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RESEARCH ARTICLE

Analysis of ITS of the rDNA to infer phylogenetic relationships among Vietnamese *Citrus* accessions

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Abstract This study focused on clarifying phylogenetic relationships among *Citrus* accessions from Vietnam. Our phylogenetic analysis based on nucleotide sequences from the ITS of the ribosomal DNA included 69 accessions belonging to *Citrus* and related (sub)genera. Maximum parsimony and Bayesian analysis confirmed a clear separation of the three 'true' *Citrus* species (*C. medica*, *C. maxima* and *C. reticulata*). Confirming recent taxonomic revisions, *Fortunella*, *Poncirus trifoliata* and *Citrus hystrix* are clustered among the accessions of subgenus *Citrus*. *C.* × *sinensis* accessions revealed a close evolutionary relationship to either *C. maxima* or *C. reticulata*, thereby confirming their involvement in its hybrid origin. Also, some other hybrid taxa and their proposed parental species were investigated and their origin could in some cases be confirmed using the ITS sequence data.

Keywords *Citrus* · Rutaceae · Phylogeny · ITS of the rDNA · Vietnam

Introduction

The genus *Citrus* is one of 33 (Swingle and Reece 1967) to 26 (Mabberley 2008a, b) genera in the subfamily Aurantioideae of the family Rutaceae. *Citrus* is divided into two subgenera: the common cultivated types of this fruit are placed in the subgenus *Citrus*, species of the subgenus *Papeda* do not bear edible fruit (Moore 2001).

The taxonomy of *Citrus* is complex and the precise number of natural species is unclear. Until the mid 1970s, *Citrus* taxonomy was based solely on morphological and geographical data, leading to two widely used classification systems. The Swingle system (Swingle and Reece 1967) is relatively simple, containing 16 species, but the Tanaka taxonomy recognizes up to 162 species (Tanaka 1977). This lack of agreement reflects differences of opinion as to what degree of difference justifies species status and Tanaka has split the genus *Citrus* into many small groups. There is no definitive work on *Citrus* taxonomy, and many scientists use a system intermediate

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between these two systems (Hodgson 1965, 1967: 36 species; Singh and Nath 1969: 31 species).

Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three (or four) 'basic' true species within the subgenus *Citrus* (as defined by Swingle): citron (*C. medica*), mandarin (*C. reticulata*), and pummelo (*C. maxima*), and sometimes lime (now generally considered a hybrid, *C.* × *aurantiifolia*). Other cultivated *Citrus* taxa within the subgenus *Citrus* are believed to be hybrids derived from these true species, species of the subgenus *Papeda*, or closely related "genera". Interestingly, the earliest plant taxonomists also believed that there were only few valid species of the subgenus *Citrus* (two species, and three varieties in Linnaeus 1753; three species Hooker 1875) and this idea has gained scientific support in recent years from molecular data (Federici et al. 1998; Nicolosi et al. 2000). For instance, morphological and molecular studies have indicated that lime (*C.* × *aurantiifolia*) and lemon (*C.* × *limon*) arose from interspecific crosses with *C. medica* (citron) as one of the parent species (Scora 1975; Barrett and Rhodes 1976; Federici et al. 1998; Nicolosi et al. 2000).

Genetic variability and relationships among cultivated taxa is complicated by several factors, like the high frequency of bud mutation and nucellar embryony, a long history of cultivation and wide cross-compatibility, leading to taxonomic ambiguities (Nicolosi et al. 2000; Moore 2001). Spontaneous or artificial hybridization and sport formation has probably played an important role in the origin of many cultivated *Citrus* taxa. The wide human-induced dispersion of *Citrus* across geographic boundaries has further facilitated "intergeneric" and intrageneric crossing leading to an immense variety of morphological forms. Interesting hybrids or sports can easily be vegetatively propagated, leading to a contrast between high levels of morphological (mainly agronomic) trait diversity versus low levels of genetic variability within taxa, as described for clementine (*C. reticulata* Blanco; Bretó et al. 2001); lemon (*C.* × *limon* (L.) Osbeck.; Gulsen and Roose 2001) and trifoliate oranges (*Poncirus trifoliata*; Fang et al. 1997).

Understanding taxonomy, phylogenetic relationships and genetic variability in *Citrus* is critical for determining genetic relationships, characterizing germplasm, establishing breeding programs and the registration of new cultivars. Vietnam, located in the South East Asian center of origin of *Citrus* (Webber 1967; Scora 1975), is a center of biodiversity for wild and cultivated *Citrus* accessions (Tanaka 1954). *Citrus* has always been one of Vietnam's most popular fruit products and as a consequence they are grown widely from the North to the South of Vietnam, leading to a high abundance of *Citrus* genetic resources.

In this study the phylogenetic relationships among 61 *Citrus* accessions collected in Vietnam were analysed using ITS sequences of the rDNA, and compared with data from 8 accessions present in the NCBI-database.

Materials and methods

Plant material

Table 1 gives an overview of the analysed accessions, their origin and institute codes. Table 2 provides the scientific names of all taxa used in this manuscript, corresponding vernacular names and their names as put forward by Mabberley (2008a, b).

