A set of primers for analyzing chloroplast DNA diversity in Citrus and related genera

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Received March 24, 2004; accepted November 20, 2004; published online April 1, 2005

Summary Chloroplast simple sequence repeat (cpSSR) markers in Citrus were developed and used to analyze chloroplast diversity of Citrus and closely related genera. Fourteen cpSSR primer pairs from the chloroplast genomes of tobacco (Nicotiana tabacum L.) and Arabidopsis were found useful for analyzing the Citrus chloroplast genome (cpDNA) and re-coded with the prefix SPCC (SSR Primers for Citrus Chloroplast). Eleven of the 14 primer pairs revealed some degree of polymorphism among 34 genotypes of Citrus, Fortunella, Poncirus and some of their hybrids, with polymorphism information content (PIC) values ranging from 0.057 to 0.732, and 18 haplotypes were identified. The cpSSR data were analyzed with NTSYS-pc software, and the genetic relationships suggested by the unweighted pair group method based on arithmetic means (UPGMA) dendrogram were congruent with previous taxonomic investigations: the results showed that all samples fell into seven major clusters, i.e., Citrus medica L., Poncirus, Fortunella, C. ichangensis Blanco, C. reticulata Swingle, C. aurantifolia (Christm.) Swingle and C. grandis (L.) Osbeck. The results of previous studies combined with our cpSSR analyses revealed that: (1) Calamondin (C. madurensis Swingle) is the result of hybridization between kumquat (Fortunella) and mandarin (C. reticulata), where kumquat acted as the female parent; (2) Ichang papeda (C. ichangensis) has a unique taxonomic status; and (3) although Bendiguangju mandarin (C. reticulata) and Satsuma mandarin (C. reticulata) are similar in fruit shape and leaf morphology, they have different maternal parents. Bendiguangju mandarin has the same cytoplasm as sweet orange (C. sinensis), whereas Satsuma mandarin has the cytoplasm of C. reticulata. Seventeen PCR products from SPCC1 and 21 from SPCC11 were cloned and sequenced. The results revealed that mononucleotide repeats as well as insertions and deletions of small segments of DNA were associated with SPCC1 polymorphism, whereas polymorphism generated by SPCC11 was essentially due to the variation in length of the mononucleotide repeats.

Keywords: cpDNA, cpSSR, genetic diversity, microsatellite, woody plant.

Introduction

Citrus, which is believed to have originated in southeast Asia (Swingle 1943, Tanaka 1977), is widely cultivated. However, the parentage and taxonomy of this genus are confusing, because of wide sexual compatibility between Citrus and related genera, high frequency of bud mutations, long history of cultivation and the occurrence of polyembryony in most of the species (Handa et al. 1986, Nicolosi et al. 2000, Pang et al. 2003). In recent decades, biochemical and molecular markers have been used in taxonomic studies of Citrus genera and species, efficiently overcoming the disadvantages of classification systems based on morphological traits (Swingle 1943, Tanaka 1977) and resulting in improved understanding of the classification status of numerous Citrus genotypes (Handa et al. 1986, Fang and Zhang 1992, Nicolosi et al. 2000, Pang et al. 2003). However, there is still some uncertainty about the parentage of genotypes, and many local varieties in southeast Asia are of uncertain origin.

Most of the research with molecular markers in Citrus has been based on total DNA or on the nuclear genome. However, the conservative nature of the chloroplast genome in higher plants makes it more useful for parentage and taxonomic studies. The chloroplast genome is small, its gene order is relatively conserved, it is non-recombinant, uni-parentally inherited and effectively haploid (Weising and Gardner 1999, Bryan et al. 1999, Provan 2000, Nicolosi et al. 2000, Provan et al. 2001, Xu et al. 2002, Vogel et al. 2003), features that could overcome the disadvantages encountered in analyses of nuclear DNA markers. Since the mid 1980s, restriction fragment length polymorphisms (RFLPs) with chloroplast heterologous probes have been used for Citrus phylogenetic studies (Green et al. 1986, Yamamoto et al. 1993), the study of chloroplast inheritance in sexual crossings (Moreira et al. 2002) and protoplast fusion (Cheng et al. 2003a). More recently, poly-
Chloroplast inheritance of... with Citrus Swingle), mandarin (Ishii and McCouch 2000), soybean (Powell et al. 1996, Xu et al. 1999), barley (Provan et al. 1999), rice (Provan et al. 1997, Bryan et al. 1999), species such as pine (Powell et al. 1995), potato (Bryan et al. 1999), and the sample concentration was adjusted to 30 ng µl⁻¹. We extracted DNA from fresh leaves according to the protocol of Cheng et al. (2003b). The quality and concentration of the DNA samples were determined spectrophotometrically, and the sample concentration was adjusted to 30 ng µl⁻¹.

