Scientia Horticulturae 129 (2011) 663–673

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

DNA content, morphometric and molecular marker analyses of *Citrus limonimedica*, *C. limon* and *C. medica* for the determination of their variability and genetic relationships within the genus *Citrus*

Diego Pessina[a], Rodolfo Gentili[a,*], Gianni Barcaccia[b], Silvia Nicolè[b], Graziano Rossi[c], Silvia Barbesti[a], Sergio Sgorbati[a]

[a] *Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano-Bicocca, Piazza della Scienza 1, I-20126 Milano, Italy*
[b] *Dipartimento di Agronomia Ambientale e Produzioni Vegetali, Università degli Studi di Padova, Campus di Agripolis, Via dell'Università 16, I-35020 Legnaro, Padova, Italy*
[c] *Dipartimento di Ecologia del Territorio, Università degli Studi di Pavia, Via S. Epifanio 14, I-26100 Pavia, Italy*

ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 13 May 2011
Accepted 16 May 2011

Keywords:
Numerical taxonomy
Molecular markers
Phylogeny
Citrus
Poncirus
DNA content

ABSTRACT

This work investigated the fingerprinting and phenotyping of *Citrus* germplasm; species selected were of historical importance belonging to *Citrus limonimedica* Lush. and its supposed ancestors, along with some other species of the *Citrus* genus. An integrated approach based on the exploitation of nuclear DNA content, morphological traits and molecular markers, such as RAPD fingerprints and ITS-based SNPs, was employed. We studied a core collection of 54 distinct accessions, including 43 genotypes of the *Citrus* species (18 species or supposed species) and 11 genotypes of the *Poncirus* genus, which was used as the reference outgroup. Morphological trait analysis and statistical analysis of DNA content and markers were useful for reconstructing a *Citrus* phylogeny. In particular, our experiments aimed at estimating the genetic variation within and the genetic relatedness among *C limon* (L.) Burm., *C. limonimedica* and *C. medica* L. to shed light on the hybrid origin hypothesis of *C. limonimedica*. The results of the multidisciplinary analyses allowed us to confirm a remarkable differentiation between *Poncirus* and *Citrus* genera and to highlight a close relationship among the three investigated *Citrus* species but a distinct difference between these three species and other species in the *Citrus* genus. RAPD fingerprints and ITS polymorphisms enabled us to point out a variation gradient between lemon and citron, with *C. limonimedica* as a possible intermediate species. Some accessions of *C. medica* and *C. limonimedica* that deviate from such a trend suggest recurrent introgression and/or hybridisation with other species of *Citrus*.

1. Introduction

The sub-genus *Citrus* sensu Swingle (1967) belongs to the *Rutaceae* family and the *Aurantioideae* subfamily. A particular fruit called hesperidium, which is a specialised berry that has a leathery exocarp and mesocarp and a segmented and juicy endocarp, characterises this taxon. From a biogeographical point of view, the centre of origin and source of the subsequent spread of *Citrus* was an area of the Asian continent characterised by tropical or subtropical climates, such as India, southern China and Japan, Indonesia, and the Philippines (Calabrese, 1992).

The history of the cultivation of *Citrus* trees is long and extremely complex, with cultivation established by 4,000 BC in Persia and the Middle East and consisting of many species and cultivars (Calabrese, 1992; Webber, 1967). Cultivation of *Citrus* plants has spread over the world with diverse utilisations, such as for fruit growth, pharmacologic use, cosmetics, and ornamental plants (Lota et al., 1999; Lee et al., 2006).

Over time, hybridisation between species and related genera, bud mutations and apomixis (e.g. adventitious embryony) have played an important role in the evolution of the numerous *Citrus* species and cultivars (Federici et al., 1998). The origins of many species of *Citrus* are poorly known or often based only on historical information. For this reason, the scientific interest in *Citrus* has increased in recent years, especially with regard to its taxonomy and phylogeny, with the aim of characterising and conserving its germplasm (Nicolosi et al., 2000; Araujo et al., 2003; Mabberley, 2004; Moreira Novelli et al., 2004; Deng et al., 2007; Pang et al., 2007; Barkley et al., 2009; Bayer et al., 2009; Jena et al., 2009). The classification systems proposed by Swingle and Reece (1967) and Tanaka (1977) and further phylogenetic analyses, starting from those of Barrett and Rhodes (1976) by means of a numerical

* Corresponding author. Tel.: +39 02 6448 2700; fax: +39 02 6448 2996.
E-mail address: rodolfo.gentili@unimib.it (R. Gentili).

0304-4238/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2011.05.012

Table 1
List of *Poncirus trifoliata* and *Citrus* taxa collected for the study.

N.	Tintori's collection code	Species	Author	Cultivar	Abbreviation
1	FC1	*P. trifoliata*	(L.) Raf.		Ptri1
2	FC1	*P. trifoliata*	(L.) Raf.		Ptri2
3	FC2	*C. limon*	(L.) Burm.f.	Amalphitanum	Clim3
4	FC1	*P. trifoliata*	(L.) Raf.		Ptri4
5	FC1	*P. trifoliata*	(L.) Raf.		Ptri5
6	FC1	*P. trifoliata*	(L.) Raf.		Ptri6
7	FC1	*P. trifoliata*	(L.) Raf.		Ptri7
8	FC1	*P. trifoliata*	(L.) Raf.		Ptri8
9	FC1	*P. trifoliata*	(L.) Raf.		Ptri9
10	FC1	*P. trifoliata*	(L.) Raf.		Ptri10
11	FC1	*P. trifoliata*	(L.) Raf.		Ptri11
12	71	*C. mitis*	Blanco	Foliis variegatis1	Cmit14
13	FC3	*C. medica*	L.	Maxima	Cmed15
14	FC3	*C. medica*	L.	Maxima	Cmed16
15	37	*C. aurantium*	L.		Caur17
16	71A	*C. mitis*	Blanco	Foliis variegatis2	Cmit18
17	1G	*C. limon*	(L.) Burm.f.	Mellarosa	Clim19
18	11I	*C. limon*	(L.) Burm.f.	Siciliano	Clim20
19	12	*C. meyeri*	Yu Tanaka		Cmey21
20	13	*C. volkameriana*	Ten. & Pasq.		Cvol22
21	91	*C. lumia*	Risso. & Poit.	Pyriformis	Clum23
22	37B	*C. aurantium*	L.	Cordifolia	Caur24
23	43	*C. aurantium*	L.	Salicifolia	Caur25
24	45	*C. aurantium*	L.	Foliis variegatis	Caur26
25	18	*C. limonimedica*	Lush.	Maxima	Clme27
26	56	*C. bergamia*	Risso & Poit.		Cber29
27	84C	*C. jambhiri*	Lush.		Cjam30
28	4	*C. limon*	(L.) Burm.f.	Sfusato amalfitano	Clim31
29	31	*C. medica*	L.	Salò	Cmed32
30	16C	*C. limonimedica*	Lush.	Piretto	Clme33
31	11Z	*C. limon*	(L.) Burm.f.	Vaniglia	Clim34
32	25	*C. limonimedica*	Lush.	Sanctus Dominicus	Clme35
33	33	*C. medica*	L.	Aurantiata	Cmed36
34	2	*C. limon*	(L.) Burm.f.	Femminello	Clim37
35	30	*C. medica*	L.	Corsican	Cmed38
36	17	*C. limonimedica*	Lush.	Paradisi	Clme39
37	70	*C. mitis*	Blanco		Cmit40
38	70	*C. mitis*	Blanco		Cmit41
39	70	*C. mitis*	Blanco		Cmit42
40	FC4	*C. limonimedica*	Lush.	Pigmentata	Clme43
41	27	*C. medica*	L.	Diamante	Cmed44
42	16	*C. limonimedica*	Lush.	Florentina	Clme45
43	84	*P. trifoliata*	(L.) Raf.		Ptri46
44	FC5	*C. medica*	(Hoola van Nooten) Swingle	*var. sarcodactylis*	Cmed47
45	74	*C. aurantifolia*	(Christm.) Swing.	La Vallette	Cauf48
46	77	*C. limettioides*	Tan.	Pursha	Cpur49
47	53	*C. sinensis*	(L.) Osb.		Csin50
48	77	*C. limettioides*	Tan.	Pursha	Cpur51
49	78	*C. hystrix*	D.C.		Chys52
50	56	*C. bergamia*	Risso & Poit.		Cber53
51	60	*C. myrtifolia*	Raf.		Cmyr54
52	34	*C. grandis*	(L.) Osb.		Cgra55
53	37	*C. aurantium*	L.		Caur59
54	26	*C.medica*	L.		Cmed60

