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ORIGINAL ARTICLE

Untangling the hybrid origin of the Chinese tea roses: evidence from DNA sequences of single-copy nuclear and chloroplast genes

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Abstract The tea-scented China roses largely correspond to the three recognized double-petaled *Rosa odorata* (Andrews) Sweet (Rosoideae, Rosaceae) varieties, which are the ancestors of modern hybrid tea roses and had a definite and permanent influence on the evolution of modern garden roses. Here the hypothesis of a hybrid origin of the tea-scented China roses between *R. odorata* var. *gigantea* and *R. chinensis* was tested. Two single-copy nuclear genes of the cytosolic glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the chloroplast-expressed glutamine synthetase (*ncpGS*) together with two plastid loci (*trnL-F* and *psbA-trnH*) were sequenced for representative accessions of four *R. odorata* varieties, *R. chinensis*, and 28 other *Rosa* species. Phylogenetic relationships were estimated from two nuclear loci using maximum parsimony and Bayesian analyses, and a haplotype network was constructed on the combined plastid data using NETWORK. For *GAPDH* and *ncpGS* loci, the clonal sequences of the three double-petaled varieties were clustered into two clades, one clade with *R. odorata* var. *gigantea*, and the other with partial sequences of *R. chinensis*, which suggested that the tea-scented China roses were hybrids between *R. odorata* var. *gigantea* and *R. chinensis*. Two plastid loci suggested that *R. odorata* var. *gigantea* could be the maternal parent and *R. chinensis* the paternal parent.

Keywords *Rosa odorata* · Hybridization · *GAPDH* · *ncpGs* · *psbA-trnH* · *trnL-F*

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Introduction

Hybridization has long been considered as an important mechanism in plant speciation (Grant 1981; Abbott 1992; Rieseberg and Wendel 1993; Arnold 1997; Rieseberg 1997; Rieseberg and Carney 1998; Hegarty and Hiscock 2005). The barriers to gene flow among *Rosa* L. species are weak (Atienza et al. 2005), and hybridization has contributed greatly to species diversity of this genus (Rehder 1940; Wissemann and Ritz 2005). Numerous fertile interspecific rose hybrids have been reported (Fagerlind 1944, 1951, 1958; Grustafsson 1944; Lewis and Basye 1961; Roberts 1977; Atienza et al. 2005; Bruun 2005; Joly et al. 2006; Mercure and Bruneau 2008). The whole section *Caninae* seems to be of hybrid origin (Zielinsky 1985; Wissemann 1999), and several species of this section have

been demonstrated to be hybrids (Wissemann 1999; Schanzer and Vagina 2007; Schanzer and Kutlunina 2010; Ritz and Wissemann 2011).

Interspecific hybridizations have also played an important role in the origin of modern garden roses (Gudin 2000). According to Wylie (1954), the modern garden roses originated from repeated hybridizations among more than ten *Rosa* species, of which seven made major contributions: *R. chinensis* Jacq., *R. odorata* var. *gigantea* (Crépin) Rehder & E.H. Wilson, *R. moschata* Herrm., *R. luciae* Franchet & Rochebrune var. *luciae*, *R. multiflora* Thunb., *R. gallica* L., and *R. foetida* Herrm. Introduction of Chinese roses into Europe and their subsequent hybridization with European roses formed the foundation of modern rose breeding. Chinese roses introduced characters of recurrent flowering, tea scent, and multiple floral colors into modern roses. The fascinating history of modern roses has to date been studied primarily by morphological analyses and written records. Molecular study of the issue is badly needed.

The modern hybrid tea roses are the most important members of the modern roses. The tea scent of hybrid tea roses is mainly due to 3,5-dimethoxytoluene (DMT), a scent compound known primarily from *R. odorata* var. *gigantea* (Joichi et al. 2005; Scalliet et al. 2008). Three tea-scented China roses (Parsons' Pink China, Hume's Blush Tea-Scented China, and Parks' Yellow Tea-Scented China) of possible origin in Yunnan province of China were introduced into Europe around 1800, crossed with other roses, and produced modern hybrid tea roses (Hurst 1941; Wylie 1954; Joichi et al. 2005). These tea-scented Chinas are still found in the Yunnan province of China and largely correspond to the three recognized double-petaled *R. odorata* (Andrews) Sweet varieties.

Rosa odorata is one of the three members of *Rosa* sect. *Chinenses* (Ku and Robertson 2003) and was first described as a variety of *R. indica* (Andrews 1810). Sweet (1818) subsequently treated it as a separate species. Four varieties of this species are currently recognized: *R. odorata* var. *gigantea*, var. *odorata*, var. *erubescens* (Focke) T. T. Yu & T. C. Ku, var. *pseudindica* (Lindley) Rehder (Table 1; Ku and Robertson 2003). *Rosa odorata* var. *gigantea* ($2n = 2x = 14$; Jian et al. 2010) has single-petaled, white to creamy-white flowers. It is naturally distributed in the Yunnan province of China and adjacent regions of Myanmar, Thailand, and Vietnam (Ku and Robertson 2003). The other three varieties (aside from the typical variety) have double- to semi-double-petaled flowers, are found mainly in human-disturbed areas in the Yunnan province of China, and are occasionally cultivated in other areas. *Rosa odorata* var. *odorata* ($2n = 2x = 14$; Jian et al. 2010) has white to pinkish flowers and is distributed in the Yunnan province of China; this variety is also cultivated elsewhere. *Rosa odorata* var. *erubescens* ($2n = 2x = 14$, or $2n = 3x = 21$; Jian et al. 2010) has pink to pale pink flowers and is distributed in the northwestern Yunnan province. *Rosa odorata* var. *pseudindica* ($2n = 2x = 14$; Jian et al. 2010) has yellow to orange flowers and is distributed in the northwestern Yunnan province. Based on a close reading of Hurst's (1941) descriptions, these three double-petaled varieties correspond to three of the four "Stud Chinas" introduced from China into Europe around 1800, which had a definite and permanent influence on the evolution of modern garden roses. According to Hurst's descriptions,