Selection of primers from published cpSSR markers

Twenty-two previously reported chloroplast primer pairs (Powell et al. 1995, Provan et al. 1997, Bryan et al. 1999, Weising and Gardner 1999, Xu et al. 2002) were synthesized (Table 2). Among these, 14 pairs were derived from the tobacco chloroplast genome database (Bryan et al. 1999, Weising and Gardner 1999), five from the rice database (Ishii and McCouch 2000, Xu et al. 2002) and three from Pinus thunbergii Parl. (Powell et al. 1995, Xu et al. 2002). The leaf DNA samples were amplified with the 22 primers, with annealing temperatures as reported in the references (or ± 1–5 °C) (Table 2). After verifying amplification of the DNAs on an agarose gel, PCR products represented by intense bands were separated on 6.0% polyacrylamide gels. When intense bands were also visible on the polyacrylamide gels, the corresponding primer was recoded with the SPCC prefix (SSR Primer for Citrus Chloroplast).

Microsatellite analysis and cpSSR primer construction based on the chloroplast sequence database of Arabidopsis thaliana

The chloroplast sequence database of Arabidopsis thaliana L. Heynh. (AP00423) was obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and surveyed for mononucleotide repeat loci. Twenty-two poly(A) and 19 poly(T) mononucleotide residue repeat (n ≥ 10) loci were obtained. After elimination of the intra-gene repeats, primers were designed with program Primer 3.0 (http://bioinformatics.hzau.edu.cn/primer/primer3.html). The criteria applied in primer design were those described by Weising and Gardner (1999), including the absence of self-annealing; dimer and hairpin formation capacities; and an expected PCR size range from 80 to 300 bp in A. thaliana. Eleven Arabidopsis cpSSR primer pairs (ARC1 to ARCP11) were synthesized. The procedures for primer selection and recoding were as mentioned previously. All primer pairs were synthesized by Biosis Company (Shanghai, China).

PCR amplification and silver staining

The PCR reactions were performed in a PTC-200 thermostymer (MJ Research, Waltham, MA) in 20-µl reaction mixtures containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.8 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 0.2 µM of each primer and 60 ng of sample DNA. The amplification program consisted of an initial denaturing cycle at 94 °C for 3 min; 32 cycles of 1 min (denaturing) at 94 °C, 40 s (annealing) at corresponding temperatures (Table 2); 1 min (elongation) at 72 °C; and one final cycle of 5 min at 72 °C. Samples were then stored at 4 °C. Five-µl aliquot fractions of the PCR products were run on a 1.8% agarose gel. The products were analyzed on 6.0% (w/v) denaturing polyacrylamide gels, and the gels were silver-stained according to the protocol in the technical manual of silver sequence DNA staining reagents (Promega, Madison, WI).

Allele scoring and data analysis

Following previously reported microsatellite marker analysis procedures (Agrama and Tuinstra 2003, Vogel et al. 2003, Rohrer et al. 2004), the most intense bands of each cpSSR locus on the polyacrylamide gel were scored as 1 for presence...
and 0 for absence. Approximate range of allele sizes was estimated by reference to the 100-bp DNA ladder (Table 3). The extent of marker diversity information was evaluated by calculating the polymorphic information content (PIC) value as:

\[
PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2
\]

where \(P_{ij}\) is frequency of the \(j\)th pattern for marker \(i\) and the summation extends over \(n\) patterns. Genetic relationships among the haplotypes based on SPCC-amplified fragment sizes were analyzed with NTSYS-pc Version 2.10 software (Rohlf 2000). A similarity matrix was generated by calculating the proportion of bands shared by each pair of accessions (Jaccard coefficient), and a dendrogram was constructed using the unweighted pair group method based on arithmetic means (UPGMA).