Note: Samples with "FC" code come from Floricoltura Chiaravalli private collection.

taxonomy approach, revealed that there were only three true species within the *Citrus* genus: *C. medica* L. (citron), *C. reticulata* Blanco (mandarin) and *C. grandis* (L.) Osb. (pummelo).

Within the genus, *C. limonimedica* Lush. has a very uncertain origin; it was described as a species by Lushington (1910), and the description was made exclusively considering the cultivated specimens. The few *C. limonimedica* cultivars known in Italy have been grown from the XVII century, during Medici's government in Florence, and have always been used as ornamental plants (Nati, 1674; Volkamer, 1708–1714; Targioni-Tozzetti, 1853). Their great morphological variability, especially concerning fruits, has led to the hypothesis that *C. limonimedica* was a result of hybridisation between *C. limon* (L.) Burm. f. (lemon) and *C. medica* L. (citron) (Tanaka, 1954; Carpenter and Reece, 1969; Barcaccia et al., 2008). Some authors and/or *Citrus* official collections consider *C. limonimedica* to be identical to the famous Hebrew citron (Lota et al., 1999; http://www.plantnames.unimelb.edu.au/new/Citrus_2.html). Other classifications keep C. *limonimedica* and *C. medica* L. var. *ethrog* Engl. as distinct entities (Galeotti and Tintori, 2000; http://www.oscartintori.it/indice.pdf). Conversely, Calabrese (2002) hypothesised that *C. limonimedica* is likely a hybrid between citron and orange.

To investigate the genetic diversity and phylogenetic relationships in *Citrus*, several molecular marker systems were employed, such as ISSRs (inter simple sequence repeats), RAPD (random amplified polymorphic DNAs), SCARs (sequence-characterised amplified regions), and SNPs (single nucleotide polymorphisms), from either nuclear or chloroplast genomes (Fang and Roose, 1997; Federici et al., 1998; Nicolosi et al., 2000; Deng et al., 2007).

Table 2
FCM values of the DNA content of selected *Poncirus* and *Citrus* specimens. Significant differences of the absolute DNA content according to ANOVA were found between the two genus *Poncirus* and *Citrus*. *C. limon*, *C. medica*, and *C. limonimedica* groups showed similar DNA contents. Other *Citrus* species groups, such as *C. mitis*, *C. aurantium*, and *C. bergamia*, were significantly different ($p < 0.05$) with respect to the *C. limon*, *C. medica* and *C. limonimedica* groups. *C. aurantifolia* cv. "La Valletta" and *C. aurantium* cv. "Cordifolia" showed DNA contents likely corresponding to a triploid and tetraploid genome (1.21 pg and 1.47 pg, respectively).

Sample	Sample mean channel	Mean CRBCs	Sample/CRBCs ratio	DNA content (pg/nucleo)	Mean – SE
Ptri1	238	759	0.31	0.73	
Ptri5	233	736	0.32	0.74	
Ptri6	241	754	0.32	0.74	
Ptri7	243	755	0.32	0.75	
Ptri10	239	738	0.32	0.75	
Ptri9	238	734	0.32	0.76	
Ptri2	243	747	0.33	0.76	
Ptri11	246	754	0.33	0.76	
Ptri4	250	752	0.33	0.77	
Ptri8	241	724	0.33	0.78	
Ptri46	193	585	0.33	0.77	
Mean Poncirus					0.76 ± 0.0051
Clim19	233	708	0.33	0.77	
Clim34	196	591	0.33	0.77	
Clim31	230	681	0.34	0.79	
Clim3	200	561	0.36	0.83	
Clim37	178	494	0.36	0.84	
Clim20	195	540	0.36	0.84	
					0.81 ± 0.0122
Cmed16	227	663	0.34	0.80	
Cmed60	234	681	0.34	0.80	
Cmed15	202	578	0.35	0.81	
Cmed38	206	588	0.35	0.82	
Cmed32	196	559	0.35	0.82	
Cmed36	203	577	0.35	0.82	
Cmed44	217	611	0.36	0.83	
Cmed47	283	769	0.37	0.86	
					0.82 ± 0.0065
Clme43	198	575	0.34	0.80	
Clme39	189	544	0.35	0.81	
Clme35	198	768	0.35	0.82	
Clme45	201	572	0.35	0.82	
Clme33	200	561	0.36	0.83	
Clme27	249	686	0.36	0.85	
					0.82 ± 0.0055
Caur17	176	524	0.34	0.78	
Caur25	183	538	0.34	0.79	
Caur59	204	599	0.34	0.79	
Caur26	197	573	0.34	0.80	
					0.79 ± 0.0027
Cmit42	192	548	0.35	0.82	
Cmit18	189	537	0.35	0.82	
Cmit40	212	564	0.38	0.88	
Cmit41	240	622	0.39	0.90	
Cmit14	215	516	0.42	0.97	
					0.876 ± 0.0225
Cber29	197	604	0.33	0.76	
Cber53	180	524	0.34	0.80	
Cpur49	197	572	0.34	0.80	
Cpu451	194	561	0.35	0.81	
					0.80 ± 0.0008
Polyploid samples					
Cauf48‴	286	552	0.52	1.21	
Caur24‴	355	564	0.63	1.47	
Other samples					
Cdel13	225	664	0.34	0.79	
Cgra55	210	596	0.35	0.82	
Chys52	210	567	0.37	0.86	
Cjam30	199	571	0.35	0.81	
Clum23	215	589	0.37	0.85	
Cmyr54	207	523	0.40	0.92	
Csin50	198	561	0.35	0.82	
Cwol22	189	576	0.33	0.76	
Cmey21	194	564	0.34	0.80	
Mean Citrus					0.84 ± 0.0180

In the present study, the genetic diversity and the genetic differentiation and relationships among the considered *Citrus* species were inferred using an integrated approach based on the analysis of 17 morphological traits and molecular markers by means of genomic RAPD fingerprints and nuclear ITS (internal transcribed spacer)-derived SNPs. RAPD analysis has already been successfully used for population genetics in different plant species (Kump and Javornik, 1996; Barcaccia et al., 1997; Chan and Sun, 1997) and the nuclear marker ITS1 has been widely used for estimating genetic diversity (Baldwin et al., 1995) and parentage analysis within the *Citrus* genus (Xu et al., 2006).

The main objectives of this study were (i) to explore possible differences in the DNA content of *Citrus* and *Poncirus* genera and within *Citrus* accessions by flow cytometry analysis; (ii) to verify the relationships between *Citrus* and *Poncirus* genera and within the genus *Citrus* on the basis of phenotypic traits and molecular markers; (iii) to investigate the phylogenetic relationships among *C. limonimedica* and its supposed ancestors, *C. limon* and *C. medica*, by means of DNA polymorphisms.

