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*

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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

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0304-4238/$ – see front matter
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).