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Isolation and characterization of ten microsatellite loci for wild *Citrus japonica* (Rutaceae)

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Introduction

The genus of *Citrus* Linnaeus (Rutaceae) includes several widely cultivated species, such as *C. sinensis* (L.) Osbeck, *C. limon* (L.) Burm.f. and *C. maxima* (Burm.) Merr., which are top ranked among all the fruit crops (Mabberley 1997). *Citrus* is believed to be native to Southeast Asia (Scora 1975; Gmitter and Hu 1990) and has been cultivated for fruit for over 4000 years (Webber *et al.* 1967). The long cultivation history, together with a great number of intraspecific and interspecific hybrids in *Citrus*, makes the genetic background extremely complex.

The *C. japonica* is native to South China, with wild populations distributed in broad-leaved evergreen forests and cultivated forms widely grown in warm parts of China (Zhang *et al.* 2008). It is a species complex, integrated from the formerly segregated genus *Fortunella*, consisting of *F. hindsii* (Champ. ex Benth.) Swingle, *F. venosa* (Champ. ex Benth.) Huang, *F. margarita* (Lour.) Swingle, *F. japonica* (Thunb.) Swingle and *F. bawangica* Huang (Zhang *et al.* 2008). Despite this taxonomic revision, a considerable amount of variation in fruit size within some wild populations still exists. Further comprehensive field and molecular studies throughout the complex are needed. Recently, due to human activities and environmental changes, wild populations of *C. japonica* have been dramatically declining (Huang *et al.* 2010). Evaluating the genetic structure of these wild populations is urgent for their conservation. Additionally, various cultivated forms of *C. japonica* have important ornamental and medicinal, as well as food values. However, their origins have never been identified. Microsatellites have been proven to be efficient genetic markers for taxon delimitation, genetic structure analysis and detection of origins of cultivars. In the present study, a set of polymorphic microsatellite markers for wild *C. japonica* complex was developed, which will support subsequent studies.

Materials and methods

Genomic DNA was extracted from silica-gel-dried leaf tissue of one *C. japonica* individual (former *F. hindsii*) using a modified CTAB method (Doyle 1991). Microsatellite loci were isolated using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol from an enriched $(TG)_n$ (Zane *et al.* 2002). Detailed procedures followed Duan *et al.* (2012).

PCR primers were designed for 52 sequences using the program Primer Premier 5.0 (Lalitha 2000). Of the 52 primers, 16 produced clear bands of amplification products with expected sizes on 1% agarose gel. Each forward primer of these was labelled with one of the fluorescent dyes HEX, FAM, ROX or TAMRA (Invitrogen, Shanghai, China) for further screening. The polymorphism was tested in 50 *C. japonica* individuals (10 former *F. venosa* individuals and 40 former *F. hindsii* individuals) from five sites. The PCR was performed in a 20 μL mixture containing 40 ng of genomic DNA, 0.3 μL dNTPs, 0.3 μM of each primer, 2 μL of 10× PCR buffer, and 0.6 U *Taq* DNA polymerase (Takara, Dalian, China). The amplification conditions were as follows: initial denaturation at 94°C for 5 min; subsequent 35 cycles of denaturing at 94°C for 40 s, annealing at a primer-specific optimal temperature for 40 s (table 1), extension at 72°C 40 s, and a final extension at 72°C for 10 min. The PCR products were scanned by an ABI Prism 3100 Genetic Analyser (Invitrogen), using an internal size standard GeneScan™500 LIZ (Applied Biosystems, Foster City, USA). Allele binning and calling were done using GeneMarker ver. 2.4.0 (Holland and Parson 2011).

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Table 1. Characteristics of 10 microsatellite loci identified from *Citrus japonica*.