2. Materials and methods

2.1. Samples

Morphological and DNA analyses were performed on 54 accessions of historical cultivars belonging to *Citrus* and *Poncirus* genera from different Italian farms and private collections (Table 1). Most of the cultivars employed in this study come from Oscar Tintori's private farm (Pescia, Province of Pistoia, Italy; http://www.oscartintori.it/indice.pdf), where several *Citrus* cultivars belonging to Medici's Gardens (Boboli Gardens of Florence) have been collected and conserved, after their recent inventory (Galeotti and Tintori, 2000). Some cultivars come from the Floricoltura Chiaravalli private collection (named with "FC" code in Table 1).

2.2. Flow cytometric analysis of DNA content

Nuclei were extracted from approximately 0.2 g of young leaves. Fresh tissue was finely chopped with a razor blade in a Petri dish placed on ice and containing 1 mL of 10 mM PBS buffer, pH 7.2–7.4, supplemented with dithiothreitol (DTT, 1 mg/mL) and Triton X100 (0.3%) detergents. The nuclear suspension was filtered through a 15-μm pore nylon mesh.

As an internal standard, freshly stored chicken red blood cells (CRBCs) (Sgorbati et al., 1986) were added at an adequate concentration to 1 mL of the filtered nuclear suspension. To obtain the absolute DNA content of *Citrus* and *Poncirus*, 1 mL of the nuclear suspension containing the internal standard was stained with 1 mL of a PBS staining solution containing 200 μg/mL propidium iodide (PI, Sigma–Aldrich) (100 μg/mL final concentration) and 100 μg/mL RNase 10^{-3} U (50 μg/mL final concentration) and analysed with flow cytometry. The DNA content measurement of leaf nuclei was performed with an Apogee A40 (Nanogen) flow cytometer. Excitation was at 488 nm, and the PI fluorescence was collected above 570 nm. Typically, 4,000–5,000 nuclei were analysed for each sample. Coefficients of variation of the DNA peaks were mostly around 2.5–4.0%. In order to obtain the best sample/internal standard ratio, we carried out a routine check of the instrument performance and of the preparation protocol, repeating the preparation and analysis of samples when the DNA peak CVs were larger than 4%.

Data obtained were analysed by ANOVA to statistically compare DNA mean values and assess DNA content differences among species (Table 2).

2.3. Numerical taxonomy

The seventeen morphological traits of the *Citrus* and *Poncirus* specimens evaluated in this study are shown in Table 3. The data matrix obtained by these 17 morphological traits was standardised and then analysed according to numerical taxonomy using Past 1.94 software (Hammer et al., 2001). A dendrogram was computed on the basis of the unweighted pair-group method (UPGMA) and using the Euclidean distance coefficient.

2.4. DNA extraction

Genomic DNA was isolated from 0.1 g of frozen young leaves using the Nucleon PhytoPure plant DNA extraction kit (Amersham Biosciences) according to the instructions provided, with small adjustments. The pelleted DNA was washed twice with 70% ethanol, dried and resuspended in 80–100 mL TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). The DNA was then stored at −20 °C until used.

2.5. RAPD markers

Amplifications of RAPD fragments generated from random decameric primers were carried out following the protocol outlined in (Barcaccia et al., 1997) in a 25-μL total reaction volume containing 10× reaction buffer (Pharmacia; 100 mM Tris–HCl pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl), 0.3 μM dNTPs in equimolar ratio, 1 U of *Taq* DNA polymerase (Pharmacia), 1 μM each of forward and reverse primers and 30 ng of genomic DNA. Several different primers (Series Band C, Operon Technology, Alameda, CA, USA) were tested, and those that gave the best performance in terms of number of bands per reaction were selected (see supplementary materials, Table 1S).

PCR reactions were performed in a 9700 Thermal Cycler (Perkin Elmer) under the following temperature profile: 95 °C for 5 min to denature the genomic DNA, 3 cycles at 95 °C for 2 min for denaturation of templates, 35 °C for 1 min for the annealing of primers, and 72 °C for 2 min for the elongation of primers, 37 cycles at 94 °C for 15 s, 36 °C for 30 s, 72 °C for 1 min and 72 °C for 10 min. The rates of temperature changes adopted for heating and cooling were +1 °C/2.9 s and −1 °C/2.4 s, respectively.

The complete reaction mixture for each sample was loaded on 2% agarose gels stained with 0.5 μg/mL of ethidium bromide and run in TBE buffer at 150 V for 120 min (Sambrook et al., 1989). A 100-bp DNA Ladder producing 12 bands from 100 bp to 2000 bp was used as reference. PCR products were visualised using a UV transilluminator, and the gel images were acquired and analysed with a DC120 camera (Kodak).

2.6. ITS

Amplification reactions of the nuclear ITS1 region using universal primer pairs ITS5 and ITS2 (Table 1S) were performed in 25 μL containing 10× PCR buffer (100 mM Tris–HCl pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl), 0.5 U of *Taq* DNA polymerase, 0.2 mM dNTPs, 0.2 μM of each primer and 15 ng of genomic DNA. PCR reactions were carried out using a GeneAmp PCR System 9700 (Applied Biosystems) with the following cycling parameters: 5 min denaturation step at 95 °C; 40 cycles of 30 s at 95 °C, 1 min at 56 °C, 1.30 min; and 7 min at 72 °C for the elongation step.

Amplified products were purified using a clean-up method containing exonuclease I and shrimp alkaline phosphatase (Amersham) and then directly sequenced bidirectionally with forward and reverse primers according to the rhodamine terminator cycle sequencing kit (ABI PRISM Applied Biosystems).

Table 3
Morphological traits and data used in numerical taxonomic analysis. Only the values of *C. limon*, *C. medica*, and *C. limonimedica* are shown.