In total 51 accessions belonging to *Citrus* subgenus *Citrus* were collected: 1 *C.* × *paradisi* (grapefruit: G), 11 *C. maxima* (pummelo: P), 5 *C. medica* (citron: C), 3 *C.* × *aurantiifolia* (lime: L), 1 *C.* × *limon* (lemon: LI), 12 *C.* × *sinensis* (sweet orange: O), 1 *C.* × *aurantium* (sour orange: OS), 15 *C. reticulata* (mandarin: M), 1 *C. reticulata* 'Clementine' (Clementine: M), 1 *C.* × *nobilis* (king mandarin: MK). Furthermore, the following 10 accessions, that have been described to belong to closely related genera or subgenera, were included in the sample set: 5 specimens of *C. hystrix* (kaffir lime: KL), belonging to the subgenus Papeda, 1 *Poncirus trifoliata* accession (PT), 3 *Fortunella* accessions (*Fortunella japonica* and *Fortunella margarita*: F) and 1 *Murraya paniculata* specimen.

Next to the 61 specimens collected in Vietnam, the NCBI database contains ITS sequence data from 6 *Citrus* accessions, and from 2 accessions from the related genus *Murraya*: *Murraya paniculata* and *Murraya koenigii*.

DNA isolation

Total cellular DNA was isolated as described by Rogers and Bendich (1988) with minor modifications.

Table 1 Overview of analyzed taxa, accession names, isolate codes, GenBank accession numbers, and their origin

Taxon	Accession name	Isolate code	GenBank accession no.	Origin
Citrus × *paradisi*	G44BC603D	STGBC603	FJ641956	SOFRI, Mekong Delta (Tien Giang) from USA
C. maxima	P12Duongl	SH-P12	FJ641947	BiRDI, eastern south Vietnam (Bien Hoa)
	PNamRoiDC	SH-TKD5	FJ641954	BiRDI, Mekong Delta (Binh Minh, Vinh Long)
	P39NuomH	SH-P39	FJ641948	FRDC-Hue, central Vietnam (Hue)
	P4RungChu	SH-P4	FJ641944	BIRDI, central of Vietnam (Nha Trang)
	P36Daxanh	SH-P36	FJ641953	BiRDI, Mekong Delta (Mr NamChuc, Ben Tre)
	P9BungHT	SH-P9	FJ641952	BiRDI, Mekong Delta (Ha Tien)
	P47PhucTr	SH-P47	FJ641951	FRDC-Hue, central Vietnam (Hue)
	P46SuuDH	SH-P46	FJ641950	BiRDI, midland region north Vietnam (Doan Hung)
	P19ThanhT	SH-P19	FJ641949	FRDC-Hue, central Vietnam (Hue)
	P10BangLu	SH-P10	FJ641946	BiRDI, midland region north Vietnam (Doan Hung)
	P8DoanHun	SH-P8b	FJ641945	PQFC, midland region north Vietnam (Doan Hung)
	C. maxima1	/	AM398229.1	
	C. maxima2	/	AM398228.1	
C. medica	Cmedica1	/	AM260544.1	
C. medica 'Fingered'	C29PhatMT	FX79	FJ641967	PQFC, central region of Vietnam (Nghe An)
	C66PhatHN	SH-L66	FJ641968	RIFAV, north Vietnam (Ha Noi)
	Cmedica2	/	AM260543.1	
C. medica 'Etrog'	C34YenSP	SH-L34	FJ641962	BiRDI, northwest Vietnam (Sa Pa)
	C18YenThanh	SH-L18	FJ641969	RIFAV, north Vietnam (Ha Noi)
	C42Yenr	SH-L42	FJ641965	BiRDI, northwest Vietnam (Sa Pa)
C. × *aurantiifolia*	L56GiayHN	SH-L56	FJ641964	RIFAV, Ha Noi
	L63Ta38	F38	FJ641955	PQFC, north Vietnam (Bac Giang)
	L39To17	F17	FJ641966	PQFC, central region of Vietnam (Nghe An)
C. × *limon*	LI59Tuthoi	F29	FJ641963	PQFC, central region of Vietnam (Nghe An)
C. × *sinensis*	O1ValenciaNH	SH-O1	FJ641911	BiRDI, central Vietnam (Nghe An)
	O5ValenciaSB	SH-O5	FJ641912	BiRDI, Mekong Delta, imported from USA
	O33ValenciaDT	SH-O33	FJ641913	SOFRI, Mekong Delta, imported from USA
	O3MatTG	STG-14	FJ641941	SOFRI, Mekong Delta (Tien Giang)
	O35MatDT	SH-O35	FJ641942	BiRDI, Mekong Delta (Dong Thap)
	O41MatDC	SH-41	FJ641940	BiRDI, Mekong Delta (Can Tho)
	O11Hamlin	F75	FJ641914	PQFC, central Vietnam (Nghe An)
	O42Xadoai	X1	FJ641918	ASINC, northern central Vietnam
	O43Xadoai	X2	FJ641919	ASINC, northern central Vietnam
	O44Xadoai	X3	FJ641920	ASINC, northern central Vietnam
	O45Xadoai	X4	FJ641921	ASINC, northern central Vietnam
	O46Xadoai	X5	FJ641922	ASINC, northern central Vietnam
C. × *aurantium*	OSArizona	SH-OS1	FJ641910	BiRDI, collected in Tucson USA
	Caurant		EF590763.1	Voucher USDA PI128347