Locus	Primer sequences (5′–3′)	Motif	T_m(°C)	Expected size (bp)	Obtained size (bp)	N_A	H_O	H_E	GenBank accession no.
CY01	F: <FAM>CGTCTTCCTTCCTTACTT R: ATCGGTGAAAATAGCAAC	$(TG)_{13}$	51	121	121–137	7	0.90	0.71	KF030884
CY05	F: <HEX>ACCAAATCACTGAACAAAT R: ACATGAGGGACCTTCTTAG	$(TC)_{10}$	50	239	240–242	2	0.06	0.12	KF030885
CY07	F: <ROX>ATAATCAAACTCCCTCGTG R: TACAAAATTGGACAGCAA	$(TG)_{10}$	50	147	139–149	3	0.16	0.19	KF030886
CY13	F: <TAMRA>TGTCGCTCATCATTAGGT R: CCAATTCATTTCAAACCC	$(AC)_9$	49	271	299–325	8	0.81	0.80	KF030887
CY19	F: <FAM>CGCATTGAAAGTCTGTGGT R: ATCTGAAGGCTTCTGTGGC	$(GAA)_7$	49	372	367–377	4	0.52	0.59	KF030888
CY23	F: <TAMRA>GTGAATGAAAGAACCCAT R: CAAAGCGACTGACAACTA	$(TG)_9$	49	249	250–254	3	0.38	0.54	KF030889
CY37	F: <FAM>CCAATACCCAGTTCCAAG R: CTACCTCTTCCCTTTTCT	$(GAT)_4 \ldots (AAG)_5$	50	318	313–322	4	0.62	0.61	KF030890
CY40	F: <HEX>ACAGCGAGATCATTGAGT R: TATCGTTATTTACGTGGG	$(GAA)_8$	50	120	109–115	3	0.16	0.30	KF030891
CY48	F: <HEX>CTGAGGTGGGACTGGTGT R: CATCTCGCAGAAAGTAAAA	$(TGA)_3 \ldots (TTG)_5(TTC)_3$	49	309	307–310	2	0.02	0.02	KF030892
CY51	F: <ROX>ACAAAAGCACAAAAGGCCAAA R: AGATGAATGCGTGTAGTAAGC	$(AAT)_4(CT)_8$	49	261	268–274	4	0.44	0.69	KF030893

T_m, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. Each forward primer was 5′ fluorescently labelled with HEX, FAM, ROX or TAMRA.

The presence of null alleles for each locus was tested by Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004). The number of the alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were determined for each locus in the whole samples using FSTAT 2.9.3.2 (Goudet 1995). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for each locus were also tested using FSTAT 2.9.3.2.

Results and discussion

Characteristics of the microsatellite loci of *C. japonica* are shown in table 1. Ten of the 16 loci were found to be polymorphic while the other six primers had no polymorphism (two primers) or multiple bands difficult to interpret (four primers). The number of alleles per locus ranged from 2 to 8, with an average of 4. All the alleles fell within the expected size range (table 1). For each locus, the observed and expected heterozygosity showed a range from 0.02 to 0.90 and from 0.02 to 0.80, respectively. Null alleles were revealed in three of the 10 loci (CY05, CY19 and CY40). No locus pair showed significant LD. Significant deviations from HWE ($P < 0.05$) were detected in six primers (CY05, CY07, CY19, CY23, CY40 and CY48), probably due to null alleles.

The *C. japonica* complex, with both wild populations and various cultivars (Zhang *et al.* 2008), provides a great model to elucidate the relationship between cultivars and wild populations. Assessment of genetic diversity by molecular markers is important not only for crop improvement efforts, but also for efficient management and conservation of plant genetic resources (Manifesto *et al.* 2001). However, little is known about the genetic variation in wild or cultivated populations of *C. japonica* (Chen 2013). Polymorphic microsatellite markers are valuable tools in evaluating population genetic structure and in detecting origins of cultivars. For example, analyses based on 24 microsatellite markers revealed that among 370 *Citrus* accessions there were a few naturally occurring species and most other types arose through various hybridization events (Barkley *et al.* 2006). Gao and Innan (2008), using 60 microsatellites, demonstrated that the two subspecies of cultivated rice *Oryza sativa*, *indica* and *japonica* did not come from completely independent domestication and there was at least partial sharing of their ancestral populations or recent gene flow between them. Thus, the polymorphic microsatellite loci reported here will be suitable for population genetics studies of this species complex. They could be used in the future investigation of genetic diversity and population genetic patterns over both wide and cultivated populations. Further, we expect that these markers will facilitate further studies on the origin of cultivars, as well as on the efficient conservation and sustainable exploitation of *C. japonica* germplasms.

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