N	Morphological traits	Trait scoring	Samples																			
			Clim03	*Clim19*	*Clim20*	*Clim31*	*Clim34*	*Clim37*	*Cmed15*	*Cmed32*	*Cmed36*	*Cmed38*	*Cmed44*	*Cmed47*	*Cmed60*	*Cmed16*	*Clme27*	*Clme33*	*Clme35*	*Clme39*	*Clme43*	*Clme45*
1	*Shoot colour*	Green = 1; purplish = 2	2	1	2	2	2	1	2	2	2	1	2	2	2	2	2	2	2	2	1	2
2	*Leaf color*	Light green = 1; variegated = 2; marked green = 3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
3	*Leaf consistency*	Soft = 1; turgid = 2; coriaceous = 3	3	2	2	2	2	2	3	2	3	2	2	1	3	3	2	2	2	2	3	1
4	*Leaf size*	Small = 1 (<3 cm); mean = 2 (3–6 cm); big = 3 (6–9 cm); very big = 4 (>9 cm)	2	3	2	3	2	3	3	3	3	2	2	3	3	4	3	3	1	2	2	2
5	*Leaf shape*	Lanceolate = 1; elliptic = 2; trifoliate = 3	1	2	1	1	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2
6	*Apex shape*	Acute = 1; obovate = 2	1	2	1	1	1	2	2	1	2	2	2	2	2	2	2	1	2	1	1	1
7	*Petiole shape*	Not winged = 0; intermediate = 1; winged = 2	0	0	0	0	0	0	1	2	0	0	0	0	0	1	0	0	1	0	0	0
8	*Bud colour*	White = 0; pinkish = 1; purplish = 2	1	1	2	1	n.d.	1	0	2	1	0	2	2	2	0	2	2	1	2	2	2
9	*Flower type*	Mandarin = 1; orange = 2; lemon = 3; cedar = 4; poncirus = 5	3	3	2	3	3	3	4	4	4	3	3	4	4	4	4	3	3	3	3	3
10	*Flowering*	Uniferous = 1; biferous = 2; reflowering = 3; continous = 4	2	2	1	1	n.d.	3	3	2	1	3	3	3	2	3	1	1	3	3	1	3
11	*Fruit colour*	Light yellow = 1; marked yellow = 2; light orange = 3; marked orange = 4; blood red = 5	2	2	2	1	2	2	2	2	4	3	2	3	2	2	2	2	1	2	3	2
12	*Fruit size*	Little = 1 (<4 cm); mean = 2 (4–8 cm); big = 3 (8–12 cm); very big = 4 (>12 cm)	2	3	2	2	2	3	3	3	4	4	4	3	3	4	4	3	1	3	3	2
13	*Fruit characteristics*	Ellipsoid = 1; subspheric = 2; ovoid = 3; conic-elipsoid; other = 5	3	2	3	3	2	3	3	3	3	2	3	5	3	3	4	3	4	3	3	3
14	*Umbo*	Not present = 0; little marked = 1; very marked = 2	2	1	1	2	1	1	1	1	2	1	2	0	1	1	2	2	1	1	2	2
15	*Stiyle residual*	Not present = 0; present = 1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
16	*Thorns*	Not present = 0; little marked = 1; very marked = 2	2	0	1	0	1	1	0	0	0	2	1	0	2	1	2	0	0	0	1	1
17	*Leaf*	Deciduous = 0; evergreen = 1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

n.d.: not determined.

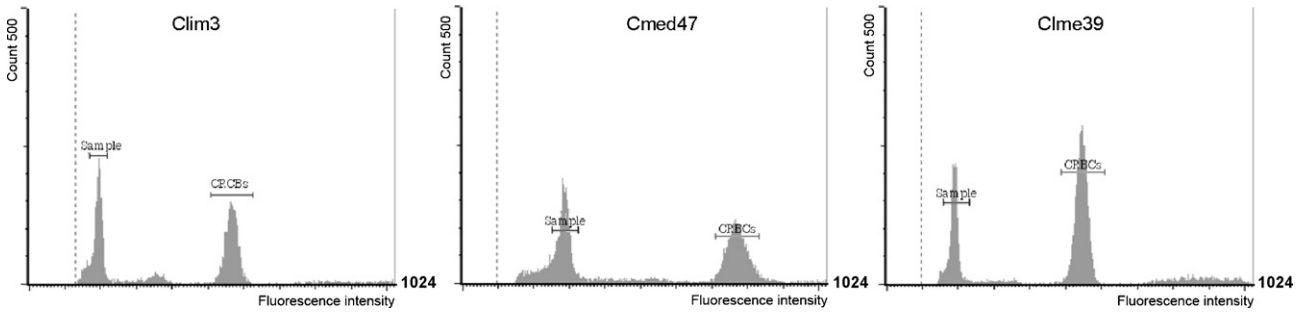

Fig. 1. Flow cytometry data of the DNA content analysis of *Citrus* samples. The DNA fluorescence signals of nuclei released from *Citrus* leaves and internal standard were distributed along 1024 fluorescence intensity channels. The ratio between the DNA fluorescence of *Citrus* samples (Clim3, *C. limon*; Cmed47, *C. medica*; and Clme39, *C. limonimedica*) and chicken red blood cells (CRBCs) as an internal standard was used for the absolute DNA content calculation (sample DNA fluorescence intensity/CRBCs DNA fluorescent intensity × 2.33 pg DNA).

The nucleotide sequences of the ITS1 region have been deposited in the NCBI's database under accession numbers from HQ660683 to HQ660702.

2.7. Phylogenetic analysis

DNA fingerprint analysis was performed visually; the detection of the bands derived from each primer was performed including all amplicons of the DNA fingerprints by scoring them from the longest to the shortest one. To provide a quantitative measure of relatedness between accessions, each band was scored for its presence (1) or absence (0) in each genome. Data obtained were submitted to hierarchical cluster analysis, and the dendrogram was computed on the basis of the unweighted pair-group method (UPGMA) using the similarity coefficient of Dice. RAPD phylogenetic analyses of all samples were carried out with 4 primer combinations (OP-R8, OP-Q10, OP-Q4 and OP-B18).

In addition, integrated data matrices of morphological traits and RAPD markers were completed with six primer combinations (OP-R8, OP-Q10, OP-Q4; OP-B18, OP-G15 and OP-R15) for the three species *C. limon*, *C. medica* and *C. limonimedica*. These data were used to perform a cluster analysis (UPGMA, Rho coefficient) and a principal coordinates analysis (PCA) with the NTSYS software (Rholf, 2000).

In morphologic and RAPD phylogenetic analyses, in accordance with McDade (1997), we used the UPGMA method instead of the neighbor-joining one, as the first may be useful in testing hypotheses of species with hybrid origin and thus may help to make progress in detecting hybrids and parents.

The ITS1 sequences were visualised and manually edited using the Sequencer 4.8 program, and a sequence similarity search was carried out by querying the GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were performed with SeAl v2.0 software, and phylogenetic and molecular evolutionary analyses were conducted using *Phylip* software (Felsenstein, 1989). Parsimony analysis (Swofford et al., 1996) was performed using close-neighbour interchange, uniform weighting, all positions containing alignment gaps data (i.e. gaps characters) were treated as 5th base and 1,000 bootstrap replicates as internal support of clades. The close-neighbour interchange initial trees were selected using random additional trees with 10 replications each and a search level of 2.

The significance of the correlation observed between the matrix of Euclidean genetic distances, estimated on the basis of morphological traits, and the matrix of Dice's genetic similarities, calculated using molecular marker polymorphims, was assayed by the Mantel's matrix correspondence test (Mantel, 1967).

3. Results

3.1. Genome size

A typical flow cytometric histogram of the DNA content analysis of *C. limon*, *C. medica* and *C. limonimedica* specimens with respect to their internal standard (CRBCs, 2.33 pg) for the absolute DNA content determination is presented in Fig. 1. The 2C DNA content values of the 55 specimens are shown in Table 2. *P. trifoliata* had lower values (mean 0.76 pg) with respect to *Citrus* specimens (mean 0.84 pg), and one-way variance analysis (ANOVA) showed that this difference was significant ($F = 26.992$, $P < 0.001$). No differences among the mean DNA contents (ANOVA) of *C. limon*, *C. limonimedica* and *C. medica* were found ($F = 0.653$, $P = 0.533$). Within the *Citrus* genus, significant differences, according to ANOVA, among the *C. aurantium* and *C. mitis* species and the group formed by *C. limon*, *C. medica* and *C. limonimedica* were found ($P < 0.05$ for the two comparisons). In all comparisons variance between the groups is higher than the variance within groups.

Among the *Citrus* species, a higher 2C DNA content was reported for Cauf48 (1.21 pg, *C. aurantifolia* cv. "La Vallette") and Caur24 (1.47 pg, *C. aurantium* cv. "Cordifolia"), corresponding to a triploid and tetraploid condition, respectively.