Table 1 continued

Taxon	Accession name	Isolate code	GenBank accession no.	Origin
C. reticulata	M11WalyTangerine	FX84	FJ641928	PQFC, central Vietnam (new variety from USA)
	M26BopBoH	SH-26	FJ641916	RIFAV, north Vietnam (Bac Giang)
	M14TaMB	F26	FJ641930	PQFC, north Vietnam (Thanh Hoa)
	M25ChuSa	F11	FJ641915	PQ FC, northwest Vietnam (Lang Son)
	M30Cleopatra	FX9	FJ641917	PQFC, central Vietnam (Nghe An)
	M9VangNLo	F14	FJ641936	PQFC, northwest Vietnam (Yen Bai)
	M31Lua	F13	FJ641939	PQFC, northwest Vietnam (Yen Bai)
	M24Chum	SH-24	FJ641932	RIFAV, north Vietnam (Ha Giang)
	M33VangLS	F87	FJ641933	PQFC, northeast Vietnam (Lang Son)
	M21QHongNhieu	SH-21	FJ641937	FRDC-Hue, Central Vietnam (Hue)
	M22CHongNhieu	SH-22	FJ641938	BiRDI, central Vietnam (Nghe An)
	M47DuongNL	SH-M47	FJ641934	BiRDI, Mekong Delta (Can Tho)
	M23AnhSon	SH-M23	FJ641931	RIFAV, Ha Noi
	M13VisaND	FV64	FJ641929	PQ FC, central of Vietnam (Nghe An)
	M37DuongD	SH-M37	FJ641926	BiRDI, Mekong Delta (Dong Thap)
	Creticul	/	AM398230.1	
C. reticulata 'clementine'	M4ClemenD	FX78	FJ641935	PQFC, central region of Vietnam (Nghe An)
C. × *nobilis*	MK8Camsan	SH-MK8	FJ641927	SOFRI, Mekong Delta (Tien Giang)
C. hystrix	KL14Truc	SH-L14	FJ641957	BiRDI, Mekong Delta (Chau Doc)
	KL14CTrucDC	SH-L14C	FJ641958	BiRDI, Mekong Delta (Chau Doc)
	KL16TrucTL	SH-L16	FJ641959	BiRDI, collected in Chaingmai, Thailand
	KL20TrucHT	SH-L20	FJ641960	BiRDI, Mekong Delta (Ha Tien)
	KL21TrucHT	SH-L21	FJ641961	BiRDI, Mekong Delta (Ha Tien)
Fortunella japonica	F43Quat	SH-F43	FJ641923	BiRDI, North of Vietnam (Ha Noi)
Fortunella margarita	F42Nagami	SH-F42	FJ641924	BiRDI, Mekong Delta. Imported from USA
Fortunella sp.	F44HanhMK	SH-F44	FJ641925	BiRDI, Mekong Delta (Can Tho)
Poncirus trifoliata	PT41Cambal	SH-O41	FJ641943	RIFAV, north Vietnam (Ha Noi)
Murraya paniculata	MurrayaOK	SH-Mu	FJ641970	BiRDI, Mekong Delta
	Mpanicu	/	AJ879085.1	
Murraya koenigii	Mkoenig	/	AJ879084.1	

Young leaves from fully expanded and mature plants were collected and maintained at low temperature in polyethylene bags. In the laboratory, the leaves were washed in distilled water, ethanol 70% and ground using the Retsch mixer mill model MM 200. Each sample was suspended in 1.0 ml of DNA extraction buffer. After incubation at 65°C for 30 min with occasional vigorous shaking, the samples were centrifuged at 13,000*g* for 10 min. The supernatant was collected and an equal volume (about 700 µl) of isopropanol was added. The samples were mixed, and placed on ice (or −20°C) for 2 h. The samples were centrifuged at 13,000*g* for 10 min and the supernatant was discarded. After addition of 400 µl of TE Buffer and 5 µl RNase the samples were incubated at 37°C for 20 min. 400 µl of CTAB Buffer was added and the samples were transferred to a warm water bath at 65°C for 15 min. Afterwards, an equal volume of isoamyl alcohol:chloroform (24:1) was added, and the samples were centrifuged at 13,000*g*. To the aqueous phase (upper phase) two volumes of 96% ethanol were added. After incubation at room temperature for 5 min the samples were centrifuged for 5 min (10,000*g*). The pellet was then washed twice

Table 2 Scientific names of all taxa used in this manuscript, corresponding vernacular names and their name as put forward by Mabberley (2008a, b)

