Intraspecific genetic variation of ITS regions between two karyotypes in *Ranunculus cantoniensis* (Ranunculaceae)

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Genetic variations in *Ranunculus cantoniensis* DC. were examined through nucleotide sequence of ITS regions on five Korean and Japanese populations and compared with previous karyological research and RAPD analysis results. All examined individuals had 648 base pairs in their ITS regions. The lengths of ITS-1 and ITS-2 were 254 bp and 264 bp, respectively and 5.8S was 130 bp. Twenty sites were variable and fifteen sites were genetically informative. They were clearly separated into two groups by neighbor-joining tree. One group coincided with the Atsuki group and the other with the Matsuyama group of karyological classification. Variations of nucleotide sequence analyses were very useful in illustrating intraspecific genetic differentiation of *R. cantoniensis*.

The buttercup plants (*Ranunculus* L.) have shown intraspecific variations of karyotype among different populations (Fujishima and Kurita, 1974; Kang et al., 1996; Okada and Tamura, 1977; Tamura, 1963). Since Kurita’s study (1955), several researchers (Fujishima, 1983; 1984; 1985a; b; Kang et al., 1996; Okada and Tamura, 1977) have reported the karyotype variations of *Ranunculus cantoniensis* (2n = 32) in Japan and Korea. The species have been subdivided into

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five groups as Atsuki group, Makikaneyama group, Matsuyama group, Hagi group and Particular group \(2n=33\) by Fujishima (1984) based on chromosome shapes, as well as the presence, loci and size of the satellite.

Fujishima (1984; 1985a) speculated that Atsuki group was the most primitive among these five karyological groups of *R. cantoniensis*, based upon comparison of their karyotypes. Genetic evidence regarding these karyological variations were examined using RAPD analysis from eight Korean and one Japanese populations (Kang et al., 1998).

Molecular phylogenetic studies, meanwhile, had demonstrated that ITS regions of nuclear ribosomal DNA were very useful to reconstruct phylogeny at various taxonomic levels (Baldwin, 1992; Hershkovitz and Lewis, 1996; Hershkovitz and Zimmer, 1996; Suh et al. 2000). Especially, ITS regions have been claimed to provide enough phylogenetic signals at interspecific and intergenetic levels because their rates of divergence are considerably rapid in comparison to protein or rDNA coding genes (Baldwin et al., 1995). To clarify the genetic differentiation of the populations of *R. cantoniensis*, we investigated intraspecific variation in the nucleotide sequences of internal transcribed spacer of nrDNA among five populations from Korea and Japan.

**Materials and Methods**

**Plant materials and DNA extraction**

We studied karyotype of all individuals from each population of *R. cantoniensis* in previous study (Kang et al., 1996). Based on the karyotype classification of Fujishima (1984) the populations of Kurye (KR), Byunsan (BS) and Higashi-Hiroshima (HH) were determined to Atsuki Group, while Sunchon (SC) and Hybrid (Hy) populations were determined to Matsuyama Group. Here, hybrid is tried to breed with individual whose karyotypes were mixed and cultivated for five years in the same space at greenhouse of Han Nam University. In order to survey nrDNA variation of *R. cantoniensis*, one individual for each population was selected to examine the intraspecific variation in the nucleotide sequences of internal transcribed spacer of nrDNA (Table 1). DNA was extracted from fresh leaf material using the QIAquick genomic DNA extraction kit following the manufacturer instructions (QIagen). Fresh leaf tissues were powdered in liquid nitrogen and kept in 70°C deep freezer until DNA extraction. DNA was stored in TE buffer at -20°C until use.
Table 1. Populations of *Ranunculus cantoniensis* examined for nucleotide sequence variation of internal transcribed spacer (ITS) of nrDNA.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Population</th>
<th>Population size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. cantoniensis</em></td>
<td>KR: Kurye, Chonbuk Prov., Korea</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>BS: Byunsan, Chonbuk Prov., Korea</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>HH: Higashi-Hiroshima, Hiroshima Pref., Japan</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Hy: Cult. Han Nam Univ., Korea</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>SC: Korea, Sunchon, Chonnam Prov., Korea</td>
<td>26</td>
</tr>
</tbody>
</table>

