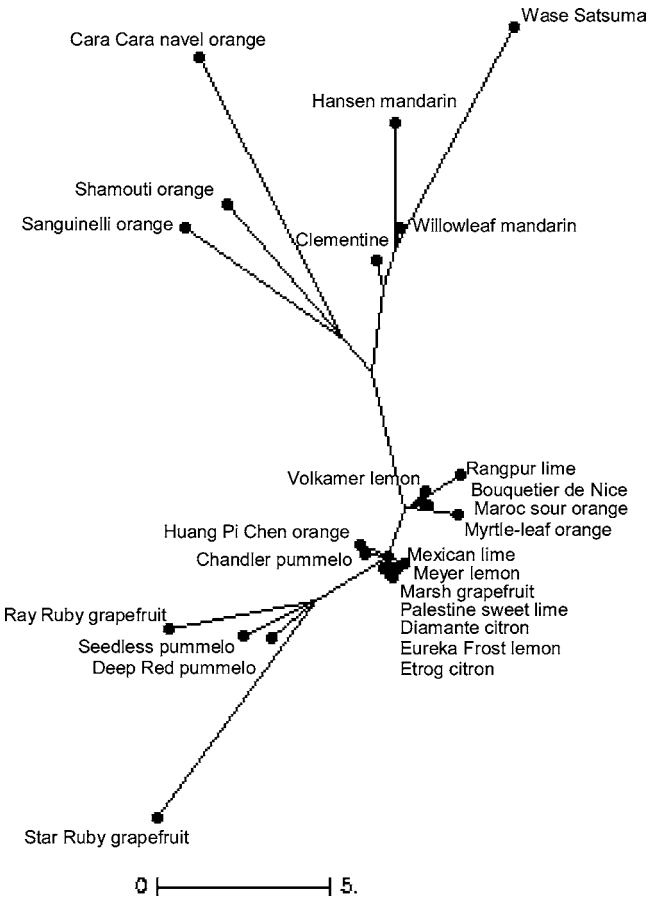

Figure 2. Diversity of carotenoid profiles of 25 citrus genotypes on the basis of carotenoid contents. The tree was constructed according to the neighbor-joining method using Euclidian distances.

DISCUSSION

Genotypic Component of Carotenoid Content Diversity. We analyzed the carotenoid composition of the 25 genotypes to evaluate the contribution of genotype diversity to carotenoid profiles. Because the samplings were quite high (45 fruits per genotype) and several extraction/saponification/HPLC analyses were performed for each genotype, we can conclude that the contribution of the genotype diversity to the variance of each carotenoid is very high. Therefore, the carotenoid diversity is highly influenced by genetic factors when other sources of variation such as growing conditions, geographical origin, fruit maturity, and method of analysis are minimized. Then, we can analyze more accurately the relationships between genetic and carotenoid diversities.

Relationships between the Organization of Carotenoid Contents and Genetic Diversity. Twenty-five genotypes were evaluated on the basis of the carotenoids detected in juices. The classification obtained allowed us to distribute the genotypes in three clusters. Cluster 1 of **Figure 1** contained the three mandarins (*C. reticulata*), cluster 2 the two citrons (*C. medica*), and cluster 3 two of the three pummelos (*C. maxima*). These three species being the three basic taxa of cultivated *Citrus*, our results suggest that, as for other phenotypical traits, the general evolution of cultivated *Citrus* has been the main factor