Table 1 The main morphological characters, distribution information, and chromosome number of varieties of *R. odorata* and *R. chinensis*, with respective names taken from Hurst's (1941) descriptions

Species name	Chromosome number	Petal type	Petal color	Distribution	Names from Hurst's descriptions
R. odorata var. *odorata*	$2n = 2x = 14$	Double or semi-double	White or pinkish	Widely cultivated elsewhere	Hume's Blush Tea-scented China
R. odorata var. *pseudindica*	$2n = 2x = 14$	Double	Yellow or orange	NW Yunnan in China	Parks' Yellow Tea-Scented China
R. odorata var. *erubescens*	$2n = 2x$, $3x = 14$, 21	Double	Pale pink	NW Yunnan in China	Parsons' Pink China
R. odorata var. *gigantea*	$2n = 2x = 14$	Single	White	Yunnan in China; Myanmar; N Thailand; N Vietnam	
R. chinensis var. *spontanea*	–	Single	Red	Native in Guizhou, Hubei, Sichuan	
R. chinensis 'Yue yuehong'	$2n = 2x$, $3x$, $4x = 14$, 21, 28	Double or semi-double	Variable	Widely cultivated elsewhere	Slater's Crimson China = *R. chinensis* var. *semperflorens*

Hume's Blush Tea-scented China is identical with *R. odorata* var. *odorata*; Parks' Yellow Tea-Scented China is probably *R. odorata* var. *pseudindica*; and Parsons' Pink China should be *R. odorata* var. *erubescens* (Table 1). Analyzing approximately 30 morphological characters, three tea-scented China roses are hypothesized to be hybrids between *R. odorata* var. *gigantea* and *R. chinensis* (Hurst 1941; Wylie 1954). However, this hypothesis has not been tested by molecular data.

Low-copy nuclear genes have been widely used to address reticulate evolution (e.g., Cronn et al. 1999; Ferguson and Sang 2001; Cronn and Wendel 2003; Doyle et al. 2003; Howarth and Baum 2005; Popp et al. 2005; Joly and Bruneau 2006; Lihová et al. 2006; Poke et al. 2006; Mercure and Bruneau 2008; Yi et al. 2008; Frajman et al. 2009; Grusz et al. 2009). Multiple low-copy nuclear genes including a ferrodoxin-NADP reductase precursor gene (*FENR*), *GAPDH*, malate synthase (*MS*), and triose phosphate isomerase (*TPI*) have been successfully used to infer hybridization events in *Rosa* (Joly et al. 2006; Joly and Bruneau 2006; Mercure and Bruneau 2008). To elucidate the hybrid origin of the three tea-scented China roses, two single-copy nuclear genes of *GAPDH* and *ncpGS* and two non-coding plastid loci of *trnL-F* and *psbA-trnH* were sequenced for representative samples of four *R. odorata* varieties, *R. chinensis*, and 28 other *Rosa* species.

Materials and methods

Plant material

Eleven individuals in total from the four varieties of *R. odorata* were sampled (including three individuals of *R. odorata* var. *odorata*, two *R. odorata* var. *erubescens*, one *R. odorata* var. *pseudindica*, and five *R. odorata* var. *gigantea*). To represent the other putative parental species, we collected two accessions of *R. chinensis* var. *spontanea* (Rehder & E. H. Wilson) T. T. Yu & T. C. Ku (the wild type of *R. chinensis*) and one ancient Chinese rose cultivar, *R. chinensis* 'Yue yuehong.' Five of the seven major wild ancestors of garden roses (Wylie 1954) were also sampled in this study. Because the phylogenetic relationships among species of *Rosa* have not yet been fully resolved (Wissemann and Ritz 2005; Joly and Bruneau 2006; Bruneau et al. 2007), 30 species of *Rosa* in total were sampled to avoid missing the real parental species, covering eight of the ten sections of subgen. *Rosa* and one species from subgen. *Platyrhodon* (Hurst) Rehder. Voucher information and GenBank accession numbers are listed in Table 2. Voucher specimens are deposited in the herbarium of the Kunming Institute of Botany (KUN), Chinese Academy of Sciences.

Molecular methods

Total DNA was extracted from fresh or silica gel-dried leaf material using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987) modified as follows: 1.0 mg polyvinylpyrrolidone (PVP, molecular weight 30,000) was added when grinding leaf material, then 3/4 volume of a sugar-removing buffer (Tris-HCl 200 mM, EDTA 50 mM, 8% NaCl, 1% PVP, 1% β-mercaptoethanol) was added prior to incubations on ice for 10 min; low-speed centrifugation at 5,000 rpm was applied before incubation at 65°C; three to four chloroform-isoamyl alcohol (24:1) extractions were undertaken (until no impurities were recovered in the central layer); DNA was precipitated with 1/2 volumes of NaCl (5 mol/L) and 2/3 volumes of isopropanol.

The *GAPDH* region was sequenced for all of the 30 species sampled. The *ncpGS*, *trnL-F*, and *psbA-trnH* markers were sequenced for all the accessions of the four varieties of *R. odorata*, and the six other most closely related species inferred by *GAPDH* data: *R. chinensis* (*R. chinensis* 'Yue yuehong,' two *R. chinensis* var. *spontanea* accessions), *R. rubus* Lévl. et Vant., *R. luciae* var. *luciae*, *R. longicuspis* Bertol., *R. multiflora*, and *R. xanthina* Lindl.

The *GAPDH* gene was amplified with primers GPDX7F and GPDX11R (Joly et al. 2006), while GScp687f and GScp994r primers (Emshwiller and Doyle 1999) were used to amplify the *ncpGS* gene. The *trnL-F* region was amplified with the universal primers "c" and "f" (Taberlet et al. 1991), and the *psbA-trnH* region was amplified with trnH (GUG) and psbA primers (Hamilton 1999).

PCR amplifications were carried out in 25 µl reactions containing 50–100 ng of DNA template, 0.1–0.2 µM of each primer, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (TaKaRa, Dalian, China).

