Phylogeny of Japanese species of *Sanguisorba* (Rosaceae) based on RFLPs of PCR-amplified cpDNA fragments

MISAKO MISHIMA¹ and MOTOMI ITO²

¹Department of Biology, Faculty of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192-03, Japan; ²Department of Biology, Faculty of Science, Chiba University, Chiba 263, Japan

Abstract. The RFLPs of the PCR-amplified cpDNA of seven Japanese species of *Sanguisorba* were investigated. Twelve restriction site mutations were phylogenetically informative. On the basis of Wagner parsimony analysis, a single most parsimonious tree with two clades was generated: one clade comprising *S. albiflora*, *S. hakusanensis*, *S. japonensis*, and *S. stipulata*, and the other comprising *S. obtusa*, *S. officinalis*, and *S. tenuifolia*. Morphologically, however, *S. obtusa* in the latter clade closely resembles the former four species of the other clade. Three possibilities are discussed to explain such a discrepancy between the species relationships supported by morphological evidence and those suggested by molecular evidence. Molecular evidence further does not support close relationship among *S. albiflora*, *S. obtusa*, and *S. hakusanensis* that are suggested by Nordborg earlier.

Key words: cpDNA, PCR, phylogeny, RFLPs, Rosaceae, *Sanguisorba*

Received May 8, 1996; accepted October 27, 1996

For phylogenetic analysis of plants, molecular marker has provided us a powerful tool (Soltis et al., 1992). Among several analytical methods of DNA variation, an analysis of the restriction fragment length polymorphism (RFLPs) of polymerase chain reaction (PCR) amplified products, PCR-RFLPs method, has some advantages. It requires a small amount of extract DNA, and analytical protocol is simpler than that of southern hybridization method (Liston, 1992).

In Rosaceae, *Sanguisorba* L. is unique in lacking petals as in ten other genera of the tribe Sanguisorbeae. About 25 species are known to be assigned to the genus, and they are distributed in temperate to subarctic region of the Northern Hemisphere (Nordborg, 1966). Seven species occur in Japan (Ohwi and Kitagawa, 1983): *Sanguisorba albiflora* (Makino) Makino, *S. hakusanensis* Makino, *S. japonensis* (Makino) Kudo, *S. obtusa* Maxim., *S. stipulata* Rafin, *S. officinalis* L., and *S. tenuifolia* Fisch. A few of them are clearly different from the others in distribution range and habitat. *Sanguisorba albiflora*, *S. japonensis*, *S. obtusa*, *S. hakusanensis*, and *S. stipulata* usually allopatrically occur in sub-alpine to alpine region, and the three former species are endemic to Japan. In contrast, *S. officinalis* is cosmopolitan, and *S. tenuifolia* occurs in a broad area of Eastern Asia. These two species grow in lowland to mountain areas. An analysis of phylogenetic relationships among the seven species promises to add to
the elucidation of plant speciation and development of the flora in Japan.

In this paper we report the results of an analysis of RFLPs of PCR amplified fragments of chloroplast DNA (cpDNA) in the seven species of *Sanguisorba* in Japan.

**Materials and Methods**

Seven species of *Sanguisorba* examined in this study are presented in Table 1 along with their collection data. *Agrimonia* L. (*A. nipponica* Koidz.) was used as an outgroup because it is the only genus of the tribe Sanguisorbeae in Japan apart from *Sanguisorba*. Total DNAs were extracted from fresh leaves according to CTAB method (Doyle and Doyle, 1987). Two cpDNA regions were amplified by two primer sets: *rpoC1*-195 and *rpoC2*-1364 (Liston, 1992), and *atpB* c1 and *rbcL*-NN3-2 (Hasebe et al., 1994) (Fig. 1). The former primer set amplifies most part of *rpoC1* and *rpoC2* genes, which includes the intron in *rpoC1* and the intergenic spacer between the two genes (Liston, 1992). The latter primer set amplifies most parts of *rbcL* and *atpB* genes including intergenic sequences between them (Hasebe et al., 1994). PCR-amplification was performed with Ex Taq DNA polymerase (Takara) according to manufacture’s protocol of pre-heating for 5 min at 94°C, followed by 35 cycles of denaturing for 30 sec at 96°C, annealing for 1 min at 55°C, and extension for 3 min at 70°C, with a final extension for 10 min at 70°C. The amplification products were digested with the following restriction enzymes (4-bp recognition sites): Hae III, Hha I, Msp I, Alu I, Mbo I, Afa I, Taq I, and BstU I. The digested PCR products were electrophoresed in 2.0% agarose gels, detected by ethidium bromide stain, photographed, and then restriction site mutations were investigated.