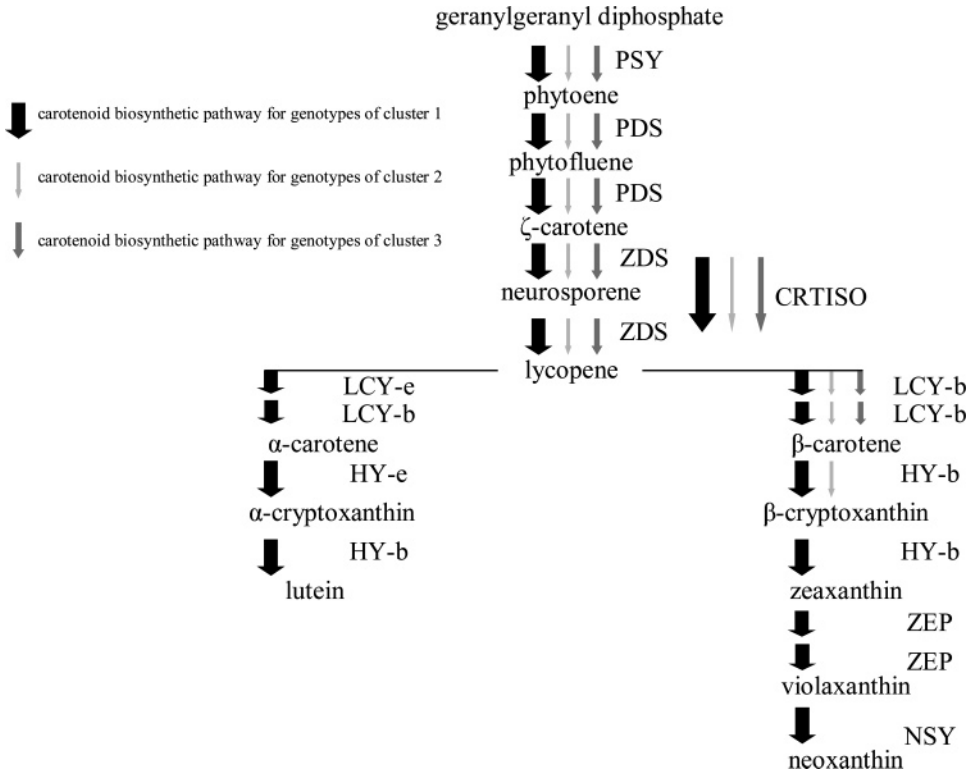

Figure 3. Carotenoid biosynthetic pathway obtained through the qualitative carotenoid composition in 25 citrus genotypes. Behaviors of genotypes of each cluster are represented by different arrows. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCY-b, lycopene β-cyclase; LCY-e, lycopene ε-cyclase; HY-b, β-carotene hydroxylase; HY-e, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; NSY, neoxanthin synthase.

of the organization of carotenoid diversity. Looking to secondary species, the classification obtained from qualitative data (presence−absence of 25 carotenoid components; **Figure 1**) is in agreement with previous classifications based on molecular markers, leading to phylogenetic inferences. In fact, in cluster 1, sweet oranges and sour oranges were close to mandarins. It was demonstrated that they were closely related with mandarins but possessed some alleles of pummelos (*26*). Clementine was also in cluster 1 and, according to previous studies, this genotype was found to be a hybrid between a mandarin and a sweet orange (*27*). Rangpur lime and Volkamer lemon, which belong to cluster 1, are hybrids between citron and mandarin and between citron and sour orange, probably back-crossed with mandarin, respectively (*27*). The two *C. limon* and the lime varieties were in cluster 2 close to the citron, as was found with molecular markers. Nicolosi et al. (*27*) suggested that lemons were hybrids between citron and sour orange and that Palestine sweet lime was a hybrid between a citron and a sweet orange. Similarly, grapefruits were present in cluster 3. Indeed, they were found to be hybrids between pummelo and sweet orange (*27*). Moreover, Mexican lime and Chandler pummelo, which should be expected to be included in clusters 2 and 3, respectively, were isolated. Concerning Mexican lime, one possible explanation is that Mexican lime is a hybrid between a citron and *Citrus micrantha* (*27*), a species that was not included in our study. The *C. micrantha* parent may be responsible of the position of Mexican lime on the tree of **Figure 1**. To confirm our conclusion, the same method of analysis should be applied to other genotypes.

Relationships between Carotenoid Profiles and Steps of the Biosynthetic Pathway. One purpose was to try to identify the steps of the carotenoid biosynthetic pathway involved in differential carotenoid accumulation. The behavior of genotypes of each cluster is illustrated in **Figure 3**, where each arrow shows which pigments were synthesized. Genotypes of cluster 1 produced the *cis*-violaxanthin and the lutein or the isolutein. It is important to stress that isolutein comes from lutein by oxidation that occurs in fruit (*33*). For genotypes of cluster 2, the last detected compound was β-cryptoxanthin. Concerning genotypes of cluster 3, the last detected compound was β-carotene.

For cluster 1, our results lead us to guess that the transformation of β-carotene into β-cryptoxanthin and zeaxanthin by hydroxylations catalyzed by β-carotene hydroxylase (HY-b) are key steps. The transformation of zeaxanthin into violaxanthin by zeaxanthin epoxidase (ZEP) seems also to play a major role. Indeed, the β-cryptoxanthin/*cis*-violaxanthin ratio allows the orange group to be differentiated from the mandarin group (tree of **Figure 2**). Concerning the five genotypes of cluster 2, qualitative data and especially quantitative data suggest that the most important step is the formation of phytoene from geranylgeranyl diphosphate by the action of a phytoene synthase (PSY). Actually, for all of the genotypes of cluster 2, carotenoid contents were very low. From the qualitative data, the last compound detected for the genotypes of cluster 3 was β-carotene. However, from the quantitative point of view, lycopene was present in a very large amount, which suggests the importance of the cyclization of lycopene, which is a branching point in the pathway.