For *ncpGS*, *trnL-F*, and *psbA-trnH*, PCR conditions were as follows: an initial denaturation step of 3 min at 97°C, followed by 32 cycles of 1 min at 94°C, 1 min at 50–54°C, 1 min at 72°C with a final extension step for 7 min at 72°C. The PCR conditions for *GAPDH* were as described in Joly et al. (2006). Amplicons were purified using PCR Products Purifying Kit (Sangon, Shanghai, China) and were directly sequenced with the PCR primers. Sequencing primers GPDX7Fb and GPDX11R (Joly et al. 2006) were used for *GAPDH*. Cycle sequencing was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA), with 5 ng of primer, 1.5 µL of sequencing dilution buffer, and 1 µL of cycle sequencing mix in a 10 µL reaction volume. Cycle sequencing conditions were as follows: 30 cycles of 30 s denaturation (96°C), 30 s annealing (50°C), and 4 min elongation (60°C). The sequencing products were analyzed on an ABI 3730xl DNA capillary sequencer

Table 2 Specimens included in this study

Taxonomy	Vocher	Locality	GenBank accessions			
			GAPDH	*ncpGS*	*trnL-F*	*psbA-trnH*
Subgen. *Rosa*						
Sect. *Chinenses* Candolle ex Seringe						
R. odorata (Andrews) Sweet var. *odorata*	#1, MJ-223	China, Yunnan (YN), Dali	C1, GU575157	T, GU590882	GU575127	GU575142
			C2, GU575158	C, GU590883		
			C3, GU575159			
			C4, GU575160			
			C5, GU575161			
			C6, GU575162			
			C7, GU575163			
			C8, GU575164			
			C9, GU575165			
	#2, MJ-226	China, YN, Dali	C1, GU575166	T, GU590884	GU575128	GU575143
			C2, GU575167	C, GU590885		
			C3, GU575168			
			C4, GU575169			
			C5, GU575170			
			C6, GU575171			
	#3, TC-6	China, YN, Tengchong	C1, GU575172	T, GU590886	GU575129	GU575144
			C2, GU575173	C, GU590887		
			C3, GU575174			
			C4, GU575175			
			C5, GU575176			
			C6, GU575177			
R. odorata var. *pseudindica* (Lindl.) Rehder	LJ-002	China, YN, Lijiang, Xinzhu	C1, GU575196	T, GU590892	GU575132	GU575147
			C2, GU575197	C, GU590893		
			C3, GU575198			
			C4, GU575199			
			C5, GU575200			
			C6, GU575201			
			C7, GU575202			
			C8, GU575203			

Table 2 continued

Taxonomy	Vocher	Locality	GenBank accessions			
			GAPDH	*ncpGS*	*trnL-F*	*psbA-trnH*
R. odorata var. *erubescens* (Focke) T.T. Yu & T.C. Ku	#1, MJ-255	China, YN, Lijiang	C1, GU575178	T, GU590888	GU575130	GU575145
			C2, GU575179	C, GU590889		
			C3, GU575180			
			C4, GU575181			
			C5, GU575182			
			C6, GU575183			
			C7, GU575184			
			C8, GU575185			
			C9, GU575186			
			C10, GU575187			
	#2, MJ-295	China, YN, Weixi	C1, GU575188	T, GU590890	GU575131	GU575146
			C2, GU575189	C, GU590891		
			C3, GU575190			
			C4, GU575191			
			C5, GU575192			
			C6, GU575193			
			C7, GU575194			
			C8, GU575195			
R. odorata var. *gigantea* (Crép.) Rehder & E.H. Wilson	#1, MJ-101	China, YN, Kunming	GU575204	GU590894	GU575133	GU575148
	#2, MJ-110	China, YN, Kunming	GU575205	GU590895	GU575134	GU575149
	#3, MJ-207	China, YN, Dali	GU575206	GU590896	GU575135	GU575150
	#4, MJ-375	China, YN, Wenshan	GU575207	GU590897	GU575136	GU575151
	#5, LC-080	China, YN, Yongde	GU575208	GU590898	GU575137	GU575152
R. chinensis Jacq. var. *spontanea* (Rehder & E. H. Wilson) T.T.Yu & T.C.Ku	#1, 1988237F	Quarry Botanical Garden	C1,2, GU564453	C1, GU590905	GU564451	GU564449
			C3,4, GU564454	C2, GU590906		
			C5, GU564455	C3, GU590907		
				C4, GU590908		
				C5, GU590909		
				C6, GU590910		
				C7, GU590911		
	#2, 2001226I	Quarry Botanical Garden	C1, GU564456	C1, GU590912		
			C2, GU564458	C2, GU590913		
			C3, GU564457	C3, GU590914		
				C4, GU590915		
				C5, GU590916		
				C6, GU590917		