Vernacular name	Scientific names used in this text	Names used by Mabberley (2008a, b)
Citron	*Citrus medica* L.	=
Clementine	*Citrus reticulata* Blanco 'Clementine'	=
Grapefruit	*Citrus* × *paradisi* Macfad.	*Citrus* × *aurantium* grapefruit group
Kaffir lime	*Citrus hystrix* DC.	=
King mandarin	*Citrus* × *nobilis* Lour.	*Citrus* × *aurantium* tangor group
Lemon	*Citrus* × *limon* (L.) Osbeck	=
Lime	*Citrus* × *aurantiifolia* (Christm.) Swingle	=
Mandarin	*Citrus reticulata* Blanco	=
Pummelo	*Citrus maxima* (Burm.) Merr.	=
Sour orange	*Citrus* × *aurantium* L.	*Citrus* × *aurantium* sour orange group
Sweet orange	*Citrus* × *sinensis* (L.) Osbeck	*Citrus* × *aurantium* sweet orange group
Trifoliate orange	*Poncirus trifoliata* (L.) Raf.	*Citrus trifoliata* L.
Round kumquat	*Fortunella japonica* (Thunb.) Swingle	*Citrus japonica* Thunb.
Oval kumquat	*Fortunella margarita* (Lour.) Swingle	*Citrus japonica* Thunb.
Orange jessamine	*Murraya paniculata* (L.) Jack	=
Curry leaf	*Murraya koenigii* (L.) Sprengel	*Bergera koenigii* L.
Xxx	*Microcitrus* species	*Citrus* species
Xxx	*Citrus micrantha* Wester	=

with ethanol 70%. The DNA was resuspended in 200 µl TE Buffer, applying a short incubation at 37°C. DNA samples were stored at −20°C.

DNA extraction, PCR and sequencing

PCR amplification of the ITS region, including the 5.8 S rDNA region, was performed using primers ITS-1 and ITS-4 (ITS1: 5′ TCCGTAGGTGAACCT GCGG 3′; ITS4: 5′ TCCTCCGCTTATTGATATGC 3′ as described by White et al. (1990), using a Perkin Elmer 9700 thermal cycler (Applied Biosystems corporation). Final reaction volumes of 25 µl each contained 50 ng genomic DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (Fermentas), 1× PCR buffer supplied by the manufacturer and about 2.5 mM $MgCl_2$. The amplification programme consisted of predenaturation at 94°C for 90 s; 30 cycles at 95°C for 50 s, 55°C for 70 s and 72°C for 90 s; and a final incubation at 72° for 3 min; 1 min at 30°C; and a final hold at 4°C. $MgCl_2$ concentration and annealing temperature had to be optimized for some of the samples to obtain a good amplification.

PCR products were purified by PureLink™ PCR Purification kit (Invitrogen). Purified fragments were directly sequenced with PCR primers using the ABI prism BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI prism 3130, Applied Biosystems).

Phylogenetic analyses

Next to the 61 new sequences obtained from Vietnamese accessions in the current analysis, ITS sequence data from 6 *Citrus* accessions, and from 2 accessions from related genus *Murraya* (*Murraya paniculata* and *Murraya koenigii*) was included in the dataset.

Sequences were aligned using ClustalX (Thompson et al. 1997) followed by manual adjustments using BioEdit 7.0.5.3. Phylogenetic analyses were carried out using PAUP* v4.0b10 (Swofford 2002) and MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). Parsimony analyses were performed with PAUP* v4.0 b10 (Swofford 2002) using the heuristic search option with random sequence addition (100 random

replications) and TBR branch-swapping. All characters had equal weight, and gaps were treated as missing characters. Constant and uninformative characters were removed from the data matrix. Consistency index (CI), retention index (RI) and rescaled consistency index (RC) were calculated. Support for the different clades was tested by bootstrap analysis (100 replicates using heuristic search, simple sequence addition). Bayesian analysis was run using MrBayes version 3.1.2. (Ronquist and Huelsenbeck 2003). Bayesian inference was run for 3,000,000 generations, and the first 100,000 generations were discarded as burn-in.

Results

Sequence data from the ITS region of the rDNA was analysed for 69 accessions, including 8 sequences obtained from the NCBI database. The alignment of all sequences included 703 positions (including gaps), 298 positions were variable among which 126 were parsimony-informative sites.

Parsimony analysis produced 14,778 equally parsimonious trees of 286 steps with a consistency index (CI) of 0.6538, a retention index (RI) of 0.8350, and a rescaled consistency index (RC) of 0.5460. A majority-rule consensus tree was constructed from these trees as shown in Fig. 1. The bootstrap values (100 replicates) are shown on each branch. The tree reconstructed by Bayesian analysis is shown in Fig. 2. The tree obtained is very similar to that of the parsimony method, although the separation of the subclusters is less obvious in the Bayesian tree.

The phylogenetic trees based on both maximum parsimony and Bayesian analyses show a clear separation between the three 'basic' species as proposed by Scora (1975) and Barrett and Rhodes (1976). Cluster 1 contains all *C. maxima* accessions together with *C.* × *paradisi*, 1 *C.* × *aurantiifolia* (1a), and *Poncirus trifoliata* and some of the *C.* × *sinensis* genotypes (1b). Cluster 2 combines *C. medica* with all other *C.* × *aurantiifolia* and *C.* × *limon* (2a); and *C. hystrix* (2b). *C. reticulata* and *C.* × *aurantium* are grouped (3a), together with most of the *C.* × *sinensis*. *Fortunella japonica* and *Fortunella margarita* are in 3b. Pairwise sequence divergence ranged from 0 (between multiple accessions) to 0.215 (between *Murraya koenigii* and Cmedica1) with an average of 0.038. Within Citrus, average pairwise sequence divergence was 0.030, with a maximum of 0.143 (Cmedica1 vs. 043Xadoai). No sequence divergence was found among a few mandarin accessions from cluster 3a, and among some oranges from the same cluster. 3 *C. medica* accessions (cluster 2a) also revealed 100% ITS sequence identity. Furthermore, 7 *C. maxima* members of cluster 1 have identical ITS sequences. Interestingly, ITS sequence of the grapefruit accession was 100% identical to these *C. maxima* accessions.