Polymerase chain reaction and sequencing

The ITS regions were amplified using the primers described by White *et al.* (1990). ITS is approximately 600-700 base pair (bp) long and include two spacer regions of less than 300 bp (ITS-1 and ITS-2) separated by 5.8S gene a small coding region (Hodkinson *et al.* 2000). PCR was carried out in 100 l final volume containing 0.5ng template DNA, 2.5units of Taq polymerase (Promega), 10mM Tris, pH 8.3, 50mM KCl, 1.5mM MgCl2, 0.001% gelatin, 200M for each dNTP and 0.5l of each primer. PCR primers were ITS1, ITS2, ITS3 and ITS4 designed by White *et al.* (1990). The thermal cycling parameters for ITS region comprised 3 minutes pre-denaturation at 95°C and 30 cycles, with 30 seconds denaturation at 95°C, 1 minute annealing at 55°C and 45 seconds extension at 72°C. A final extension of 10 minutes at 72°C was also included. The PCR reaction was performed on Thermocycler 2400 (PE Applied Biosystems). For the purification, PCR products were separated on a 0.8% agarose gel, and gel was eluted using QIAquick gel extraction kit (QIAGen) as described by a manufacturer. For the sequence reaction, purified PCR products were cloned into pGEM-T easy vector. Cloned DNA sequences were determined by ABI PRISM (M-377, Ver-3.0) automatic sequencer in Korea Basic Science Institute (KBSI). The DNA sequence data was analyzed using PC/Gene software.

Sequence alignment and phylogenetic analysis

The boundary of ITS-1, 5.8S and ITS-2 were determined by the comparison of previous studies (Yokota *et al.*, 1989; Baldwin, 1992; Sun *et al.*, 1994, Hershkovitz and Lewis, 1996; Odorico and Miller, 1997). The sequence divergence (K) was calculated by Kimura’s two parameter method, which takes different rates of transversions and transitions into account (Kimura, 1980). The
Table 2. Sequence characteristics of the ITS-1, ITS-2 and 5.8S regions in five populations of *Ranunculus cantoniensis*.

<table>
<thead>
<tr>
<th></th>
<th>ITS-1</th>
<th>ITS-2</th>
<th>5.8S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>254</td>
<td>264</td>
<td>130</td>
</tr>
<tr>
<td>Variable sites (%)</td>
<td>7 (35%)</td>
<td>9 (45%)</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>Inform sites (%)</td>
<td>6 (50%)</td>
<td>5 (41.7%)</td>
<td>1 (8.3%)</td>
</tr>
<tr>
<td>(G+C) %</td>
<td>49.3–50.0</td>
<td>52.7–54.9</td>
<td>51.5–53.1</td>
</tr>
</tbody>
</table>

Sequence distance values were calculated using DNADIST program (PHYLIP ver. 3.57c for IBM PC; Felsenstein, 1995). Neighbor–joining tree reconstruction (Saitou and Nei, 1987) was performed using the NEIGHBOR and DNAML programs, respectively, which were implemented in the phylogeny inference package (PHYLIP).

**Results**

Nucleotide sequence analysis was performed to seek genetic variation for four Korean populations and one Japanese population of *R. cantoniensis*. The results of genetic analysis were compared with former karyological research (Kang *et al.*, 1996) and RAPD data (Kang *et al.*, 1998). Complete and aligned DNA sequences of the ITS regions for five samples of *R. cantoniensis* were provided in Fig. 1 and their characteristics were summarized in Table 2. All examined individuals had 648 bp length ITS-1 to ITS-2 regions. The lengths of ITS-1 (254 bp), 5.8S (130 bp) and ITS-2 (264 bp) region were the same for all individual from five populations. Among the 618 sites, 20 sites were variable and 15 sites were phylogenetically informative. Nucleotide sequence distance calculated by Kimura's two parameter method (Kimura, 1980) varied from 0 to 0.0324 (from KR to SC; Table 3). Phylogenetic tree was made by neighbor–joining method based upon Kimura's two parameter method (Kimura, 1980).