Table 2 continued

Taxonomy	Vocher	Locality	GenBank accessions			
			GAPDH	*ncpGS*	*trnL-F*	*psbA-trnH*
R. chinensis 'Yue yuehong'	MJ-860	Kunming Yang Chinese Rose Gardening	GU575208- GU575217	C1, GU590899 C2, GU590900 C3, GU590901 C4, GU590902 C5, GU590903 C6, GU590904	GU575138	GU575153
Sect. *Synstylae* Candolle						
R. brunonii Lindley	Yi-10375	China, YN, Lijiang to Ludian	GU575235			
R. multiflora Thunb.			#1, DQ091172* #2, DQ091173*		DQ778870*	DQ778791*
R. longicuspis Bertol.	#1, MJ-160	China, YN, Chuxiong	GU575218	GU590918	GU575139	GU575154
	#2, GS-10	China, YN, Kunming	GU575219	GU590919	GU575140	GU575155
R. rubus Lévl. et Vant.	MJ-389	China, YN, Wenshan	GU575226	GU590920	GU575141	GU575156
R. luciae var. *luciae* Franchet & Rochebrune	MJ-900	Japan, Kyoto, Medicinal Botanical Garden	GU575237	GU590921	DQ778891*	DQ778812*
R. soulieana Crép.	MJ-572	China, YN, Weixi	GU575234			
R. helenae Rehd. et Wils.	MJ-901	Kunming Institute of Chinese Academy	GU575224			
R. setigera Michx.			DQ091174*			
Sect. *Rosa*						
R. gallica L.	MJ-858	Kunming Yang Chinese Rose Gardening	GU575236			
Sect. *Cinnamomeae* Candolle ex Seringe						
R. willmottiae Hemsl.	Yi-10736	China, Sichuan, Xiaojin	GU575227			
R. pratti Hemsl.	MJ-527	China, YN, Lijiang	GU575231			
R. rugosa Thunb.	MJ-856	Kunming Yang Chinese Rose Gardening	GU575228			
R. saturata Baker	Yi-10439	China, YN, Zhongdian	GU575230			
R. sweginowii Koehne	MJ-539	China, YN, Zhongdian	GU575229			
R. pisocarpa Gray			DQ091069*			
R. blanda Ait.			DQ091039*			
R. gymnocarpa Nutt.			DQ091047*			
Sect. *Pimpinellifoliae* Candolle ex Seringe						
R. woodii Lindl.			DQ091085*			
R. graciflora Rehder & E.H. Wilson.	MJ-413	China, YN, Lijiang Alpine Botanic Garden	GU575225			
R. omeiensis Rolfe	TC-009	China, YN, Weixi	GU575223			
R. mairei Lévl.	MJ-290	China, YN, Tengchong	GU575232			

Table 2 continued

Taxonomy	Vocher	Locality	GenBank accessions			
			GAPDH	*ncpGS*	*trnL-F*	*psbA-trnH*
R. xanthina Lindl.	MJ-853	Kunming Yang Chinese Rose Gardening	GU575233	GU590922		
	230	Maowen Rose Garden			GU564452	GU564450
Sect. *Laevigatae* Thory						
R. laevigata Michx.	Yi-10138	China, Guizhou	GU575222			
Sect. *Banksianae* Lindl.						
R. banksiae Ait.	MJ-852	Kunming Yang Chinese Rose Gardening	GU575220			
Sect. *Carolinae* Crép.						
R. nitida Willd.			DQ091055*			
R. palustris Marsh.			DQ091043*			
R. foliolosa Nutt.			DQ091067*			
Subgen. *Platyrhodon* (Hurst) Rehder						
R. roxburghii Tratt.	MJ-204	China, YN, Dali	GU575221			

* Genbank accessions reported in previous studies (Joly et al. 2006; Bruneau et al. 2007)

(Applied Biosystems). For the *GAPDH* and *ncpGS* genes, PCR products of all accessions of double-petaled varieties of *R. odorata* and three accessions of *R. chinensis* could not be sequenced directly. Thus, the PGEM®-T Vector System from Promega was used for cloning of the *GAPDH* gene, according to the manufacturer's instructions. Three to ten clones per individual were sequenced. In order to avoid cloning, the method of Chen et al. (2008) was used to sequence *ncpGS* gene. A pair of type-specific primers, T-type (5′-GCCAGGTTTTCCTCTTGAT-3′) and C-type (5′-GCCAGGTTTTCCTCTTGAC-3′), were designed to terminate at the first single nucleotide polymorphism site of *ncpGS* alignment in order to separate the two types from each accession of double-petaled *R. odorata.*

Data analyses

Sequence data were assembled using Sequencher Version 4.4 (Gene Codes, Ann Arbor, MI, USA). Alignment of sequences was initially performed using Clustal X version 1.81 (Thompson et al. 1997) and then manually adjusted using BioEdit version 7.0.1 (Hall 1999). All data matrices have been deposited in TreeBASE (study number SN4919).

The DNA sequence recombination is a problem when cloning products of PCR reactions in which multiple alleles or paralogous gene copies have been amplified (Cronn et al. 2002; Kelly et al. 2010; Russell et al. 2010). Multiple methods have been developed to detect recombination events, however these programs are prone to (1) low power: recombination could be detected only when a few recombination events exist in the data set; (2) a high rate of false positives (Kelly et al. 2010). The fragments of the clonal sequences for *GAPDH* generated in this study were small with 433–822 bp, and 48 clonal sequences were generated. We thus followed the method of Russell et al. (2010) to detect chimeric sequences by eye.

Indels were treated as missing data in the two nuclear genes (*GAPDH* and *ncpGS*). Maximum parsimony (MP) analyses were conducted with PAUP*4.0b10 (Swofford 2002) using a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 1,000 random addition sequence replicates with MULTREES "on." To evaluate node confidence, bootstrap analyses (Felsenstein 1985) were conducted with 1,000 replicates using the same options as above except that the MULTREES option was "off". This method calculates more quickly and provides essentially identical bootstrap values as keeping the MULTREES option "on" (DeBry and Olmstead 2000; Bruneau et al. 2007).

Bayesian inferences (BI) were performed on *GAPDH* and *ncpGS* with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Parameter settings for models of sequence evolution

were identified using MODELTEST 3.07 (Posada and Crandall 1998). For *GAPDH* and *ncpGS*, the preferred models under the Akaike Information Criterion (AIC) of MODELTEST were the TrN + G model and K81uf model, respectively. Markov chain Monte Carlo (MCMC) (Huelsenbeck and Ronquist 2001) analyses were run for 2,000,000 generations, starting from random trees and sampling 1 out of every 100 generations. For each dataset, MCMC runs were repeated twice to avoid spurious results. Finally, the first 5,000 trees (25%) were discarded as burn-in, as determined by AWTY (Wilgenbusch et al. 2004; Nylander et al. 2008) and Tracer version 1.4 (Rambaut and Drummond 2007). The remaining trees were used to construct majority-rule consensus trees using PAUP*.

For the two combined plastid markers (*trnL-F* and *psbA-trnH*), site mutations and indels were assumed to evolve with equal possibility. A haplotype analysis was conducted to estimate the sequence similarity using DnaSP software version 5 (Librado and Rozas 2009). The haplotype network was constructed by the median-joining network (MJN) method using the program NETWORK 4.5.1.6 (available at http://www.fluxus-engineering.com; Bandelt et al. 1999).