Relationships between Biosynthetic Pathway Functionality and *Citrus* Evolutionary Process. Our results provide some evidence that at the interspecific level differences are qualitative and that it can be due to the allopatric evolution of the three taxa and a limitation of sexual recombination. Actually, genotypes of cluster 1, closely related with mandarins, synthesized all compounds and particularly *cis*-violaxanthin. For

genotypes of cluster 2, related with citrons, and for genotypes of cluster 3, related with pummelos, the biosynthesis was stopped at the level of β-cryptoxanthin and β-carotene, respectively. Lutein was not detected for genotypes of clusters 2 and 3. At the intraspecific level, differences are quantitative. It can be illustrated by differences reported for *C. sinensis* varieties. On the basis of qualitative data these genotypes were placed in cluster 1, whereas quantitative data showed strong differences. These results can be explained by mutational events that are responsible for the diversification among these genotypes.

Hypothesis on the Regulation Mechanisms at These Key Steps. Several possible mechanisms were considered to explain the regulation of the carotenoid biosynthetic pathway. One possible explanation was the existence of several isoforms of enzyme encoded by different genes or the existence of several alleles of one gene. A second explanation was a differential expression of the biosynthetic genes at the transcriptional level. Another one was a defect in one enzyme activity or in an enzyme-associated factor. Studies on tomato have demonstrated that the accumulation of lycopene during fruit ripening was controlled by transcriptional regulation with an increase of the expression of PSY and PDS genes and a down-regulation of LCY-b and LCY-e (*34*). In *Citrus*, Rodrigo et al. (*19*) and Kato et al. (*11*) also demonstrated that during fruit development differential gene expression at the transcriptional level explained differences in carotenoid accumulation. To our knowledge, only Kato et al. (*11*) studied the expression of the biosynthetic genes using several genotypes belonging to different species. These authors showed that the mechanism leading to diversity in β,β-xanthophyll composition between Satsuma mandarin and Valencia orange was due to HY-b substrate specificity and the balance expression between upstream biosynthetic genes (PSY, PDS, ZDS, and LCY-b) and downstream biosynthetic genes (HY-b and ZEP). Regulation of gene expression at the transcriptional level seems to be the major mechanism controlling the carotenoid accumulation during fruit ripening and controlling the differences in carotenoid accumulation between varieties. However, other mechanisms should be investigated to explain the differences between genotypes at mature stage. Actually, regulation at the transcriptionnal level appears to be more relevant to explain differences between genotypes that are close in phylogenetic classifications. This proposition is supported by the study of Kato et al. (*11*), who have shown that the differences between mandarin and orange were explained by this regulation. The presence of different isoforms or different alleles of the biosynthetic genes should be studied using genotypes belonging to the different clusters previously formed. For example, the existence of several isoforms of HY-b was supported by studies on tomato and pepper, where two isoforms were present depending on the tissue concerned (*35*). In *Citrus*, Kim et al. (*21*) isolated two clones, CHX1 and CHX2, encoding for HY-b that corresponded to alleles of the same gene. In addition, their results suggested that the expression of HY-b gene was not regulated at the transcriptional level during mandarin fruit ripening and that another isoform of HY-b or other enzymes involved in the carotenoid biosynthesis may be transcriptionally regulated.

Whatever the regulation mechanisms, extended experiments are required. Indeed, it would be necessary to monitor the gene expression of varieties of the same cluster to verify that carotenoid contents are dependent on the gene expression involved in the key steps. It would also be interesting to check if qualitative differences among clusters are related to the allelic diversity. For that, the estimation of the number of gene loci and their sequencing would be required.

Conclusion. Our results based on the precence or absence of carotenoid compounds showed that the 25 genotypes were included in only 3 clusters. This classification is in agreement with previous genetic studies. It stresses the fact that at the interspecific level, the organization of the diversity of carotenoid composition is linked to the global evolution process of cultivated citrus rather than to more recent mutational/human selection process. However, this selection process has been clearly efficient for the intraspecific diversification of secondary species such as *C. sinensis* and *C. paradisi*. It mainly results in quantitative variations that should be related to the modification of specific enzymatic activities (mutation of the gene of the enzymes of the biosynthetic pathway) or to the modification of gene regulation.

The genotypes of each cluster may have specific functional or regulation mechanisms with respect to carotenoid biosynthesis. The different behaviors among the three clusters lead us to identify the most important steps of the biosynthetic pathway involved in the diversification of carotenoid contents in *Citrus* species. This information will be very useful for further genomic works on the variability of the origin of carotenoid biosynthesis.

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