*R. cantoniensis* is so variable in external morphology that several intraspecific classifications have been proposed (Kang *et al.*, 1996). The five examined populations were clearly separated into two groups on the neighbor–joining tree, that is, one group from the population of KR, HH, BS and the other group from the populations of Hy and SC. From the cytological viewpoint by Kang *et al.* (1996), the former is congruent with the Japanese Atsuki group, while the
Fig 1. DNA sequence alignment of the ITS-1, 5.8S and ITS-2 regions from four Koreans and one Japanese populations of *Ranunculus cantoniensis*. The 5.8S region is written in Italic small letters. KR: Kurye, Chonbuk Prov., Korea; HH: Higashi-Hiroshima, Hiroshima Pref., Japan; Hy: Hybrid, Cult. at Han Nam University; SC: Sunchon, Chonnam Prov., Korea; BS: Byunsan, Chonbuk Prov., Korea.

latter is consistent with the Japanese Matsuyama group. This result supports the previous conclusions of cytological study (Kang *et al.*, 1996) and RAPD analysis (Kang *et al.*, 1998).

**Discussion**

The size of ITS-1, ITS-2 and 5.8S cording sequences in *R. cantoniensis* lie within the range of those reported previously for other angiosperms (ITS-1, 187-298; ITS-2, 187-252; Baldwin *et al.*, 1995; Suh *et al.*, 2000).

The results of this study proved that there were two groups of *R. cantoniensis* in Korea. That supported former studies, cytological study (Kang *et
Table 3. Genetic distance made from nucleotide sequence of ITS regions.

<table>
<thead>
<tr>
<th></th>
<th>KR</th>
<th>BS</th>
<th>HH</th>
<th>Hy</th>
<th>Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR</td>
<td>0.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS</td>
<td>0.0107</td>
<td>0.0000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH</td>
<td>0.0107</td>
<td>0.0000</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hy</td>
<td>0.0324</td>
<td>0.0213</td>
<td>0.0213</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>Sc</td>
<td>0.0324</td>
<td>0.0213</td>
<td>0.0213</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

al., 1996) and RAPD analysis study (Kang et al., 1998), that reported the existence of two karyotype and gene groups of R. cantoniensis in Korea. Compared with previous studies, analysis of nucleotide sequence of ITS regions makes a better differentiation between Atsuki and Matsuyama groups. Genetic variations among populations were much greater from RAPD (0.2513; Kang et al., 1998) than from ITS sequences (0.0324, Table 3). It was proven that analyses of nucleotide sequence of ITS regions were more suitable to detect isolation by distance among closely related population of R. cantoniensis.

Among the R. cantoniensis group in Japan (Tamura, 1978), R. silerifolius and R. chinensis exhibit interspecific chromosome polymorphisms (Fujishima, 1977, 1985a, 1985b, 1988; Fujishima and Kurita, 1974; Okada and Tamura, 1977; Okada, 1981, 1984). The distribution areas of these cytotypes are geographically separated from each other, but in some localities two cytotypes grow close to each other. Natural hybrids are rarely found, even at such localities, although artificial crossing easily results in hybrid individuals (Okada and Kubo, 1999).