Results

Sequence characterization

For *GAPDH* gene, no chimeric clonal sequence was found. The aligned matrix of *GAPDH* gene was 844 bp long with 158 variable sites, of which 76 were parsimony-informative. The aligned matrix of *ncpGS* was 706 bp long with 29 variable sites, of which 18 were parsimony-informative. The aligned matrices of *trnL-F* and *psbA-trnH* were 942 and 315 bp in length, respectively. The combined matrix of the two plastid fragments contained 11 indels and 15 substitution sites.

Phylogenetic analyses

Parsimony analyses of *GAPDH* gene resulted in 1,495 most parsimonious trees with 202 steps (consistency index, CI = 0.84; retention index, RI = 0.93). The MP strict consensus tree was largely consistent with the Bayesian tree but with lower resolution. Thus, the Bayesian tree based on *GAPDH* sequences is shown in Fig. 1. The clonal sequences of all the double-petaled accessions were

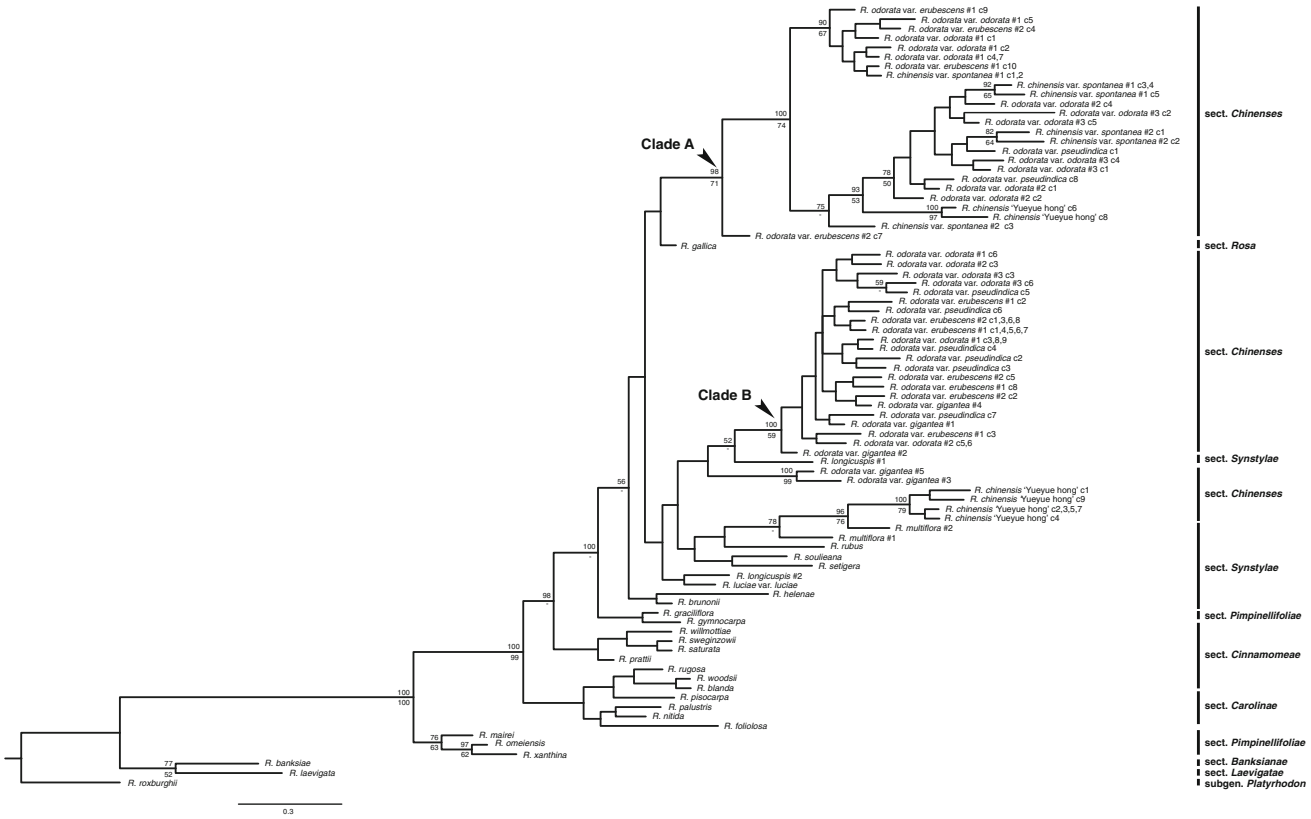

Fig. 1 Phylogram obtained from Bayesian inference analysis of the *GAPDH* data set. The PP values >50% are presented *above the branches* and the BS values >50% are shown *below the branches*. *Bars to the right* indicate the sections and subgenera of *Rosa* by Wissemann (2003)

clustered into two clades: clade A (parsimony bootstrap value, BS = 71%; Bayesian posterior probabilities, PP = 98%) and Clade B (BS = 59%; PP = 100%). Besides the accessions of double-petaled *R. odorata* varieties, Clade A also included all the clonal sequences of *R. chinensis* var. *spontanea* (#1 and #2) and two clonal sequences of *R. chinensis* 'Yue yuehong'; Clade B also included three accessions of the single-petaled *R. odorata* var. *gigantea* (#1, #2, and #4). The other two accessions of *R. odorata* var. *gigantea* (#3 and #5) formed a strongly supported clade. The remaining sequences of *R. chinensis* 'Yue yuehong' were clustered with *R. multiflora* #2 with moderate support (BS = 76%; PP = 96%).