3.2. Morphological and RAPD analysis

Numeric taxonomic analysis of the specimens belonging to the genus *Citrus* and *Poncirus* was carried out using the seventeen morphological characters shown in Table 3 (and in Table 2S; see supplementary material). The dendrogram resulting from the UPGMA clustering of the similarity matrix is presented in Fig. 2. Two major groups were formed (Dice coefficient values between −1.5 and −1.75), corresponding to *Citrus* (43 accessions) and *Poncirus* (11 accessions) genera. The *Citrus* genus was divided into clusters I and II, which in turn were divided into subclusters A and B and subclusters C and D, respectively. All three investigated species, *C. limonimedica*, *C. limon* and *C. medica* (19 accessions), were grouped in subcluster D, with the exception of accession No. 34 (*Citrus limon* cv. "Vaniglia"), which was found in subcluster C.

Regarding the whole set of the 54 *Citrus* and *Poncirus* samples, a total of 80 reproducible and reliable RAPD markers (four primers combination) were scored, with fragment sizes ranging from 270 bp to 3,500 bp across all samples, while the average number of PCR products per primer was 20 (Table 1S). There were 76 polymorphic bands (95% of the total number of bands). The primers OP-R08 and OP-Q10 detected the highest level of variability, and the percentage of polymorphic bands detected using this pair was 100%. The set of markers resulting from primers OP-R08, OP-Q10, OP-Q04 and OP-

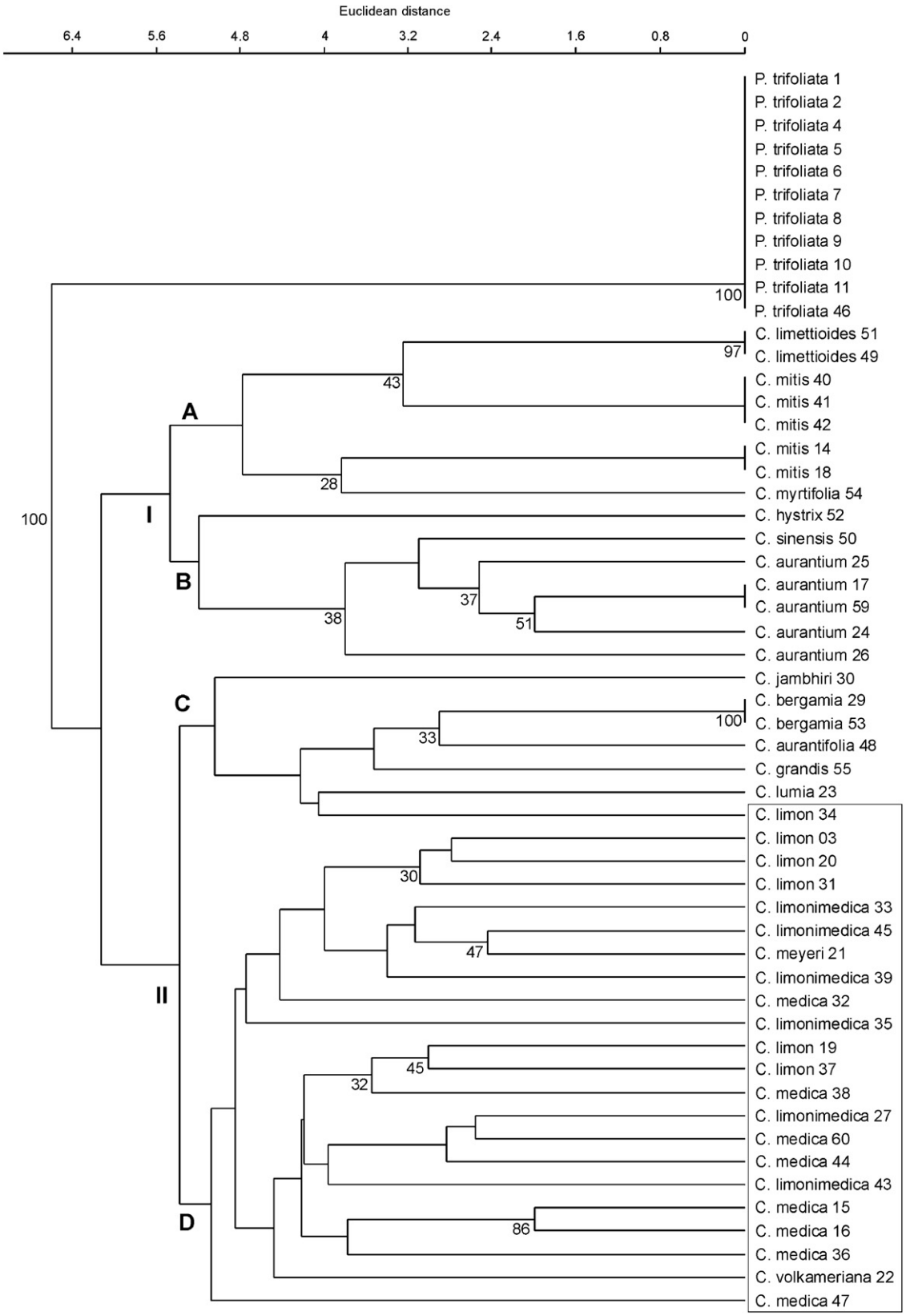

Fig. 2. UPGMA dendrogram (Euclidean coefficient) displaying the phenotypic distance estimates within the *Citrus* genus (Supplementary). *C. limon*, *C. medica* and *C. limonimedica* were grouped altogether into subcluster D, with the only exception of the accession n. 34 (*C. limon* cv. "Vaniglia"). *Poncirus trifoliata* samples were analysed for comparison as an outgroup. Bootstrap values higher than 25% are shown.