Discussion

In this study sequence data from ITS of the rDNA of 69 accessions from the genus *Citrus* and related genera were obtained and their evolution was investigated using maximum parsimony and Bayesian analyses. In contrast to previous studies the current phylogenetic analysis includes a larger number of closely related accessions of a few closely related species instead of one (or a few) specimens from different genera, which allows us to investigate relationships at a lower taxonomic level and to investigate evolutionary divergence within taxa.

The separation of the three 'true' *Citrus* (*C. medica*, *C. maxima* and *C. reticulata*) is confirmed by their grouping in three different clusters in our ITS of the rDNA sequence analysis.

As isozyme and morphological data suggested before (Barrett and Rhodes 1976; Torres et al. 1978) and could be expected based on vegetative propagation of cultivars, ITS-data reveal a close evolutionary relationship among the analysed *C. maxima* accessions. Furthermore, our data confirm a close evolutionary relationship between *C. maxima*, *C.* × *paradisi* and, although more distantly, some sweet orange accessions (*C.* × *sinensis*). cpDNA analysis of these three species has also shown these species to be very closely related (Nicolosi et al. 2000; Kyndt et al., unpublished data).

Grapefruit (*C.* × *paradisi*) has been proposed to be of hybrid origin, with pummelo as mother and sweet orange as father (Gmitter 1995; Moore 2001) and subsequent backcrossing with pummelo (Fang and Roose 1997; Herrero et al. 1996; Pang et al. 2007, Mabberley 2008a, b). This hypothesis is confirmed by our ITS sequence data, since the