Parsimony analysis of *ncpGS* gene resulted in 18 most parsimonious trees with 50 steps (CI = 0.98; RI = 0.99). The topologies produced with the BI and MP methods were largely congruent except that the one from the BI analysis had higher resolution. Only the BI tree is presented in Fig. 2. Two types of sequence were detected from each individual of the three double-petaled *R. odorata* varieties; we assigned them as types "C" and "T" in Fig. 2. These sequences were clustered into two clades (clade C and clade D) in final analyses (Fig. 2). Clade C also contained all five accessions of *R. odorata* var. *gigantea*, albeit with low bootstrap support (BS < 50%; PP = 92%). The weak support for clade C might have been caused by too few informative sites; all double-petaled varieties shared the same sequence with *R. odorata* var. *gigantea* #4 and differed from other *R. odorata* var. *gigantea* sequences by only one 1-bp indel. Clade D also contained parts of the clonal sequences of *R. chinensis* var. *spontanea* #1, all clonal sequences of *R. chinensis* var. *spontanea* #2, and some of the clonal sequences of *R. chinensis* 'Yue yuehong' (BS = 96%; PP = 100%). The other four clonal sequences of *R. chinensis* 'Yue yuehong' clustered with *R. luciae* var. *luciae* with high support (BS = 97%; PP = 100%). Four of the clonal sequences of *R. chinensis* var. *spontanea* #1 formed a separate clade with moderate support (BS = 59%; PP = 95%).

A total of 13 haplotypes were resolved from the combined data of the two plastid markers. All the double-petaled *R. odorata* accessions shared the same haplotype (H1) with *R. odorata* var. *gigantea* #3 and were differentiated from other *R. odorata* var. *gigantea* (H5, H7, H8, and H9)

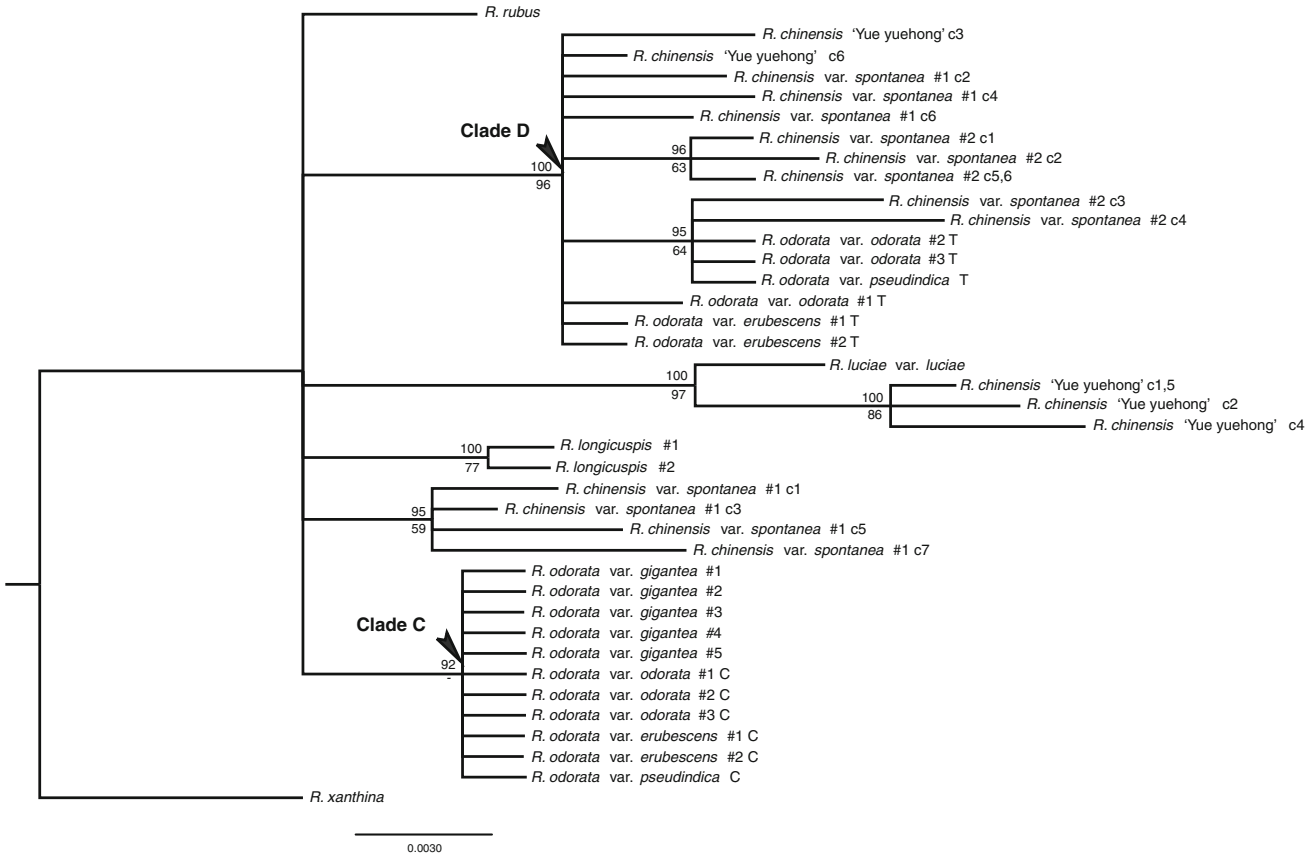

Fig. 2 Phylogram obtained from Bayesian inference analysis of the *ncpGS* sequence data. The PP values>50% are presented *above the branches* and the BS values >50% are shown *below the branches*. The capital letters "C" and "T" following accession numbers of three double-petaled varieties indicate the two type sequences detected by type-specific primers. Accession numbers(*#1–5*) and clonal sequences (*c1–c7*) are shown