DNA sequencing and sequence comparison

To verify that the PCR products were amplified chloroplast DNA and that the polymorphism was generated by mononucleotide repeats, the amplification products of SPCC1 and SPCC11 were selected for sequencing because both primer pairs were designed from different model plants and were rich in polymorphism. Seventeen PCR products of SPCC1 and 21 PCR products of SPCC11 were extracted from the corresponding polyacrylamide gels, re-amplified with the corresponding primers, separated on 1.8% agarose gels and then re-amplified with the corresponding primers, separated on 1.8% agarose gels and then re-extracted with an EZNA Gel Extraction Kit (Omega BioTek, Doraville, GA). The DNA samples obtained were ligated into the pMD 18-T Vector (Takara Biotech, Dalian, China) and sequenced by the United Gene Company (Shanghai, China). The DNA was sequenced by the United Gene Company (Shanghai, China). The similarity of sequences, with each other as well as with the primers’ original sequences from Nicotiana tabacum L. (SPCC1) and Arabidopsis thaliana (SPCC11), was checked.

Results

Designing SSR primers for amplification of Citrus chloroplast sequences

Twenty-two previously published cpSSR primers originally designed for pine (Pinus thunbergii), rice (Oryza sativa L.) and tobacco (Nicotiana tabacum) were tested. No amplification was obtained with any of the primers from pine. Four (RC3, RC5, RC6 and RC9) out of five rice primers generated bands on agarose gels, but only a smear was detected when the bands were separated on polyacrylamide gels. In contrast, eight of the 14 primers from tobacco amplified Citrus chloroplast sequences and produced intense bands when products were separated on polyacrylamide gels (Table 2), revealing some degree of polymorphism among the Citrus accessions as well as their related genera (Figures 1A and 1B). The results of the cpSSR primer screening revealed that the target sequences of the cpSSR primers from pine (a gymnosperm) and rice (a monocotyledon) are highly divergent compared with those of Citrus (a dicotyledon), whereas tobacco proved to be an ideal model for the construction of consensus cpSSR primers in another dicotyledonous plant.

Based on results with tobacco, we speculated that the chloroplast database of Arabidopsis thaliana (AP000423) may be useful for developing cpSSR primers for Citrus analysis. Arabidopsis thaliana is another dicotyledon that is widely used as a model plant; however, only a limited number of chloroplast microsatellite loci have been reported from this plant (Provan 2000). Forty-one loci were picked, based on the criteria of cpSSR primer construction described by Weising and Gardner (1999); after eliminating the unsuitable loci, 11 primer pairs were synthesized. Of the 11 primers, six produced

<table>
<thead>
<tr>
<th>Locus</th>
<th>Alleles</th>
<th>Size range (bp)</th>
<th>PIC</th>
</tr>
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<tbody>
<tr>
<td>SPCC1</td>
<td>7</td>
<td>220–240</td>
<td>0.727</td>
</tr>
<tr>
<td>SPCC2</td>
<td>2</td>
<td>800–820</td>
<td>0.493</td>
</tr>
<tr>
<td>SPCC3</td>
<td>5</td>
<td>750–820</td>
<td>0.606</td>
</tr>
<tr>
<td>SPCC4</td>
<td>3</td>
<td>140–170</td>
<td>0.166</td>
</tr>
<tr>
<td>SPCC5</td>
<td>2</td>
<td>110–120</td>
<td>0.057</td>
</tr>
<tr>
<td>SPCC6</td>
<td>2</td>
<td>85–100</td>
<td>0.160</td>
</tr>
<tr>
<td>SPCC7</td>
<td>4</td>
<td>120–150</td>
<td>0.596</td>
</tr>
<tr>
<td>SPCC8</td>
<td>1</td>
<td>100–110</td>
<td>0.000</td>
</tr>
<tr>
<td>SPCC9</td>
<td>8</td>
<td>200–250</td>
<td>0.732</td>
</tr>
<tr>
<td>SPCC10</td>
<td>2</td>
<td>220–250</td>
<td>0.251</td>
</tr>
<tr>
<td>SPCC11</td>
<td>6</td>
<td>200–220</td>
<td>0.670</td>
</tr>
<tr>
<td>SPCC12</td>
<td>1</td>
<td>200</td>
<td>0.000</td>
</tr>
<tr>
<td>SPCC13</td>
<td>3</td>
<td>120–150</td>
<td>0.528</td>
</tr>
<tr>
<td>SPCC14</td>
<td>1</td>
<td>240</td>
<td>0.000</td>
</tr>
</tbody>
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intense bands on polyacrylamide gels, showing a high degree of polymorphism (Figures 1C and 1D).