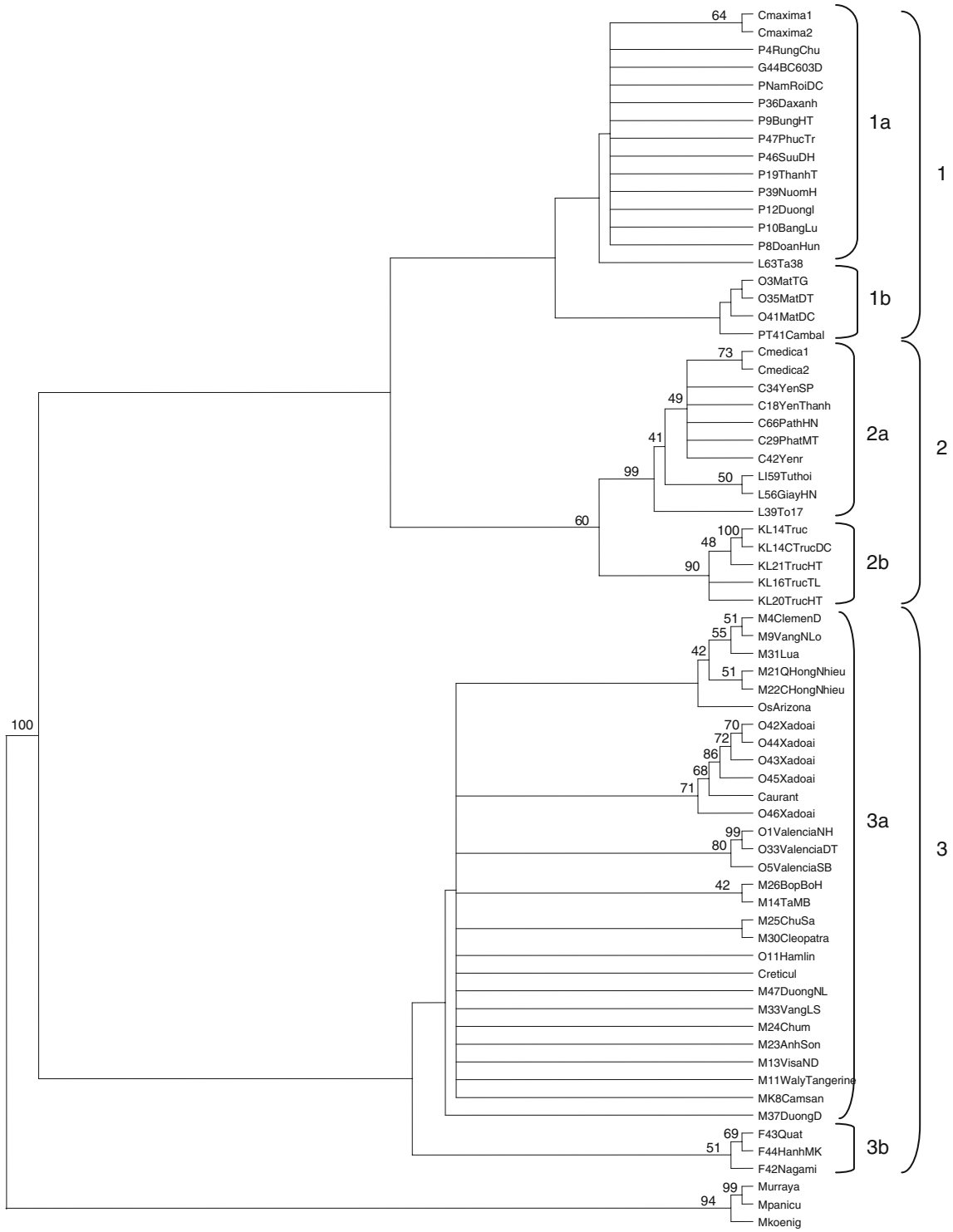

Fig. 1 Majority rule consensus tree of 14,778 maximum parsimonious trees of 69 *Citrus* accessions based on ITS of the rDNA sequence data. Tree length = 286; consistency index (*CI*) = 0.6538; retention index (*RI*) = 0.8350; rescaled consistency index (*RC*) = 0.5460. Bootstrap values above 40% are given on the nodes. The tree is rooted with the three *Murraya* accesssions

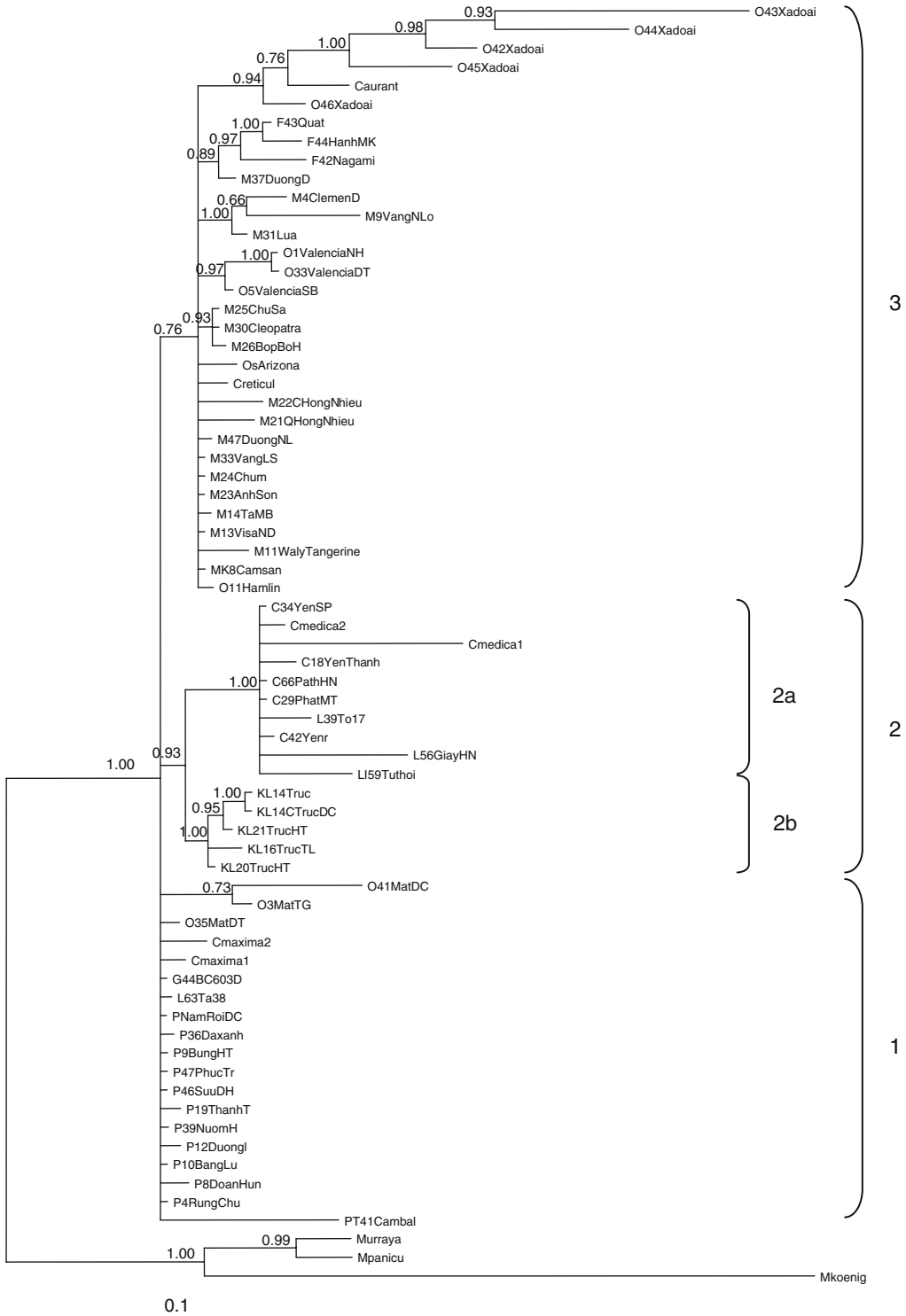

Fig. 2 Bayesian phylogenetic tree of 69 *Citrus* accessions based on ITS of the rDNA sequence data. The posterior probability is given on each node. The tree is rooted with the three *Murraya* accesssions. The *scale bar* represents branch length (number of substitutions/site)

grapefruit accession reveals 100% sequence identity with some pummelo accessions at ITS level.