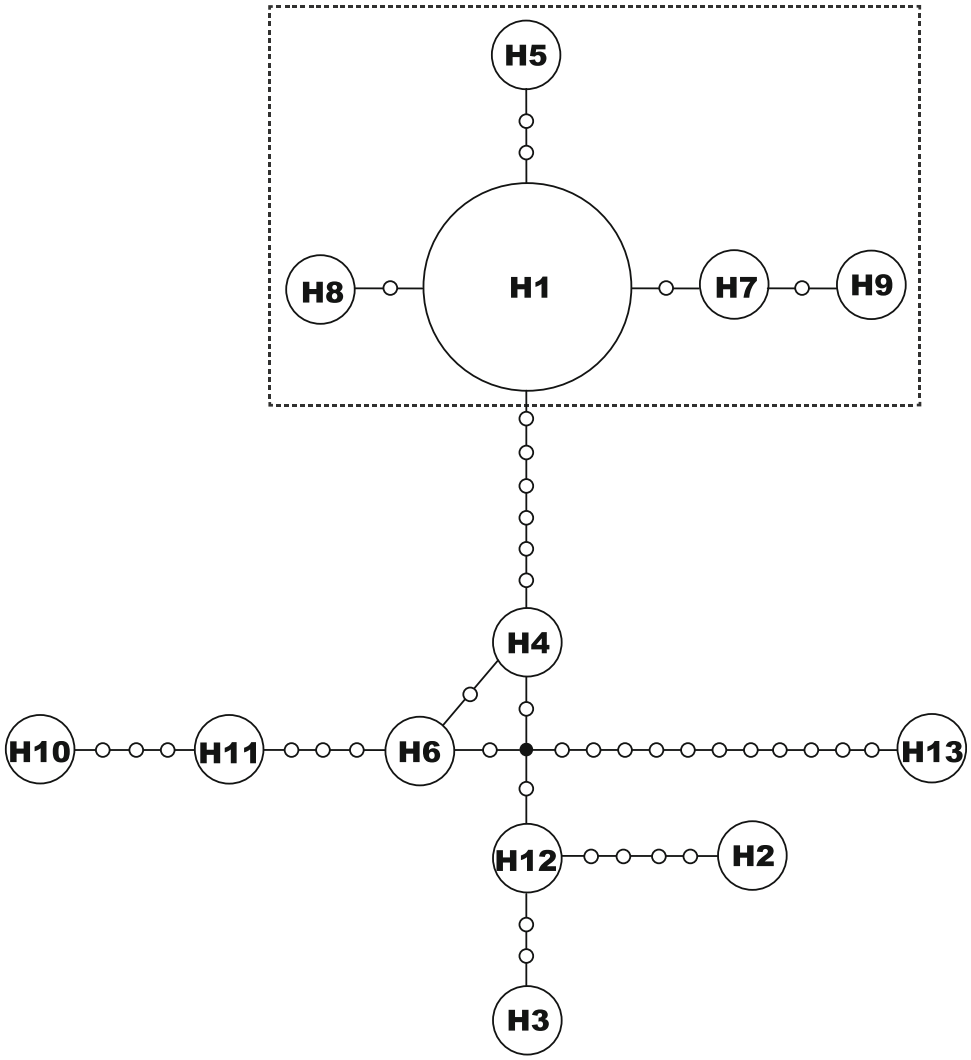

Fig. 3 Median-joining network of the 13 haplotypes detected from combined plastid data. The *size of the circles* corresponds to the frequency of each haplotype. *Small open circles* between haplotypes represent the number of mutational steps. The *small filled circle* represents the inferred intermediate haplotypes. The haplotype group enclosed by *dashed lines* is comprised of all haplotypes of the four varieties of *R. odorata*. H1: *R. odorata* var. *gigantea* #3; *R. odorata* var. *erubescens* #1, #2; *R. odorata* var. *odorata* #1, #2, #3; *R. odorata* var. *pseudindica*. H2: *R. rubus*. H3: *R. chinensis* 'Yue yuehong.' H4: *R. longicuspis* #2. H5: *R. odorata* var. *gigantea* #5. H6: *R. longicuspis* #1. H7: *R. odorata* var. *gigantea* #1. H8: *R. odorata* var. *gigantea* #4. H9: *R. odorata* var. *gigantea* #2. H10: *R. multiflora*. H11: *R. luciae* var. *luciae*. H 12: *R. chinensis* var. *spontanea* #1. H13: *R. xanthina*

accessions by only one to two steps (Fig. 3). The two *R. chinensis* haplotypes, H3 and H12, differentiated from each other by only two steps (Fig. 3).The four *R. odorata* varieties were separated from the two accessions of *R. chinensis* by many more steps.

Discussion

Hybrid origin of the tea-scented China roses

Because three tea-scented China roses are believed to correspond to the three double-petaled *R. odorata* varieties, six individuals representing three double-petaled *R. odorata* varieties were collected across their distribution regions in the Yunnan province of China and were included to address the hypothesis of a hybrid origin of the tea-scented China roses. For each of two nuclear loci of *GAPDH* and *ncpGS*, the clonal sequences from each accession of the three double-petaled varieties fell into two distinct groups: one was related to *R. chinensis*, and the other was related to *R. odorata* var. *gigantea*. These results suggest that the three double-petaled varieties of *R. odorata* are hybrids of *R. odorata* var. *gigantea* and *R. chinensis*.

Because the chloroplast DNA of *Rosa* is maternally inherited (Corriveau and Coleman 1988), the plastid regions could be used to detect the maternal parents of the hybrids in this genus. Based on the haplotype analyses of the combined *trnL-F* and *psbA-trnH* data, the double-petaled *R. odorata* varieties shared an identical haplotype with *R. odorata* var. *gigantea* #3 and displayed close relationships with other *R. odorata* var. *gigantea* samples. This result indicates that *R. odorata* var. *gigantea* could be the maternal parent of the three double-petaled *R. odorata*.

The three double-petaled varieties of *R. odorata* share some morphological characters with both *R. odorata* var. *gigantea* and *R. chinensis* (Ku and Robertson 2003; Scalliet et al. 2008; author's observations; Table 3). The four

Table 3 Morphological comparison among three double-petaled *R. odorata*, *R. odorata* var. *gigantea* and *R. chinensis* cultivars and *R. chinensis* var. *spontanea*

Characters	*R. odorata*		*R. chinensis*	
	Three double-petaled varieties	var. *gigantea* (wild type)	Varieties or cultivars	var. *spontanea* (wild type)
Shrub or liana	Liana	Liana	Shrub	Shrub
Number of leaflets	5–7	5–7	3–5, rare 7	3–5
Flowers fragrant or not	Fragrant	Fragrant	Slightly fragrant or not	Fragrant
Scent compound	DMT	DMT		TMB
Length of pedicel	Often more than 2.5 cm	Usually 1–2 cm	Often more than 2.5 cm	Often more than 2.5 cm
Flower colors	White, yellow to orange, and pink	White to creamy-white	Variable	Red
Petals	Double to semi-double	Single	Double to semi-double	Single
Number of flowers	1 or 2–3	Mostly 1	Often 4–5, rare 1	Often 1
Sepals	Entire or rarely slightly incised	Mostly entire	Entire or few pinnate lobes	Often entire or rare few lobes