Polymorphism in chloroplast microsatellite loci of Citrus

Eleven cpSSR primer pairs showed some degree of polymorphism among the analyzed Citrus accessions and related genera (Table 3). The number of alleles and PIC values among the 34 genotypes detected at 14 chloroplast microsatellite loci ranged from 1 to 8 (mean = 3.54) and 0 to 0.732 (SPCC9) (mean = 0.384), respectively.

Cluster analysis of chloroplast SSR haplotypes


These results agree with studies based on cpDNA PCR-RFLP analysis by Nicolosi et al. (2000). They also support the three species classification system of Citrus (Citron (C. medica), tangerine (C. reticulata) and pummelo (C. grandis)) suggested by Scora (1975) and Barrett and Rhodes (1976) based on phylogenetic studies.

Previous studies revealed that C. jambhiri, C. sinensis, C. aurantium and C. paradisi are of hybrid origin. Our results verified that C. reticulata is the maternal parent of C. jambhiri and C. grandis is the female parent of C. sinensis, C. aurantium and C. paradisi, which also agrees with the conclusion of Nicolosi et al. (2000). Likewise, C. limon and C. grandis were classified in the same cluster (Nicolosi et al 2000).

The mandarin-like C. madurensis originated in China, but its taxonomy is uncertain. Although C. madurensis is mandarin-like in most respects, it also has similarities in morphology.
with kumquat. The cpSSR analyses revealed that *C. madurensis* was located in a cluster with *Fortunella* spp. (Figure 2) and it shared the same loci sequence for SPCC1 and SPCC11 (Figures 3 and 4), indicating that *Fortunella* is the maternal parent of *C. madurensis*, which agrees with a previous hypothesis that *C. madurensis* is a natural hybrid between *C. reticulata* and *Fortunella* (Swingle 1943).

*Citrus reticulata* cv. ‘Tankan’ is an old variety originating in southeastern China and still cultivated commercially. Tanaka (1954) considered it to be a natural tangor. Analysis of ‘Tankan’ by random amplified polymorphic DNA (RAPD) and sequence characterized amplified region (SCAR) (Nicolosi et al. 2000) revealed that it was genetically similar to *C. reticulata* and shared a few *C. grandis* DNA bands. The cpSSR analyses located ‘Tankan’ in the *C. reticulata* cluster, suggesting that *C. reticulata* was the female parent that gave rise to this variety.

*Citrus ichangensis* formed a distinct cluster, in contrast with previous reports based on analyses of chloroplast proteins of the large and small subunits (Handa et al. 1986) and chloroplast DNA PCR-RFLPs (Nicolosi et al. 2000). These results suggested that *C. ichangensis* was closer to *Fortunella* than to *C. grandis* and *C. reticulata*. Our cpSSR data also revealed that *C. ichangensis* has a complicated chloroplast composition, and further detailed studies are needed to clarify the taxonomic status of *C. ichangensis*. Kuigan is a native variety in the Three Gorges region of China, and Huyou is a local variety of Zhejiang province, but both genotypes share the same cpSSR hypolotype. In addition, although both Bendiguangju and Satsuma mandarin have identical geographical origin in Zhejiang province, they are morphologically similar and belong to the same cluster based on isoenzyme and nuclear DNA markers (Fang 1990, Hu 1998), our cpSSR data indicate that these genotypes differ in chloroplast genome (cpDNA) composition: Satsuma belonged to *C. reticulata*, whereas Bendiguangju was in the *C. sinensis* cluster (Figure 2). According to Nicolosi et al. (2000), *C. aurantifolia* belongs to the *C. microcarpha* Wester. cluster. Although we did not include *C. microcarpha* in our study, we found that *C. aurantifolia* formed a unique cluster.

