The Complete Chloroplast Genome Sequence and Intra-Species Diversity of *Rhus chinensis*

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ABSTRACT *Rhus chinensis* is a shrub widely distributed in Asia. It has been used for traditional medicine and ecological restoration. Here, we report the complete chloroplast genome sequence of two *R. chinensis* genotypes collected from China and Korea. The assembled chloroplast genome of Chinese *R. chinensis* is 149,094 bp long, consisting of a large single copy (97,246 bp), a small single copy (18,644 bp) and a pair of inverted repeats (16,602 bp). Gene annotation revealed 77 protein coding genes, 30 tRNA genes, and 4 rRNA genes. A phylogenomic analysis of the chloroplast genomes with 11 known complete chloroplast genomes clarified the relationship of *R. chinensis* with the other plant species in the Sapindales order. A comparative chloroplast genome analysis identified 170 SNPs and 85 InDels at intra-species level of *R. chinensis* between Chinese and Korean collections. Based on the sequence diversity between Korea and Chinese *R. chinensis* plants, we developed three DNA markers useful for genetic diversity and authentication system. The chloroplast genome information obtained in this study will contribute to enriching genetic resources and conservation of endemic *Rhus* species.

Keywords *Rhus chinensis*, Medicinal plant, Ecological restoration, DMZ, Chloroplast genome sequence, Molecular marker

INTRODUCTION

Rhus chinensis is a deciduous shrub belonging to the family Anacardiaceae and distributed widely in Asia including India, Vietnam, China, Korea, and Japan (Min and Barford 2008). It contains various pharmacologically active constituents and insect-induced galls, which have been used for medicinal purposes (Min and Barford 2008; Djakpo and Yao 2010). In addition, *R. chinensis* is used as revegetation plant for ecological restoration owing to its cold tolerance and easy multiplication by both seed and clonal propagation (Nam *et al.* 2004; Lim and Oh 2015). The diverse utilization of this shrub led to international

seed trading which in turn resulted in contamination and destruction of endemic population of *R. chinensis*. Although the generic and infrageneric delimitation of *Rhus* species remains controversial, very limited genetic resources are available for *R. chinensis* (Young 1978; Miller *et al.* 2001; Yi *et al.* 2004; Ma *et al.* 2013; Lee *et al.* 2016). In this study, we have characterized the complete chloroplast genome of Chinese *R. chinensis* and compared with Korean *R. chinensis* chloroplast genome for estimating genetic diversity and developing marker for authentication.

Chloroplast DNA has a characteristic of exhibiting variations at intra-species level and thus chloroplast DNA has widely been used for classification among populations.

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Chloroplast genomes are uni-parentally inherited and contain rare recombination and mutation events (Cheng *et al.* 2005; Dong *et al.* 2012). These features make chloroplast genomes good candidate for studying evolution at inter- or intra-species level. For instance, intra-species researches had been conducted using chloroplast DNA in various plant species including *Panax ginseng*, *Pedicularis chamissonis*, *Primula cuneifolia*, *Tellima grandiflora*, and *Tiarella trifoliata* (Soltis *et al.* 1991; Soltis *et al.* 1992; Fujii *et al.* 1995; Sewell *et al.* 1996; Fujii *et al.* 1997; Kim *et al.* 2015a; Joh *et al.* 2017). Chloroplast genome was also applied to taxonomic studies of *Rhus* and marker development to classify species (Miller *et al.* 2001; Lee *et al.* 2004; Yi *et al.* 2004; Yi *et al.* 2007). However, high resolution markers have not been developed for distinguishing *Rhus* species at both inter- and intra-species level.

In this study, we assembled the complete chloroplast genome of two *R. chinensis* genotypes from China and Korea. In addition, we have conducted a comparative phylogenomic analysis of Chinese and Korean *R. chinensis* along with 11 species belonged in the Sapindales order. We also developed polymorphic DNA markers derived from chloroplast genomes to practically apply for authentication and genetic diversity among *R. chinensis* collections.