Data are from Ku and Robertson (2003), Scalliet et al. (2008), and author's observation

DMT 3,5-Dimethoxytoluene, *TMB* 1,3,5-trimethoxybenzene

varieties of *R. odorata* are all lianas with five to seven leaflets and have the same scent compounds (Scalliet et al. 2008). They mainly differentiate from one another by double- (semi-double) or single-petaled flowers, floral color, and the size of the petals (Ku and Robertson 2003). At the same time, the three double-petaled varieties share several morphological characters with *R. chinensis* including longer pedicel, more flowers, and multiple floral colors, which are absent in *R. odorata* var. *gigantea*. Hurst (1941) also drew similar conclusions after analyzing 31 morphological characters in *R. odorata* var. *odorata*. He found that 11 characters of this variety were under the influence of *R. chinensis*, and the remaining 20 were under the influence of *R. odorata* var. *gigantea*. The three double-petaled varieties have been treated as infraspecific taxa of *R. chinensis* or *R. gigantea* (*R. odorata* var. *gigantea*) by different botanists (Rehder 1949; Ku and Robertson 2003), which further suggests the morphological similarity among them. Consistent with the molecular results, the morphological characters also suggest three double-petaled *R. odorata* varieties are hybrids between *R. odorata* var. *gigantea* and *R. chinensis*. This means that the hypothesis of three tea-scented China roses being hybrids between *R. chinensis* or *R. odorata* var. *gigantea* was proved by our molecular data.

Who are the paternal parents of the tea-scented China roses?

The molecular data of *GAPDH* and *ncpGS* seem to support that *R. chinensis* var. *spontanea* rather than the *R. chinensis* cultivar examined is the closest relative to the three double-petaled varieties of *R. odorata* (Figs. 1, 2). However, *R. chinensis* var. *spontanea*, the wild type of *R. chinensis*, has been reported to be distributed in the provinces of Hubei, Sichuan, and Guizhou in China, while *R. odorata* var. *gigantea* is naturally distributed in the Yunnan province of China (Ku and Robertson 2003). The allopatric distribution of these two taxa precludes the possibility of direct hybridization between them in the field. At the same time, it is still not clear whether wild *R. chinensis* (*R. chinensis* var. *spontanea*) exists in the field. We failed to collect *R. chinensis* var. *spontanea* in the field and couldn't find specimens of this variety after carefully checking *Rosa* specimens in most Chinese herbaria. The *R. chinensis* var. *spontanea* accessions included in this study were introduced from China into the Royal Botanical Garden Edinburgh. More field work should be carried out to verify the existence of *R. chinensis* var. *spontanea*.

Notably, the three double-petaled *R. odorata* varieties have double-petaled flowers with three different colors, which are absent in both *R. odorata* var. *gigantea* and *R. chinensis* var. *spontanea*. The character "double-petaled vs. single-petaled" is a monogenic controlled character, with single-petaled being recessive (Debener and Mattiesch 1999; Crespel et al. 2002; Debener and Linde 2009), and the additional petals result from the homeotic transformation of stamens into petals (Debener et al. 2003). It is thus unlikely that the doubled petals of the three double-petaled varieties directly evolved from the hybridization between single-petaled *R. odorata* var. *gigantea* and *R. chinensis* var. *spontanea*. Moreover, it seems less likely that the hybridization between *R. odorata* var. *gigantea* (white to creamy-white flowers) and *R. chinensis* var. *spontanea*

(red flowers) directly produced three different colors in the three double-petaled varieties of *R. odorata*. Characters of doubled petals and three floral colors of double-petaled varieties of *R. odorata* exist in cultivars of *R. chinensis*, which is widely cultivated in the Yunnan province and close to naturally occurring *R. odorata* var. *gigantea*. Summarizing these data, the three double-petaled varieties of *R. odorata* are more probably hybrids between naturally distributed *R. odorata* var. *gigantea* and different local cultivars of *R. chinensis*. Based on our data, we could not reject the hypothesis that the double-petaled varieties of *R. odorata* are garden hybrids that were kept by local farmers.

The possible hybrid origin of *R. chinensis* 'Yue yuehong'

Rosa chinensis 'Yue yuehong' is an ancient cultivar of *R. chinensis*. This variety was introduced to England in 1789 and was named Slater's Crimson China (Krüssmann 1981, 1982). This cultivar was first figured by Curtis (1794) and treated as *R. chinensis* var. *semperflorens* by Koehne (1893). One sample of this cultivar included in this study contains two types of sequences at *GAPDH* and *ncpGS* loci. One type of sequence clusters with *R. chinensis* var. *spontanea* and the three double-petaled varieties of *R. odorata*, and another type clusters with *R. multiflora* in *GAPDH* data and with *R. luciae* var. *luciae* in *ncpGS* data (with limited sampling). Moreover, the chloroplast haplotype of this cultivar is close to the *R. chinensis* var. *spontanea* haplotype. Molecular data thus support a possible hybrid origin of this cultivar. *Rosa chinensis* var. *spontanea* is probably the maternal parent of the first hybrid product because all the nuclear markers do not support the same paternal parent. This cultivar probably results from multiple hybridization events involving *R. multiflora* and *R. luciae* var. *luciae*. *Rosa chinensis* has been cultivated in China for more than 2,000 years and many cultivars have been bred (Fei et al. 2008). Wylie (1954) documented the origin of the cultivars bred in Europe but ignored the origin of the cultivars coming from China. He treated Slater's Crimson China as a direct derivation from *R. chinensis*. More samples are needed to clearly infer the origin of *R. chinensis* 'Yue yuehong' and other ancient *R. chinensis* cultivars.

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