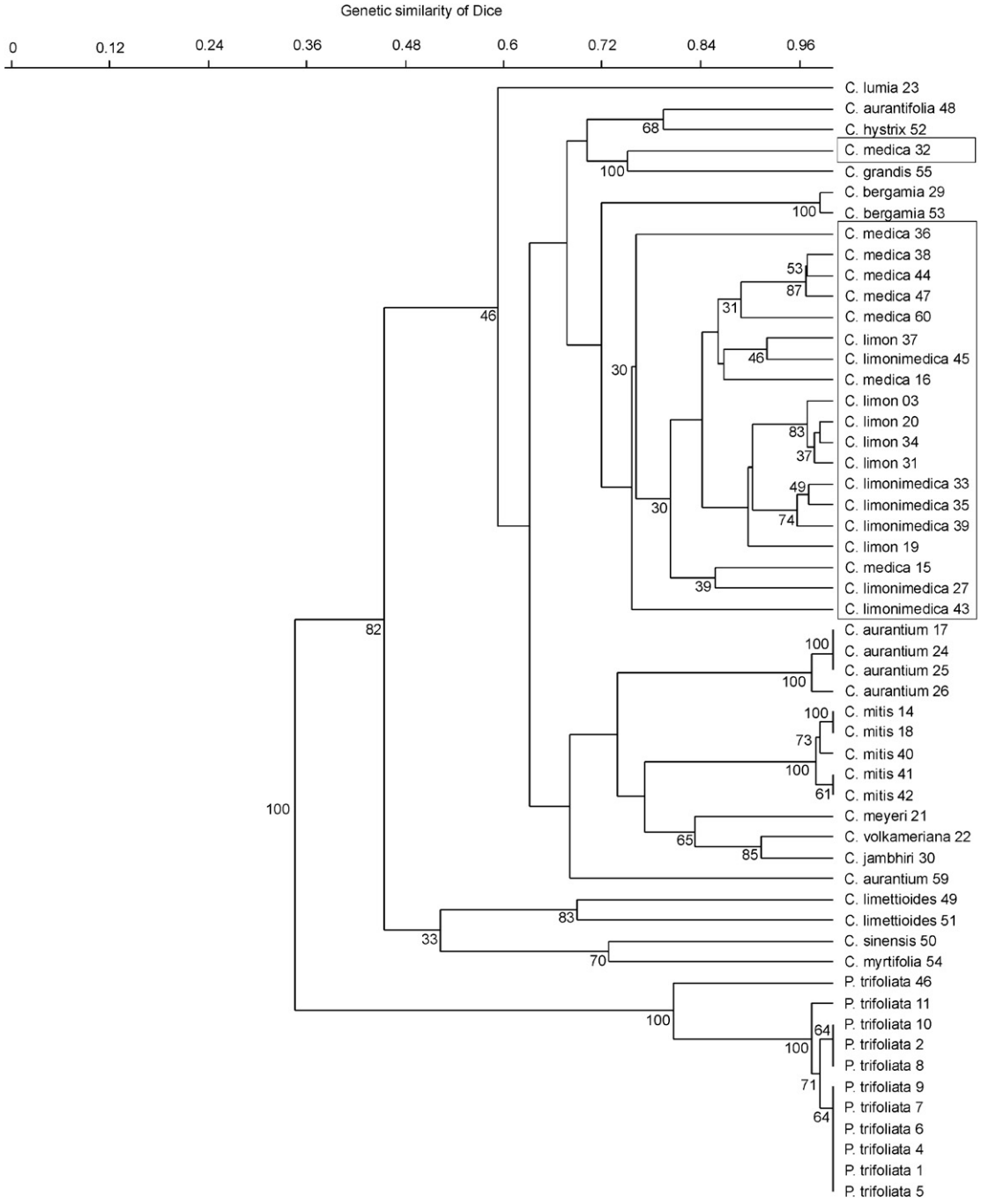

Fig. 3. UPGMA dendrogram (dice coefficient) displaying the genetic similarity estimates according to RAPD within the *Citrus* genus. *C. limon*, *C. medica* and *C. limonimedica* were grouped altogether in a distinct cluster, with the only exception of the accession Cmed32 (*C. medica* cv. "Salò"). *Poncirus trifoliata* samples were analysed for comparison as an outgroup. Bootstrap values higher than 25% are reported.

B18 was selected for the cluster analysis. The UPGMA dendrogram based on the genetic similarity matrix showed a clear separation between the *Poncirus* and *Citrus* genera (Fig. 3).

Among the *Citrus* accessions, the three selected species, *C. limon*, *C. medica* and *C. limonimedica,* were found to be interrelated and to form a distinctive cluster, with the exception of the accession Cmed32 (*C. medica* cv. "Salò"). The species *C. aurantium*, *C. mitis*, and *C. bergamia* separated into distinctive clusters.

3.3. Integrated data set (20 samples)

Integrated PCA analysis resulting from morphological and RAPD (six primer combinations: see supplementary materials, Table 1S) data matrices showed a clear separation of the three considered *Citrus* species in three corresponding distinct groups (Fig. 4). *C. limonimedica* accessions were plotted in the middle position with respect to the other two species.

3.4. ITS1

The complete sequences of ITS1 were determined for the twenty accessions of *C. limon*, *C. medica* and *C. limonimedica*. The length of the ITS1 ranged between 332 bp and 359 bp because of the presence of a 29-bp long in/del in the three accessions Cmed32, Clme43 and Clme39. The overall transition/transversion bias was $R = 21.311$, and all positions containing alignment gaps and miss-

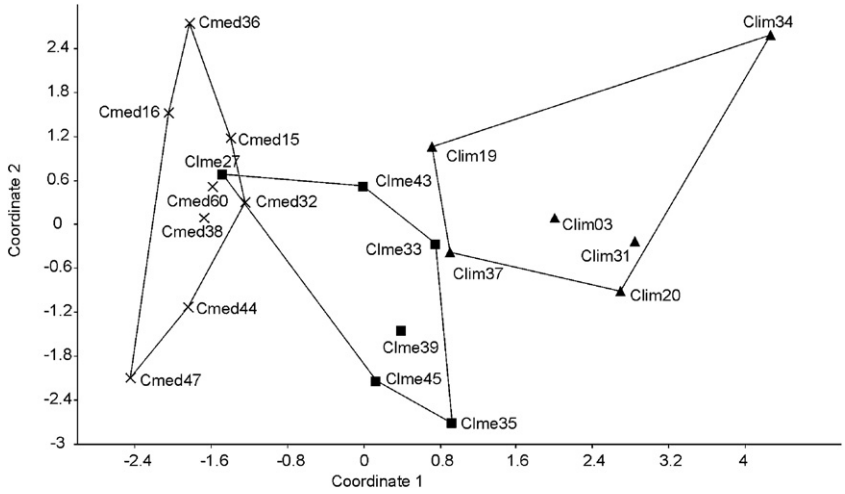

Fig. 4. Scattergram according to PCA analysis (integrated matrix from morphological and RAPD marker data) of *C. limon*, *C. medica* and *C. limonimedica*.

ing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). DNA sequences of the ITS1 region from selected plants were used to construct phylogenetic trees using a parsimony algorithm. The clustering result showed that there is not a clear separation between the three *Citrus* species, and this finding emphasised the existence of two major clusters, but with very low bootstrap values (Fig. 5). The former cluster grouped almost all the *C. medica*, while in the latter one the accessions of *C. limonimedica* is mostly related to *C. limon*.

4. Discussion

Numerous studies based on different techniques have been carried out to unravel the complex taxonomy and phylogeny of *Citrus* and related genera, which nevertheless remain largely controversial (Nicolosi et al., 2000). As stressed by Barrett and Rhodes (1976), the main problem responsible for the confusion in *Citrus* taxonomy is due to the fact that some biotypes are facultative apomicts. This reproductive behaviour yields progeny with a range of sexual variants plus a single clonal population. Other factors, such as intrageneric and intergeneric hybridisation, polyploidy and bud mutations, have produced further uncertainty (Araujo et al., 2003; Jena et al., 2009). For these reasons, a high number of *Citrus* species (*sensu lato*) at present are considered or inferred to be of hybrid origin (Federici et al., 1998).

In this study, we adopted an integrated approach to verify a possible relationship between the historical cultivars of *C. limonimedica* and its supposed ancestors, *C. medica* and *C. limon*, in relation to