The mandarin (*C. reticulata*) cluster is not well resolved, as also seen in other molecular data analyses (Federici et al. 1998; Barkley et al. 2006). Sour oranges (*C.* × *aurantium*) and most of the sweet oranges (*C.* × *sinensis*) cluster among the mandarins, confirming the mandarins as one of their parental species (Barrett and Rhodes 1976) as suggested by previous molecular data (Nicolosi et al. 2000; Barkley et al. 2006; Pang et al. 2007).

Sweet orange (*C.* × *sinensis*) is thought to be a natural hybrid between predominantly *C. reticulata* and some *C. maxima* traits (Scora 1975; Barrett and Rhodes 1976). Molecular data already confirmed that the chloroplast genome of sweet orange is derived from pummelo (Green et al. 1986; Nicolosi et al. 2000; Barkley et al. 2006; Kyndt et al., unpublished data). This ITS-sequence analysis and the chloroplast PCR-RFLP study of Jena et al. (2009) suggest that *C.* × *sinensis* has a polyphyletic origin. While some Vietnamese sweet orange genotypes are closely related with pummelo, others are grouped with mandarin. Most probably *C.* × *sinensis* originated from one or a few hybridization events between pummelo as maternal parent and mandarin as father and subsequent backcrosses with one of these parents. It has to be noted that some well-established *C.* × *sinensis* cultivars ('Xadoai' and 'Valencia') are highly supported in the phylogenetic trees, and are found within the mandarin group.

Citrus medica is grouped with the proposed hybrid species *C.* × *limon* (lemon) and *C.* × *aurantiifolia* (lime), which is consistent with the fact that the citron has been proposed to be the paternal ancestor of several hybrids in *Citrus* (Federici et al. 1998; Nicolosi et al. 2000). While Barrett and Rhodes (1976) proposed that lime arose from a trihybrid "intergeneric" cross involving *C. medica*, *C. maxima* and a "*Microcitrus*" species, RAPD and SCAR markers (Nicolosi et al. 2000) suggested that limes resulted from a cross between *C. micrantha* (subgenus *Papeda*) and *C. medica* (male parent). Isozyme analyses (Torres et al. 1978) found low diversity between seven cultivars of lime (*C.* × *aurantiifolia*) and this was suggested to be due to its apomictic perpetuation (Barrett and Rhodes 1976). However, this is contradicted by the current study, where one lime accession clusters with pummelo and all others with citron, suggesting *C. maxima* and *C. medica* as parent species of *C.* × *aurantiifolia*.

Lemons (*C.* × *limon*) are thought to be natural hybrids of *C. medica* and lime (Scora 1975; Barrett and Rhodes 1976) or a hybrid of citron and sour orange (Gulsen and Roose 2001). Lemon is clustered among the citron-lime group (cluster 2a in Fig. 1), suggesting that lime is indeed one of the ancestors of lemon (Scora 1975; Barrett and Rhodes 1976). No relationship with sour orange is seen in our study. Although our ITS data confirm a close relationship between *C.* × *limon* and *C. medica*, and thereby confirm the involvement of the latter species in its hybrid origin, no clear-cut conclusions can be drawn about the other hypothetic ancestor species of this hybrid taxon.

ITS data shows a close evolutionary relationship between *Fortunella* and *Citrus* spp., although their morphology is very different. This observation agrees with previous molecular studies (Green et al. 1986; Pang et al. 2007), where some analyses even showed a nested clustering of *Fortunella* in *Citrus* (Herrero et al. 1996; Federici et al. 1998; Nicolosi et al. 2000; Pang et al. 2003; Barkley et al. 2006). Also in our analysis *Fortunella* spp. are clustered within *Citrus*, close to the *C. reticulata* group, confirming their recent reclassification as *Citrus japonica* (Mabberley 2008a, b). The same is true for *Poncirus trifoliata*, which is clustered within *Citrus*, and is now called *Citrus trifoliata* (Mabberley 2008a, b).

While Herrero et al. (1996) and Federici et al. (1998) find *C. hystrix* (kaffir lime, subgenus *Papeda*) clustered with *C. maxima*, Nicolosi et al. (2000) and our ITS data suggest that *C. hystrix* is closer to *C. medica*, *C.* × *limon* and *C.* × *aurantiifolia*. Generally, these observations demonstrate that the subdivision of the subgenera *Citrus* and *Papeda*, as proposed by Swingle and Reece (1967), based on the abundant presence of acridic oil in the fruit and the very broadly winged petioles in subgenus Papeda is not confirmed by molecular data. Based on all observations we can hypothesize that *C. hystrix* is a probable (grand)parent or sister species of *C.* × *aurantiifolia* or *C. maxima* and subsequently diverged independently from the subgenus *Citrus*.