The restriction site data were analyzed by Wagner parsimony method using PAUP 3.1 (Swofford, 1993). The most parsimonious tree was searched for with the Exhaustive option. The tree was rooted using *Agrimonia*
FIG. 1. Primer sets used and cpDNA regions amplified by PCR. A. rpoCl-195 and rpoC2-1364. B. atpB-cl and rbcl-NN3-2.

ṭnipponica as an outgroup. To evaluate the confidence intervals, the bootstrap method (Felsenstein, 1985) was employed with one thousand replicates.

Results

The primer set of rpoCl-195 and rpoC2-1364 amplified an approximately 4,100 bp, and the product of another primer set of atpB-cl and rbcl-NN3-2 was an approximately 3,800 bp. No length variation in each region was found among all the species examined.

The digestion with eight restriction enzymes resulted in about 104 restriction sites. Among them, 12 unique restriction sites exhibited interspecific variation and five of them were phylogenetically informative (Table 2). Fig. 2 shows Hha I-digestion patterns of the fragment amplified by the primer set of atpB-cl and rbcl-NN3-2. Wagner parsimony analysis using all the information summarized in Table 2 resulted in a single most parsimonious tree (Fig. 3). It is 12-step tree with a consistency index (C. I.) of 1. In this tree, seven species are divided into two clades: one comprises Sanguisorba albiflora, S. hakusanensis, S. japonensis, and S. stipulata, which are defined by four synapomorphies and supported by 100% boot-
TABLE 2. Chloroplast DNA restriction site mutations in *Sanguisorba*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Regions</th>
<th>Fragment sizes (Kb)</th>
<th>Species*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bust UI</td>
<td><em>rpoC1:rpoC2</em></td>
<td>1.45 = 0.75 + 0.70 0.59 = 0.35 + 0.24</td>
<td>1–3, 6 1–7</td>
</tr>
<tr>
<td>Hae III</td>
<td><em>atpB-c1:rbcL-NN3-2</em></td>
<td>0.22 + 0.83 = 1.05 0.92 = 0.14 + 0.78</td>
<td>1–3, 6 1–3, 6</td>
</tr>
<tr>
<td>Hha I</td>
<td><em>atpB-c1:rbcL-NN3-2</em></td>
<td>0.95 = 0.65 + 0.30</td>
<td>4, 5, 7</td>
</tr>
<tr>
<td>Msp I</td>
<td><em>atpB-c1:rbcL-NN3-2</em></td>
<td>0.21 + 0.33 = 0.54</td>
<td>1–7</td>
</tr>
<tr>
<td>Taq I</td>
<td><em>rpoC1:rpoC2</em></td>
<td>0.20 + 0.85 = 1.05</td>
<td>1–7</td>
</tr>
<tr>
<td></td>
<td><em>atpB-c1:rbcL-NN3-2</em></td>
<td>0.17 + 0.17 = 0.34</td>
<td>1–7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.22 + 0.22 = 0.44</td>
<td>1–7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.64 + 0.17 = 0.81</td>
<td>1–7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.44 = 0.25 + 0.19</td>
<td>1–7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.58 = 0.43 + 0.15</td>
<td>1–3, 6</td>
</tr>
</tbody>
</table>

*Species having the derived mutations. The numbers correspond to the species listed in Table 1.

FIG. 2. Electrophoretogram showing digestion patterns by restriction enzyme Hha I of cpDNA fragment (3,800 bp) in seven species of *Sanguisorba* and *Agrimonia nipponica*. cpDNA fragment was amplified by PCR with the primer set, *atpB-c1* and *rbcL-NN3-2*. m: Molecular size standards (Hind III digestion of λ-DNA); 1: *S. albiflora*; 2: *S. hakusanensis*; 3: *S. japonensis*; 4: *S. obtusa*; 5: *S. officinalis*; 6: *S. stipulata*; 7: *S. tenuifolia*; 8: *A. nipponica*.

strap reproductive probabilities; the other comprises *S. obtusa*, *S. officinalis*, and *S. tenuifolia*, which has a single synapomorphy and is supported by 68% bootstrap reproductive probabilities. The relationships among the
MISHIMA & ITO: Phylogeny of *Sanguisorba*

**Discussion**

Results of the present analysis show that the *Sanguisorba* species investigated are divided into two clades consisting of four species and three species, respectively. PCR-RFLPs method of cpDNA in *Sanguisorba*, therefore, is likely to be available in distinguishing one group of species from the other.