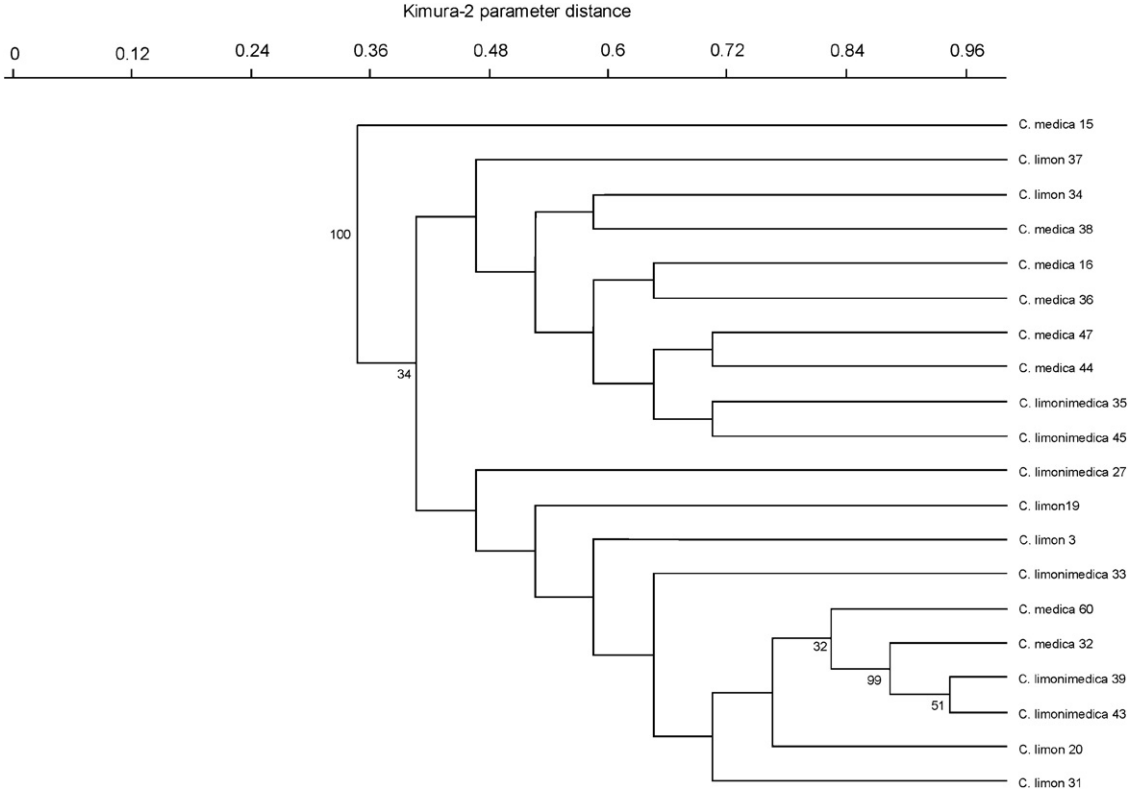

Fig. 5. Maximum parsimony rooted tree of 20 genotypes within the *Citrus* genus: *C. medica* (8 accessions), *C. limon* (6 accessions) and *C. limonimedica* (6 accessions). The tree was constructed using ITS1 nucleotide sequences. The numbers for each interior branch indicate bootstrap support of 1,000 replicates.

other *Citrus* taxa using the related *Poncirus* genus as reference for genome size, morphology and molecular variability.

4.1. DNA content

We obtained a high-resolution measurement of the DNA content of 11 accessions of *P. trifoliata* (2C DNA content 0.76 pg, 0.015 sd), which differs significantly from that of the *Citrus* complex (about 10% difference). This result, together with morphological and molecular data (Fig. 3), supports a marked taxonomic difference, at least at the genus level.

Concerning the complex *C. limonimedica*, *C. medica* and *C. limon*, our analysis did not show any significant difference in terms of DNA content, in contrast with data reported by Ollitrault et al. (1994), which highlighted differences in DNA content between *C. medica* and *C. limon*. In particular, the DNA content value of *Citrus medica* (0.82 pg) is the same of that (0.814 pg) reported by Ollitrault et al. (1994), but the DNA content value for *Citrus limon* (0.81) differs somewhat from that found by Ollitrault (0.78 pg), probably because of the different cultivars used in the analysis. Our DNA content values compare very nicely with those reported for *Citrus limon* (0.80 pg) and *Poncirus trifoliata* (0.77 pg) in Kayim et al. (1998), cited in references and also reported in the Kew Gardens plant DNA data base. As far as we know for poliploid, Kayim et al. (1998) reports a 2C DNA content value of 1.27 pg for *Fortunella hindsii*, considered as a value lower than expected for a tetraploid species.

4.2. Relationships between Citrus and Poncirus genera and within the genus Citrus

RAPD marker and numeric taxonomy data regarding all the examined 54 samples were in agreement, as supported by the significant matrix correlation ($r = 0.639$), and produced UPGMA trees showing a clear separation between the *Citrus* and *Poncirus* genera (Federici et al., 1998).

Within the *Citrus* genus, such analyses clearly differentiated species with sub-elliptic (lemon-shaped) fruit (*C. grandis*, *C. jambhiri*, *C. limon*, *C. limonimedica*, *C. medica* and *C. meyeri*) from those with flattened (mandarin-shaped) fruit at the ends (*C. limettioides* 'Pursha', *C. mitis* and *C. myrtifolia*) or sub-spherical (orange-shaped) fruit (*C. aurantium* and *C. sinensis*). As previously shown by Xie et al. (2008), our results confirm the common lineage of lemon-shaped species. Regarding *C. hystrix*, which Deng et al. (2007) assigned to the sub-genus *Papeda*, our morphological analysis put the species in a separate subcluster, confirming its ancient origin (Federici et al., 1998; Xie et al., 2008). Instead, by means of RAPD markers, *C. hystrix* groups together with *C. aurantifolia*, a result previously obtained by Nicolosi et al. (2000) and Federici et al. (1998), indicating a probable common lineage.

C. medica is considered by several authors to be a basal species of Indian origin and has always acted as male parent in the origin of several hybrids/cultivars (Federici et al., 1998; Nicolosi et al., 2000; Jena et al., 2009), but its exact phylogenetic position is uncertain. Chloroplast (Nicolosi et al., 2000; Araujo et al., 2003) and nuclear (Nicolosi et al., 2000; Uzun et al., 2009) markers have produced different results; with the former, *C. medica* is very distant from *C. limon*, while with the latter, the two species are in the same cluster. In our analysis, it seems confirmed that *C. medica* is closely related to *C. limon*. As in the studies of Federici et al. (1998) and Xie et al. (2008), based on RAPD/RFLP and AFLP, it is likely that *C. limon* originated from *C. medica*.

4.3. Relationships among C. limonimedica and its supposed ancestors

Regarding *C. limonimedica*, our integrated morphological and RAPD data showed that this lemon-shaped species is strictly linked to its hypothesised parental species, confirming the likely hybrid origin of *C. limonimedica* from *C. medica* and *C. limon*. The PCA scattergram showed a marked differentiation among these species, with *C. limonimedica* in the middle position between *C. limon* and *C. medica*. In addition, both DNA content and phylogenetic analysis based on the ITS sequences showed poor differentiation between the three species, and this result could be due to the very recent origin of *C. limonimedica* from *C. limon* and *C. medica*. In fact, in both clusters formed in the parsimony tree from ITS sequences, it is clear that some accessions of *C. limonimedica* are more closely related to *C. medica*, while others are more tightly associated to *C. limon*. This is likely because of hybridisation followed by repetitive introgression events in the hybrid species.

5. Conclusions

We demonstrated that the combined use of molecular, morphological and cytometric parameters could improve the discriminating power and help resolve complex taxonomic entities in phylogenetic studies. For the vast majority of cases, the origin of *Citrus* species, both wild relatives and derived hybrids, is a complicated and unsolved puzzle. In this study, numeric taxonomy and molecular marker methods have been exploited as an integrated tool to shed light on the hybrid origin of the historical *C. limonimedica* species from *C. limon* and *C. medica*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2011.05.012.

References

Araujo, E.F., Queiroz, L.P., Machado, M.A., 2003. What is *Citrus*? Taxonomic implications from a study of cp-DNA evolution in the tribe Citreae (Rutaceae subfamily Aurantioideae). Org. Divers. Evol. 3, 55–62.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J., 1995. The ITS region of the nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot. Gard. 82, 247–277.

Barcaccia, G., Mazzucato, A., Belardinelli, M., Pezzotti, M., Lucretti, S., Falcinelli, M., 1997. Inheritance of parental genomes in progenies of *Poa pratensis* L. from sexual and apomictic genotypes as assessed by RAPD markers and flow cytometry. Theor. Appl. Genet. 95, 516–524.