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References

Barkley NA, Roose ML, Krueger RR, Federici CT (2006) Assessing genetic diversity and population structure in a *Citrus* germplasm collection utilizing simple sequence repeat markers (SSRs). Theor Appl Genet 112:1519–1531

Barrett HC, Rhodes AM (1976) A numerical taxonomic study of the affinity relationships in cultivated *Citrus* and its close relatives. Syst Bot 1:105–136

Bretó MP, Ruiz C, Pina JA, Asins MJ (2001) The diversification of *Citrus clementina* Hort. Ex Tan., a vegetatively propagated crop species. Mol Phyl Evol 21:285–293

Fang DQ, Roose ML (1997) Identification of closely related *Citrus* cultivars with inter-simple sequence repeats markers. Theor Appl Genet 95:408–417

Fang DQ, Roose ML, Krueger RR, Federici CT (1997) Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers. Theor Appl Genet 95:211–219

Federici CT, Fang DQ, Scora RW, Roose ML (1998) Phylogenetic relationships within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theor Appl Genet 94:812–822

Gmitter FG Jr (1995) Origin, evolution and breeding of the grapefruit. Plant Breed Rev 13:345–363

Green RM, Vardi A, Galune E (1986) The plastome of *Citrus*. Physical map, variation among *Citrus* cultivars and species and comparison with related genera. Theor Appl Genet 72:170–177

Gulsen O, Roose ML (2001) Lemons: diversity and relationships with selected *Citrus* genotypes as measured with nuclear genome markers. J Amer Soc Hort Sci 126:309–317

Herrero R, Asins MJ, Carbonell EA, Navarro L (1996) Genetic diversity in the orange subfamily Aurantioideae. I. Intraspecies and intragenus genetic variability. Theor Appl Genet 92:599–609

Hodgson RW (1965) Taxonomy and nomenclature in the *Citrus* fruits. In: Krishnamurthi S (ed) Advances in agriculture sciences and their applications. Agric. Coll. Res. Inst., Coimbatore, pp 317–331

Hodgson RW (1967) Horticultural varieties of *Citrus*. In: Reuther W, Webber HJ, Batchelor LD (eds) The *Citrus* industry. Vol. I. history world distribution, botany, and varieties. Univ of California, Div of Agricultural Sci, Berkeley, pp 431–591

Hooker JD (1875) Rutaceae. In: Reeve L (ed) The flora of British India. London, pp 484–517

Jena SN, Kumar S, Nair NK (2009) Molecular phylogeny in Indian *Citrus* L. (Rutaceae) inferred through PCR-RFLP and trnL-trnF sequence data of chloroplast DNA. Scientia Hort 119:403–416. doi:10.1016/j.scienta.2008.08.030

Linnaeus C (1753) Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Holmiae

Mabberley DJ (2008a) 21. *CITRUS* Linnaeus, Sp. Pl. 2: 782. 1753. Flora China 11:90–96

Mabberley DJ (2008b) Mabberley's plant book: a portable dictionary of plants, 3rd edn. Cambridge University Press, Avon

Moore GF (2001) Orange and lemons: clues to the taxonomy of *Citrus* from molecular markers. Trends Genet 17:536–540

Nicolosi E, Deng ZN, 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 100:1155–1166

Pang XM, Hu DG, Deng XX (2003) Phylogenetic relationships among *Citrus* and its relatives as revealed by SSR markers. Acta Genet Sin 30:81–87

Pang XM, Hu CG, Deng XX (2007) Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. Genet Resour Crop Evol 54:429–436

Rogers SO, Bendich AJB (1988) Extraction of DNA from plant tissues Plant molecular Biology Manual, vol A6. Kluwer Academic Publishers, Dordrecht, pp 1–10 printed in Belgium

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 12:1572–1574

Scora RW (1975) On the history and origin of *Citrus*. Bull Torrey Bot Club 102:369–375

Singh R, Nath N (1969) Practical approach to the classification of *Citrus*. Proceedings of 1st international *Citrus* symposium, pp 435–440

Swingle WT, Reece PC (1967) The botany of *Citrus* and its wild relatives. In: Reuther W, Webber HJ, Batchelor LD (eds) The *Citrus* industry. Vol. I. History, world distribution, botany, and varieties. Univ of California, Div of Agricultural Sci, Berkeley, pp 190–430

Swofford DL (2002) PAUP. Phylogenetic analysis using parsimony (* and other methods). Version 4.0. Sinauer Associates, Boston

Tanaka T (1954) Species problem in *Citrus*. A critical study of wild and cultivated units of *Citrus*, based upon field studies in their native homes. Japanese Society for the Promotion of Science, Ueno

Tanaka T (1977) Fundamental discussion of *Citrus* classification. Stud Citrol 14:1–6

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Torres AM, Soost RK, Diedenhofen U (1978) Leaf isozymes as genetic markers in *Citrus*. Am J Bot 65:869–881

Webber HJ (1967) History and development of the *Citrus* industry. In: Reuther W, Webber HJ, Batchelor LD (eds) The *Citrus* industry. Vol. I. history world distribution, botany, and varieties. Univ of California, Div of Agricultural Sci, Berkeley, pp 1–39

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322