MATERIALS AND METHODS

Plant materials and genome sequencing

Seeds of wild Chinese *R. chinensis* were obtained from Shandong and Henan provinces in China. We also collected seven *R. chinensis* samples in Korea (Table 1): two samples

from demilitarized zone (DMZ) between South and North Korea and five samples from other locations in South Korea. We sequenced plants collected from Yang-gu province in Korea and Shandong and Henan provinces in China as representatives of the collections of Korea and China. The genomic DNAs were extracted from the leaf tissues or seeds of collected plants using a modified cetyltrimethylammonium bromide (CTAB) method (Allen *et al.* 2006) and quantified using Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA). Paired-end (PE) sequencing was conducted using Illumina MiSeq platform by LabGenomics (www.labgenomics.co.kr, Seongnam, Korea).

Chloroplast genome assembly

Raw PE reads were trimmed and *de novo* assembled using the method as described by Kim *et al.* (2015a, 2015b). From initial assembly, contigs representing chloroplast genome sequences were extracted, ordered, and merged to generate a single contig sequence using the reference chloroplast genome *Acer buergerianum* ssp. *ningpoense* (KF753631, Yang *et al.* 2014). The assembled sequence was manually corrected and gap-filled by a series of PE read mapping. The assembled chloroplast genome was annotated using GeSeq (<https://chlorobox.mpimp-golm.mpg.de/geseq-app.html>) and manually curated using the Artemis annotation tool (Rutherford *et al.* 2000).

Phylogenetic analysis

Phylogenetic analysis was carried out using multiple sequence alignments of 13 complete chloroplast genome

Table 1. Sample collections of *R. chinensis* used in this study.

Accession nos.	Countries	Collected locations	Collected year
1	China	Shandong and Henan provinces	2014
2	Korea	Yang-gu, Gangwon-do	2014
3	Korea	Hwacheon, Gangwon-do	2014
4	Korea	Mt.Jiri, Sancheong, Kyungsangnam-do,	2014
5	Korea	Han-taek Botanical Garden, Yong-in, Kyungki-do	2015
6	Korea	Mt. Kariwang, Jeong-seon, Gangwon-do	2015
7	Korea	Gwangreung Botanical Garden, Korea National Arboretum, Po-cheon, Gyeonggi-do	2015
8	Korea	Gwangreung Botanical Garden, Korea National Arboretum, Po-cheon, Gyeonggi-do	2015

sequences (*Acer burgerianum* ssp. *ningpoense*, KF753631; *Acer davidii*, NC_030331; *Acer miaotaiense*, NC_030343; *Azadirachta indica*, NC_023792; *Boswellia sacra*, KT934315; *Citrus aurantiifolia*, KJ865401; *Citrus sinensis*, DQ864733; *Dipteronia sinensis*, NC_029338; *R. chinensis* (China), MF351625; *R. chinensis* (Korea), NC_033535; *Sapindus mukorossi*, KM454982; *Spondias bahiensis*, KU756561; *Spondias tuberosa*, KU756562) that belong to the Sapindales order. Phylogenetic tree was constructed using MEGA6.0 (Tamura *et al.* 2013) with the parameters of neighbor-joining method and 1000 bootstrap replicates.

Comparison of intra-species level and development of molecular marker

Sequence variations were identified by mVISTA program (<http://genome.lbl.gov/vista/mvista/submit.shtml>) and the polymorphic sites were arranged by MAFFT program (<http://mafft.cbrc.jp/alignment/software>). The two types of molecular markers, InDel and SNP, were developed based on the polymorphic sites in chloroplast genomes of two *R. chinensis*. The primers were designed using Primer-blast tool in NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). PCR reactions were performed in 25 µL final volume which is composed of 20 ng of template DNA, 1x *Taq* buffer, 2.5 mM dNTP, 10 pmol of each primer, and 2 unit/µL *Taq* DNA polymerase (Vivagen, Korea). PCR conditions were as follows: 5 minutes at 95°C, 35 cycles of 30 seconds at 95°C, 30 seconds at 56°C, and 30 seconds at 72°C, and 5 minutes at 72°C as final extension. PCR amplicons were inspected using 3% agarose gel including Inclone™ Safe Gel stain. Then they were visualized under UV trans-illuminator and a gel documentation system.