The dendrogram (Figure 2) shows that the chloroplast haplotypes reflect the geographic locations of their genotypes. For example, Cleopatra mandarin, originating in India, had a different haplotype than mandarins originating in China. Furthermore, hybrid genotypes such as Kuigan, Huyou, Bendiguangju, Eureka lemon, Tankan, Rough lemon, Murcott tangor and Calamondin usually had novel chloroplast haplotypes, from which it could be deduced that chloroplast introgressive hybridization exists among the inter-crossing *Citrus* species. To date, no cpDNA geographic diversity or transfer in *Citrus* has been reported, although studies have been conducted on a wide range of taxa, including the eucalypts (McKinnon et al. 2004) and beech (Vettori et al. 2004).
Nature of the polymorphism revealed by sequencing

Sequence alignments of the cloned SPCC1 and SPCC11 products suggest that both microsatellite- and non-microsatellite-derived polymorphisms exist in chloroplast SSR loci of Citrus. Most of the polymorphic cpSSR primers detected the typical continuous pattern of variation in length, meaning that the variation in the number of mononucleotide repeats was the most probable cause of polymorphism. Alignment of the cloned sequences of the amplification products of SPCC11 with the original primer motif sequence of Arabidopsis revealed that a variable poly(T) was the direct cause of the polymorphism (Figure 3). The results of SPCC1 showed a discontinuous array of allele sizes, and alignment of the cloned sequences of its amplification products proved that both cpSSR and small segments of DNA insertion or deletion were the main cause of polymorphism.

In accessions P. trifoliata, F. hindsii and C. madurensis, a small 7-bp insert in positions 86 to 93 and a poly(A) mononucleotide repeat region between 205 and 230 existed. In the rest of the sequences, either the poly(A) repeats or the small insertion located in positions 205 to 230 (or 86 to 93) (Figure 4) is the direct reason for the discontinuous variation in length. Usually the insertion or deletion “masks” the polymorphism arising from the microsatellite present at a locus (Bryan et al. 1999).

An interesting outcome derived from the alignment of the SPCC1 primer amplification products is that the original motif poly(T)10 residual of Nicotiana tabacum does not exist in Citrus, but a shorter poly(A) residual should be considered as the motif for Citrus cpSSR primer construction. The highly polymorphic loci SPCC3 and SPCC9 generated discontinuous variation among the DNA samples, indicating that non-cpSSR insertion or deletion events occurred. In particular, amplification with primer SPCC3 generated fragments about 700–800 bp in Citrus and related genera, whereas the original primer motif sequence in Nicotiana tabacum is only 163 bp in length, suggesting there is an insert of about 600 bp (Figure 1B).

Discussion

Development of cpSSR primers for Citrus species

Three primer pairs from Pinus and five from Oryza (RC3, RC5, RC6, RC9, RD19) failed to generate amplification products with Citrus chloroplast genomic DNA. However, eight out of 14 primers from Nicotiana tabacum developed by Bryan et al. (1999) (NTPC4, NTPC9, NTPC12, NTPC18, NTPC40) and Weising and Gardner (1999) (CCMP1 to CCMP10) produced PCR products that showed a high degree of polymorphism when analyzed on polyacrylamide gels. Weising and Gardner (1999) tested a set of 20 cpSSR primers designed from the Pinus thunbergii chloroplast genome on total DNA of Actinidia delicosa (A. Chev.) C.F. Liang & A.R. Ferg, and all failed to generate amplification products. It was therefore concluded that the conservation of mononucleotide repeat flanking regions between gymnosperms and angiosperms was poor. Our results demonstrate that there is also poor conservation of cpSSR primer target sequences between monocotyledonous and dicotyledonous plants. Such poor conservation restricts the consensus among cpSSR primers, preventing the development and utilization of this technique with unrelated species. Therefore, during primer design, the conflict between consensus and polymorphism must be considered. Some primers may generate PCR products among several unrelated species; however, because the loci are too conserved, there will be no polymorphism among unrelated species. For example, five out of the nine consensus cpSSR primers designed by Weising and Gardner (1999) could be used in Citrus and its related genera with PIC values ranging from 0 (SPCC8) to 0.596 (SPCC7) (mean = 0.196), indicating that the polymorphism for these loci is relatively poor in Citrus. On the contrary, PIC values for primers SPCC1, SPCC2 and SPCC3 designed by Bryan et al. (1999) ranged from 0.493 (SPCC2) to 0.727 (SPCC1) (mean = 0.609) showing significant polymorphism in Citrus.