Fujishima et al. (1995) supported the suggestions of Okada (1984), who reported that an amphidiploid species or R. cantoniensis (2n=32) has originated by the hybridization between diploid species, R. silerifolius (2n=16) and R. chinensis (2n=26) after polyploidization to tetraploid level. The pollen grains (and probably embryo sacs also) of the diploid hybrids R. silerifolius × R. chinensis appear practically sterile (only 1 to 5% fertility). This suggests that even if interspecific hybridization between the two species occurred in nature, there would hardly be possibilities for their continued existence (Okada and Kubo, 1999). This is different from European R. ficaria with intraspecific autopolyploidy (2x, 3x, 4x) and extensive means of vegetative propagation (Gill et al., 1972; Marchant and Brighton, 1974, Gadella, 1977; Okada, 1984). The comparison between artificial tetraploid hybrids and wild R. cantoniensis concerning
Fig. 2. Neighbor-joining tree of *Ranunculus cantoniensis* on ITS sequences (5.8S region included) using Kimura's two parameter methods. Localities are those described in Fig.1.

number and shape of somatic chromosomes and characters of middle leaflet proved these interpretations. But, paring configurations at meiotic metaphase I of PMCs (Pollen Mother Cells) showed a feature different from *R. cantoniensis* (Okada, 1989).

Interspecific hybrids between *R. silerifolius* and *R. chinensis* may not persist because of their biennial habitat and their very low fertility (Okada, 1984). But in *R. silerifolius* and *R. cantoniensis* inter-cytotypic hybrids may persist for some time in nature. The fertility of inter-cytotypic hybrids of *R. cantoniensis* is about 90% (Okada, 1984: Okada and Kubo, 1999).

The genus *Ranunculus* consists of about 600 species and includes great diversity (Tamura, 1995) in reproductive strategies, including self incompatibility in *R. japonicus* and partial self pollination and partial apomixis in *R. auricomus* (Steinbach and Gottsberger, 1994). The reason why the variations of nucleotide sequences occurred in this species needs to be further studied by concerning Okada's report (1984) and comparing nucleotide sequences for the phylogenetic systematics among *R. cantoniensis* and its allied species.

In the previous reports (Park, 1974; Lee, 1980), *R. cantoniensis* has been observed within the restricted area, southern part of the Korean Peninsula.
including Cheju Isl. and geographically independent region. The Atsuki group
distributes in the western region, and the Matsuyama group in the eastern part
of the Korean Peninsula. Fujishima et al. (1995) and we (in this study), however,
could find another population in the middle part of the Korean Peninsula.
Hence, there is a possibility that a new gene group might be found in the Ko-
rean Peninsula.

Even though we could not find regular variation of external morphological
characters in *R. cantoniensis*, genetic differentiations of *R. cantoniensis* has kept
occurring in their gene levels. Based on this study, we conclude that analysis
of nucleotide sequences is very useful to identify genetic variation in *R.
cantoniensis*

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털개구리미나리의 핵형 변이에 관한
ITS region 염기서열 분석

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(日本 廣島大學 大学院 國際協力研究科1, 생명공학연구소3, 한남대학교 생물학과3)

적 요

한국산 털개구리미나리 4개 지역 개체군과 일본산 1개 지역 개체군의 ITS 염기서열을 이용하여 유전적 분화양상을 조사하였고, 그 결과를 세포학적 분화양상 및 RAPD에 의한 유전분석 결과와 비교하였다. 조사된 모든 개체의 ITS region의 길이는 648 base pair로 동일하였고, 이중 ITS-1은 254bp, ITS-2는 264bp 그리고 5.8S는 130bp로 각각 조사되었다. 총 648sites 중 20개의 부위가 한 개이상의 염기에서 차이가 났으며, 계통학적으로 정보를 갖는 부위는 15 부위였다. 조사된 개체군은 Neighbor-joining 분석에서 명확히 2group으로 구분되는데 이는 세포학적 결과에서의 Atsuki group 및 Matsuyama group과 일치하였다. 종내의 유전적 군집분화 양상을 파악하는데 핵형 분석, RAPDs 분석방법 그리고 염기서열 분석방법이 모두 매우 유용한 것으로 생각된다.

주요어: 털개구리미나리, ITS 염기서열, Neighbor-joining, 군집분화양상

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