PCR reaction for high resolution melting (HRM) analysis against SNP target was performed in a 20 µL final volume which consists of 20 ng of template DNA, 1x *Taq* buffer, 2.5 mM dNTP, 10 pmol of each primer, 2 unit/µL *Taq* DNA polymerase (Vivagen, Korea), and fluorescent dye SYTO 9 (Roche Diagnostics). PCR conditions were as follows: 5 minutes at 95°C, 45 cycles of 30 seconds at 95°C, 30 seconds at 56°C, and 30 seconds at 72°C, and 5 minutes at 72°C as final extension. Then, HRM analysis was conducted using LightCycler 480 (Roche Applied

Science). HRM conditions were as follows: 1 minute at 95°C, 1 minute at 40°C, 5 seconds at 70°C, and the temperature increased up to 90°C, then decreased to 40°C as fluorescence acquisition.

RESULTS

Complete chloroplast genome and intra-species polymorphism between Korean and Chinese *R. chinensis*

The assembled chloroplast genome of Chinese *R. chinensis* is a circular molecule of 149,094 bp long and has typical quadripartite structure consisting of a large single copy (LSC) region of 97,246 bp small single copy (SSC) region of 18,644 bp and a pair of inverted repeats (IRa and IRb) of 16,602 bp (Fig. 1, Table 2). A total of 111 genes, including 77 protein coding genes, 30 tRNA genes, and 4 rRNA genes were annotated (Table 3) and the GC content of the chloroplast genome was 37.86%.

Comparison of the chloroplast genome of Korean and Chinese *R. chinensis* revealed a total length 83 bp differences. Chinese *R. chinensis* is 364 bp longer than Korean *R. chinensis* in LSC region, but 139 bp and 3 bp shorter in IR and SSC regions, respectively. We identified 170 SNPs and 85 InDels between two *R. chinensis* chloroplast genome sequences derived from Chinese and Korean collections (NC_033535, Lee *et al.* 2016). More intra-species polymorphic sites were found at intergenic regions than genic regions: 99 SNPs and 78 InDels were found at intergenic regions; 71 SNPs and 7 InDels were identified from genic regions.

Phylogenomic analysis of *R. chinensis* based on chloroplast genome

Phylogenomic analysis was conducted using two complete chloroplast genomes *R. chinensis* and 11 complete chloroplast genome sequences in the Sapindales order. This results showed, as expected, that *R. chinensis* was grouped with *Spondias* species in the Anacardiaceae family (Fig. 2). In addition, a group including *R. chinensis* showed a close sister relationship with species belonging to the Burseraceae and Sapindaceae family in the Sapindales order.

Fig. 1. Chloroplast genome map of *R. chinensis* and intra-species polymorphic DNA markers. (a) Chloroplast genome map of *R. chinensis* generated using OGDRAW (<http://ogdraw.mpimp-golm.mpg.de/>). Genes transcribed clockwise and counterclockwise are indicated on the outside and inside of the large circle, respectively. The four parts of the chloroplast genome and GC content are indicated on the inner circle. Red and blue bars in the inner circle indicate intra-species InDels and SNPs identified between Chinese and Korean *R. chinensis* chloroplast genome sequences. Black arrows represent the target regions for InDel markers and the purple arrow indicates SNP used to differentiate between Chinese and Korean *R. chinensis*.

Table 2. Summary of NGS data and chloroplast genomes of two *Rhus chinensis* collections.

Collected locations	Raw data bases (bp)	Cp genome coverage (x)	Cp length (bp)	LSC length (bp)	IR length (bp)	SSC length (bp)
China	4,199,252,923	162	149,094	97,246	16,602	18,644
Korea (Yang-gu) ²⁾	6,251,344,649	96	149,011	96,882	16,741	18,647

²⁾reported by Lee *et al.* (2016).

Table 3. Genes annotated in *R. chinensis* chloroplast genome.