Barcaccia, G., Pessina, D., Nicole', S., Gentili, R., Rossi, G., Barbesti, S., Sgorbati, S., 2008. Molecular analysis and flow cytometry evaluation of nuclear DNA in *Citrus limonimedica* Lush, to assess genetic variability and to trace phylogenetic relationship among lemon and cedar. Cytometry Part A 73A, 67.

Barkley, N.A., Krueger, R.P., Federici, C.T., Roose, M.L., 2009. What phylogeny and gene genealogy analyses reveal about homoplasy in *Citrus* microsatellite alleles. Plant Syst. Evol. 282, 71–86.

Barrett, H.C., Rhodes, A.M., 1976. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. Syst. Bot. 1, 105–136.

Bayer, R.J., Mabberley, D.J., Morton, C., Miller, C.H., Sharma, I.K., Pfeil, B.E., Rich, S., Hitchcock, R., Sykes, S., 2009. A molecular phylogeny of the orange subfamily (Rutaceae: Aurantioideae) using cpDNA sequences. Am. J. Bot. 96, 668–685.

Calabrese, F., 2002. Origin and history. In: Dugo, G., Di Giacomo, A. (Eds.), The genus *Citrus*. Taylor & Francis, London, pp. 1–15.

Calabrese, F., 1992. The history of *Citrus* in the Mediterranean countries and Europe. Proc. Int. Soc. Citricult. 1, 35–38.

Carpenter, J.B., Reece, P.C., 1969. Catalog of Genera, Species, and Subordinate Taxa in the Orange subfamily Aurantioideae (Rutaceae). Crops Research, Agricultural Research Service, U.S. Department of Agriculture Beltsville, Maryland, USA.

Chan, K.F., Sun, M., 1997. Genetic diversity and relationships detected by isozyme and RAPD analysis of crop and wild species of *Amaranthus*. Theor. Appl. Genet. 95, 865–873.

Deng, Z., La Malfa, S., Xie, Y., Xiong, X., Gentile, A., 2007. Identification and evaluation of chloroplast uni- and trinucleotide sequence repeats in *Citrus*. Sci. Hortic. 111, 186–192.

Fang, D.Q., Roose, M.L., 1997. Identification of closely related *Citrus* cultivars with inter-simple sequence repeat markers. Theor. Appl. Genet. 95, 408–417.

Federici, C.T., Fang, D.Q., Scora, R.W., Roose, M.L., 1998. Phylogenetic relationships within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theor. Appl. Genet. 96, 812–822.

Felsenstein, J., 1989. PHYLIP—Phylogeny Inference Package (Version 3.2). Cladistics 5, 164–166.

Galeotti, P., Tintori, G., 2000. Gli Agrumi Ornamentali. Calderini Edagricole, Firenze.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeont. Electr. 4, 1–9.
Jena, S.N., Kumar, S., Nair, N.K., 2009. Molecular phylogeny in Indian *Citrus* L (Rutaceae) inferred through PCR-RFLP and trnL-trnF sequence data of chloroplast DNA. Sci. Hortic. 119, 403–416.
Kayim, K., Koc, N.K., Rokka, V.M., 1998. Variation of the nuclear DNA content of species of subtribe *Citrinae* (Rutaceae). HortScience 33, 1247–1250.
Kump, B., Javornik, B., 1996. Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench.) populations by RAPD markers. Plant Sci. 114, 149–158.
Lee, C.W., Kim, H.S., Kim, D.H., Kim, G.O., Choi, S.Y., Kim, S.J., Chang, I.S., 2006. Cosmetic applications of selected various *Citrus* fruits. Planta Med. 72, 344.
Lota, M.L., de Rocca Serra, D., Tomi, F., Bessiere, J.M., Casanova, J., 1999. Chemical composition of peel and leaf essential oils of *Citrus medica* L. and *C. limonimedica* Lush. Flavour Fragr. J. 14, 161–166.
Lushington, A.W., 1910. The genus *Citrus*. Indian For. 36, 323–353.
Mabberley, D.J., 2004. *Citrus* (Rutaceae): a review of recent advances in etymology, systematics and medical applications. Blumea 49, 481–498.
Mantel, N., 1967. The detection of desease clustering and a generalized regression approach. Cancer Res. 27, 209–220.
McDade, L., 1997. Hybrids and phylogenetic systematics. III. Comparison with distance methods. Syst. Bot. 22, 669–683.
Moreira Novelli, V., Takita, M.A., Machado, M.A., 2004. Identification and analysis of single nucleotide polymorphisms (SNPs) in *Citrus*. Euphytica 138, 227–237.
Nati, P., 1674. Florentina phitologica observatio de malo limonia citrata-aurantia Florentiae vulgo la bizzarria. Hippolyti de Naue, Firenze.
Nicolosi, E., Deng, Z.N., Gentile, A., La Malfa, S., Continella, G., Tribulato, E., 2000. *Citrus* phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. 100, 1055–1166.
Ollitrault, P., Dambier, D., Luro, F., Duperrray, C., 1994. Nuclear genome size variation in *Citrus*. Fruits 49, 390–393.
Pang, X.M., Hu, C.G., Deng, X.X., 2007. Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. Genet. Resour. Crop. Evol. 54, 429–436.
Rholf, F.J., 2000. NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System, Version 2.1. Exter Publishing, New York.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.
Sgorbati, S., Levi, M., Sparvoli, E., Trezzi, F., Lucchini, G., 1986. Citometry and flow citometry of 4′,6-diamidino-2-phenylindole (DAPI)-stained suspensions of nuclei released from fresh and fixed tissues of plants. Physiol. Plantarum 68, 471–476.
Swingle, W.T., Reece, P.C., 1967. The botany of *Citrus* and its wild relatives. In: Reuther, W., Webber, H.J., Batchelor, L.D. (Eds.), The *Citrus* Industry, vol. 1. University of California Press, Berkeley, pp. 190–430.
Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D., Moritz, C., Mable, B. (Eds.), Molecular Systematics. , 2nd ed. Sinauer Associates, Sunderland, pp. 407–514.
Tanaka, T., 1977. Fundamental discussion of *Citrus* classification. Stud. Citrol. 14, 1–6.
Tanaka, T., 1954. Species Problem in *Citrus* Revisio Aurantiacearum. Japan Society for the Promotion of Science, Ueno, Tokyo.
Targioni-Tozzetti, O., 1853. Cenni storici sull'introduzione di varie piante nell'agricoltura e orticoltura toscana. Firenze.
Uzun, A., Yesiloglu, T., Aka-Kacar, Y., Tuzcu, O., Gulsen, O., 2009. Genetic diversity and relationships within *Citrus* and related genera based on sequence related amplified polymorphism markers (SRAPs). Sci. Hortic. 121, 306–312.
Volkamer, J.C., 1708–1714. Nurnbergische Hesperides, oder grundliche Beschreibung der Edlen Citronat-Citronen und Pomerantzen-Fruchte, 2 Voll. Nurnberg.
Webber, H.J., 1967. History and development of the *Citrus* industry. In: Reuther, W., Webber, H.J., Batchelor, L.D. (Eds.), The *Citrus* Industry, vol. 1. University of California Press, Berkeley, pp. 1–39.
Xie, R.-J., Zhou, Z.-Q., Deng, L., 2008. Taxonomic and phylogenetic relationships among the genera of the True *Citrus* Fruit Trees Group (Aurantioideae, Rutaceae) based on AFLP markers. J. Syst. Evol. 46, 682–691.
Xu, C.J., Bao, L., Zhang, B., Bei, Z.M., Ye, X.Y., Zhang, S.L., Chen, K.S., 2006. Parentage analysis of huyou (*Citrus changshanensis*) based on internal transcribed spacer sequences. Plant Breeding 125, 519–522.