In addition, we conclude that only the chloroplast genome databases of dicotyledonous plants can provide useful motifs for designing cpSSR for other dicotyledonous species. To date, cpSSR primers from Arabidopsis thaliana have rarely been cross-species amplified in woody plants. We scanned chloroplast genome microsatellite loci of Arabidopsis thaliana with a standard motif of n ≥ 10. Forty-one primer pairs were designed (not listed), 11 potentially useful primer pairs were synthesized and their good performance was demonstrated by the polymorphism detected in Citrus. Sequencing demonstrated that variation in poly(T) accounted for the polymorphism in the SPCC11 locus among the 34 tested accessions.

In higher plants, mononucleotide repeats are frequent and randomly located mainly in the intron and intergenic regions. Using the n ≥ 10 mononucleotide repeat strategy, 39 loci in Nicotiana tabacum and 69 loci in Arabidopsis thaliana were found, and when the search criterion was changed to n ≥ 8, 116 loci in Nicotiana tabacum and 123 loci in Arabidopsis thaliana were obtained (Provan et al. 2001). The sequencing results of the SPCC1 fragments suggested that the most suitable motif in Citrus was the poly(A) repeat, whereas in the corresponding original Nicotiana tabacum sequences, this poly(A)n motif was eliminated by the n ≥ 10 criterion. From these results, it can be assumed that motifs found with the n ≥ 8 criterion are the most useful for the design and application of cpSSR primers to a wide range of species.

A set of universal cpDNA primers from rice (Hiratsuka et al. 1989) has been successfully used in Citrus for CAPS analyses (Nicolosi et al. 2000, Cheng et al. 2003a). For both CAPS and chloroplast microsatellites, primers are designed from the flanking region located in intron or intergenic regions, and polymorphism in PCR products is detected by restriction analysis or directly on polyacrylamide gels. The 14 useful cpSSR primer pairs for Citrus originated either from Nicotiana or from Arabidopsis. The other eight primers from Oryza and Pinus were incapable of producing suitable PCR products. The main reason for the difference in the design of universal
Figure 3. Sequence alignment of cloned SPCC11 products.
Figure 4. Sequence alignment of cloned SPCC1 products.
primers for use in CAPS and cpSSR is the length of the amplified DNA fragments, which determines the position of target sequences. In CAPS analysis, the amplified fragments range from 1 to 3 kb, and the target sequence is usually located inside genes; however, in cpSSR analysis, the fragments are relatively short, about 100 to 300 bp, and the target sequences are usually located outside genes. In chloroplasts, the codon sequences are highly conservative, whereas the intron and intergenic regions are highly variable, thus restricting the combination of primers with their target sequences in cpSSR.

**Analyzing Citrus chloroplast polymorphism with cpSSR markers**

We demonstrated that cpSSR markers provide a new and efficient tool for Citrus cytoplasm analyses. Compared with nuclear DNA markers, chloroplast DNA markers are more reliable for Citrus taxonomic studies because of the conservation and uni-parental inheritance of the chloroplast genome in higher plants. In the mid 1980s, chloroplast RFLPs were used in Citrus phylogenetic studies (Green et al. 1986); however, only a few RFLP reports on Citrus have been published. The use of universal chloroplast primers for CAPS in Citrus (Nicolosi et al. 2000) greatly simplified the procedures of plastid genetic analyses, and the method has been successfully used in Citrus somatic cytoplasm composition analysis (Cheng et al. 2003a). However, comparing the present cpSSR study with our previous chloroplast CAPS analyses of Citrus (Cheng et al. 2003a) revealed several disadvantages of CAPS including the limited sources of universal primers, cost of the enzymes and poor resolution on agarose gels. The cpSSR method overcomes these disadvantages. First, the amplification regions are mainly located in variable mononucleotide regions, which are rich in polymorphism in the chloroplast genome. Besnard and Bervillé (2002) compared the PCR and RFLP methods in a study on olive (Olea europaea) chloroplast diversity analyses and concluded that data based on PCR reveal more polymorphisms in cpDNA and lead to more reliable results than those based on the RFLP method. In addition, the high resolution of polyacrylamide gels can reveal polymorphism at the mononucleotide level. Consequently, cpSSR seems the simplest and cheapest procedure among those presently available for the analysis of chloroplast DNA, in that neither isotopes as used in RFLPs nor the restriction enzymes as used in CAPS are required.