Gene types	Gene names
Photosystem I	<i>psaA</i> , <i>B</i> , <i>C</i> , <i>I</i> , <i>J</i>
Photosystem II	<i>psbA</i> , <i>B</i> , <i>C</i> , <i>D</i> , <i>E</i> , <i>F</i> , <i>H</i> , <i>I</i> , <i>J</i> , <i>K</i> , <i>L</i> , <i>M</i> , <i>N</i> , <i>T</i> , <i>Z</i>
Cytochrome b/f complex	<i>petA</i> , <i>B</i> , <i>D</i> , <i>G</i> , <i>N</i> , <i>L</i>
ATP synthase	<i>atpA</i> , <i>B</i> , <i>E</i> , <i>F</i> , <i>H</i> , <i>I</i>
NADH dehydrogenase	<i>ndhA</i> , <i>B</i> , <i>C</i> , <i>D</i> , <i>E</i> , <i>F</i> , <i>G</i> , <i>H</i> , <i>I</i> , <i>J</i> , <i>K</i>
RubisCO large subunit	<i>rbcL</i>
RNA polymerase	<i>rpoA</i> , <i>B</i> , <i>C1</i> , <i>C2</i>
Ribosomal proteins (SSU)	<i>rps2</i> , <i>3</i> , <i>4</i> , <i>7</i> , <i>8</i> , <i>11</i> , <i>12</i> , <i>14</i> , <i>15</i> , <i>16</i> , <i>18</i>
Ribosomal proteins (LSU)	<i>rpl2</i> , <i>14</i> , <i>16</i> , <i>20</i> , <i>23</i> , <i>32</i> , <i>33</i> , <i>36</i>
clpP, matK	<i>clpP</i> , <i>matK</i>
Other genes	<i>ccsA</i> , <i>cemA</i> , <i>accD</i>
Hypothetical chloroplast reading frames	<i>ycf1</i> , <i>2</i> , <i>3</i> , <i>4</i> , <i>15</i>
Transfer RNAs	<i>trnM</i> -CAU, <i>A</i> -UGC, <i>C</i> -GCA, <i>D</i> -GUC, <i>E</i> -UUC, <i>F</i> -GAA, <i>G</i> -GCC, <i>UCC</i> , <i>H</i> -GUC, <i>I</i> -CAU, <i>GAU</i> , <i>K</i> -UUU, <i>L</i> -CAA, <i>CAU</i> , <i>UAG</i> , <i>UAA</i> , <i>M</i> -CAU, <i>N</i> -GUU, <i>P</i> -UGG, <i>Q</i> -UUG, <i>R</i> -UCU, <i>ACG</i> , <i>S</i> -UGA, <i>GCU</i> , <i>GGA</i> , <i>T</i> -GGU, <i>V</i> -GAC, <i>UAC</i> , <i>W</i> -CCA, <i>Y</i> -GUA
Ribosomal RNAs	<i>rrn4.5</i> , <i>5</i> , <i>16</i> , <i>23</i>

Fig. 2. Phylogenetic analysis of *R. chinensis*. The tree was generated with complete chloroplast genome sequences of *R. chinensis* and species belonging to the Sapindales order by multiple alignment using MAFFT (<http://mafft.cbrc.jp/alignment/server/index.html>) and a maximum likelihood (ML) analysis using MEGA 6.0 (Tamura *et al.* 2013). Numbers in the nodes are bootstrap support values (>50%) from 1000 replicates. Chloroplast genome sequences used for this tree are: *Acer buergerianum ssp. ningpoense*, KF753631; *Acer davidii*, NC_030331; *Acer miaotaiense*, NC_030343; *Azadirachta indica*, NC_023792; *Boswellia sacra*, KT934315; *Citrus aurantiifolia*, KJ865401; *Citrus sinensis*, DQ864733; *Dipteronia sinensis*, NC_029338; *R. chinensis* (China), MF351625; *R. chinensis* (Korea), NC_033535; *Sapindus mukorossi*, KM454982; *Spondias bahiensis*, KU756561; *Spondias tuberosa*, KU756562.

Molecular markers to discriminate Korean and Chinese collections of *R. chinensis* species

Three intra-species polymorphic markers were designed from polymorphic regions (Table 4). The PCR amplicons showed 288/249 bp and 221/196 bp difference (Chinese/Korean) for two InDel markers, rh_InDel_02 and rh_InDel_03 markers, respectively. The rh_InDel_02 and rh_InDel_03 showed InDel polymorphism between Korean *R. chinensis* (Yang-gu) and Chinese collection as expected (Fig. 3a, b). However other six Korean collections were identical with Chinese collections (Fig. 3c, d). One SNP marker (rh_hrm_11) distinguished Chinese collection

from all the Korean collections based on HRM analysis although the size of PCR amplicons are same (Fig. 4).

DISCUSSION

Intra-species diversity of *R. chinensis*

R. chinensis is known as a valuable medicinal plants with remedial components (Min and Barfod 2008; Djakpo and Yao 2010). In addition, *R. chinensis* is used for ecological restoration (Nam *et al.* 2004; Lim and Oh 2015). Due to its wide utility, it is necessary to explore this plant for genetic diversity and marker development to prevent false trading

Table 4. Molecular markers developed in this study.

Type	Marker Name	Target Region		Primer Sequence	Variation	Product Size (bp)
InDel	rh_InDel_02	<i>trnS-GGA-rps4</i>	F	AGTGGTTCAAGGCGTAGCAT	39 bp	288/249
			R	ATTTGATCCGGCGATTTGGA		
	rh_InDel_03	<i>ycf1</i>	F	TGATTCGCTCGATTCGCCA	25 bp	221/196
			R	TCTGTCCTTCAATATCACGGAAC		
SNP	rh_hrm_11	<i>ycf1</i>	F	CATGTGTGCATCTCTGGGTT	G/T	191
			R	CTTCCTTTGGTCCAATTCTCGAT

Fig. 3. Two InDel-based markers to validate intra-species diversity in *R. chinensis*. (a) A 39-bp InDel in *trnS-GGA-rps4* and (c) its PCR products (Marker rh_InDel_02 in Table 4) for eight *R. chinensis* plants. (b) 25-bp InDel in *ycf1* and (d) its PCR products (Marker rh_InDel_03 in Table 4) for eight *R. chinensis* plants. *R. chinensis* collections 1: China (Shandong and Henan), 2: Korea (Yang-gu), 3: Korea (Hwacheon), 4: Korea (Mt. Jiri), 5: Korea (Han-taek Botanical Garden), 6: Korea (Mt. Kariwang), 7, 8: Korea (Gwangreung Botanical Garden), M: 100-bp DNA ladder.

Fig. 4. SNP-based marker to validate intra-species diversity in *R. chinensis*. (a) Alignment of nucleotide sequences to show the SNP (arrow) in *ycfI* gene of chloroplast of Korean and Chinese *R. chinensis* plants. (b) Agarose gel electrophoresis of the PCR products amplified by Marker rh_hrm_11 (Table 4). (c) HRM analysis of the PCR products to detect the SNP. *R. chinensis* collections 1: China (Shandong and Henan), 2: Korea (Yang-gu), 3: Korea (Hwacheon), 4: Korea (Mt. Jiri), 5: Korea (Han-taek Botanical Garden), 6: Korea (Mt. Kariwang), 7, 8: Korea (Gwangreung Botanical Garden), M: 100-bp DNA ladder.

(Khairallh and Salama 2009). Here, we generated the complete chloroplast genome of two *R. chinensis* plants collected from Korea and China. Comparative analysis of chloroplast genome of *R. chinensis* revealed 255 intra-species polymorphic sites, 170 SNPs and 85 InDels. The abundant genetic diversity would be found in the wild collections of *R. chinensis* and could be applied to phylogenetic analysis and development of molecular markers for verifying genetic diversity of *R. chinensis*.

Discrimination of *R. chinensis* collections with three molecular markers

Two InDel markers were unique for one Korean collection of *R. chinensis* (Yang-gu) from the others (Fig. 3a, b). The Yang-gu collection is derived from the DMZ region between South Korea and North Korea. The eco-system was well maintained in DMZ regions for longer than 60 recent years with less artificial interruption. Two InDel markers revealed unique genotype of Yang-gu collection among the eight collections, suggesting the Yang-gu collection are relatively isolated in the DMZ

region from others. However, it is required to expand our study by evaluating more population for solid conclusion. Meanwhile, the SNP markers could efficiently divide the melting patterns for Chinese *R. chinensis* from Korean *R. chinensis* through HRM analysis (Fig. 4).

Chloroplast genomes are valuable genomic resources for practical application of DNA markers because of the uni-parentally inherited features and conserved structure of the chloroplast genomes (Cheng *et al.* 2005; Dong *et al.* 2012, Kim *et al.* 2015a, 2015b, 2015c; Joh *et al.* 2017). Here, we report three DNA markers which can be applied for genetic diversity and practical application for authentication of the plant collections of *Rhus* species.

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