**Elucidating the classification of several Citrus genotypes**

In an isozyme study of 108 accessions of Citrus and its related genera, Fang (1990) identified five genetic sources of Citrus species during evolution: C. macroptera, C. ichangensis, C. medica, C. grandis and C. reticulata. Based on cpSSR analysis, we identified 18 chloroplast haplotypes that were divided into seven major clusters.

Both Bendiguangju mandarin and Satsuma mandarin have similar morphology, but Bendiguangju was ascribed to the Pummelo cluster, whereas Guoqing No. 4 Satsuma was ascribed to the mandarin cluster. Chinese scholars deduced that these mandarins were closely related based on the morphology of leaves and fruits and on their growing location in ancient times. This hypothesis has been verified by isozyme analysis (Fang and Zhang 1992) and RAPDs (Hu 1998). Amplified fragment-length polymorphism (AFLP) and SSR analyses of Bendiguangju and Satsuma mandarin further support the close relationship between Bendiguangju and Satsuma mandarin (Cao 2003). On the other hand, RFLP results of mitochondrial and chloroplast DNA showed that Satsuma mandarin holds a mandarin-type cytoplasm and Bendiguangju holds an orangetype cytoplasm, indicating that Bendiguangju and Satsuma mandarin are sister lines of a reciprocal cross between mandarin and sweet orange (Cao 2003), results that are further supported by our research.

Huyou pummelo is a well-known local variety in the province of Zhejiang, with a cultivation history of more than 500 years, and is still widely cultivated commercially in southeast China. There is no doubt about its hybrid origin; however, the identity of the female parent genotype is still unclear. We found that Huyou pummelo and Kuigan mandarin, an ancient variety in the Three Gorges areas, had identical chloroplast haplotype, and were located in the C. grandis cluster in the UPGMA dendrogram. Based on RAPDs analyses, Hu (1998) deduced that Kuigan is a mandarin × pummelo hybrid. Based on our data, we deduce that Huyou pummelo and Kuigan mandarin are hybrids and that pummelo was the female parent.

The finding that three genotypes of Huyou, Bendiguangju and Satsuma mandarin originated in the same region of Zhejiang province and had different chloroplast haplotypes indicates that Zhejiang province is a citrus area with high chloroplast diversity. The diverse chloroplast haplotypes might be associated with the migration history of Citrus. Zhejiang province, located near the East China Sea, was one of the most economically developed areas in China for centuries; its well-developed agriculture in ancient times led to the collection of many citrus genotypes from different areas of China as well as from other countries. The first citrus monograph was published in 1178 in Zhejiang, with 27 types of citrus recorded. Based on the hypothesis of Petit et al. (2003), the mixing areas of plant migration routes would subsequently create the diversity of chloroplast haplotypes.

Given the complex parentage and taxonomy of Citrus and related genera, it is difficult to clarify the origin and genetic relationships of these taxonomically questionable genotypes on the basis of traditional nuclear DNA markers. Although the risk of homoplasy existing in chloroplast microsatellite loci may limit the application of cpSSR markers in phylogenetic studies (Weising and Gardner 1999, Vogel et al. 2003), by combining the results of previous nuclear DNA markers with those of the present cpSSR markers, an accurate map of the origin of all genotypes can be deduced.

In summary, cpSSR has proved to be an important complimentary method in Citrus taxonomic studies. Moreover, the strategies of Citrus cpSSR primer development could be used for chloroplast studies of woody plants whose chloroplast sequences are unavailable. The high degree of chloroplast DNA polymorphism revealed by cpSSR verified that this marker is a powerful tool for the study of Citrus parentage and recent evo-
lution, as well as for the analysis of organelle inheritance in *Citrus* somatic hybrids.

**Acknowledgments**

We thank Prof. Hongwei Huang (Institute of Wuhan Botany, Chinese Academy of Sciences) for assistance in the data analyses and Prof. Gene L. Albrigo (Citrus Research and Education Center, University of Florida) for reviewing the manuscript. This research was funded by the National Science Foundation of China (NSFC: No. 30170472 and 30123001), the International Foundation for Science (IFS: No. AD/15702) and the International Plant Genetic Resources Institute (IPGRI/AM-LOA-002-2002).

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