The four species belonging to the first clade, i.e., *Sanguisorba albi\_flora*, *S. hakusanensis*, *S. japonensis*, and *S. stipulata*, have similar habitat and a few floral morphological features. They are all distributed in the sub-alpine to alpine regions (Ohwi and Kitagawa, 1983). Their floral characteristics are as follows: stamens are longer than the calyx lobes; flattened filaments are 2.5 times wider or more in the upper part than in the lower part; the stigma is tufted; flowers emit sweet smell (Nordborg, 1966; Mishima, pers. obs.). Two of the three species forming the second clade, i.e., *S. officinalis* and *S. tenuifolia*, are distinct from the above four alpine species. They have wider distribution area extending into lowland and low mountains. Their floral characteristics are as follows: the stamens are equal or a little longer than the calyx lobes; the filiform or sometimes flattened filaments are less than 2 times wider in the upper part than in the lower part; the stigma is compact; flowers have an offensive smell.

*Sanguisorba obtusa* belongs to the same clade as *S. officinalis* and *S. tenuifolia*. However, unlike the latter two lowland species but like the four alpine species, *S. obtusa* has longer and flattened filaments, tufted stigma,
and sweet smell in flowers. In other words, *S. obtusa* has the same mutation in cpDNA with the two lowland species but is morphologically more similar to the four alpine species. To explain the character evolution in Japanese *Sanguisorba*, there may be three possibilities. First, the morphological characteristics shared by *S. obtusa* and the four alpine species are apomorphies, and were produced by convergence. Second, they are pleomorphies; two lowland species having derived characteristics. Thirdly, the common features were derived by hybridization of two species in two different clades, because the chloroplast genome is inherited maternally in most angiosperms. However, several examples are known in the literature where that the trees based on chloroplast genome disagree with those based on nuclear genome (for review see Rieseberg and Soltis, 1991). In order to test the third possibility, we need to examine the phylogeny using nuclear genome.

Japanese species of *Sanguisorba* are placed in sect. *Sanguisorba* and are further divided by Nordborg (1966) into four groups of species based on the inflorescence type and the filament shape: “*S. obtusa* group” including *S. albiflora, S. hakusanensis, and S. obtusa; “*S. officinalis* group” including *S. officinalis; “*S. tenuifolia* group” including *S. tenuifolia; and “*S. canadensis* group” including *S. stipulata* and *S. japonensis*. Nordborg treated *S. albiflora* as a synonym of *S. obtusa*, and considered that *S. hakusanensis* is closely related to *S. obtusa*. However, results of cpDNA variation analysis indicate that the *S. obtusa* group *sensu* Nordborg is not monophyletic, and even suggest that it is necessary to revise the intrageneric classification of *Sanguisorba*.

We thank Drs. J. Murata and H. Setoguchi of Makino Herbarium, Tokyo Metropolitan University, for their guidance and helpful suggestion, and Drs. H. Takahashi of Hokkaido University and H. Kato of Kyoto University for providing us with some living materials. This study was partly supported by Sasakawa Scientific Research Grant from the Japan Science Society (No. 7–190).

**References**


発表者

三島美佐子1・伊藤元己2: 日本産ワレモコウ属(バラ科)の葉緑体DNAのPCR-RFLPsに基づく系統解析

日本産ワレモコウ属7種の分子データを用いた系統解析を行った。葉緑体DNAの2領域(計約8Kbp)をPCR法で増幅し、8種類の4塩基認識酵素で処理した。その結果得られた断片長多型に基づき、ヒメキンミズヒキを外群として最節約法により系統樹を構築した。その結果、一つの共有派生形質によりワレモコウ、ナガボノワレモコウ、ナンプトウウチソウが、4つの共有派生形質によりカライトソウ、タカネトウウチソウ、エゾトウウチソウ、シロバナトウウチソウが、それぞれ単系統群を形成した。ナンプトウウチソウ・カライトソウ・タカネトウウチソウ・エゾトウウチソウ・シロバナトウウチソウは高山から亜高山帯にやや遠存的に生じ、外部形態的に類似した形質状態を示す。このうちナンプトウウチソウ(岩手県早池峰山固有種)が、低地から産地に広く分布し互いにやや類似した形質状態を有するワレモコウ・ナガボノワレモコウと単系統群に含まれた事は、以下のいずれかの可能性を示している。1) 現在の高山種に見られる形態の類似した形質状態は収斂による、2) ワレモコウとナガボノワレモコウに見られる形態の類似した形質状態は派生的な状態である。3) ナンプトウウチソウは低地性2種のいずれかと、高山種のいずれかとの間の雑種起源種であり、低地性の種の葉緑体ゲノムを受け継いでいる。

(1)〒192-03 東京都八王子市南大沢1-1 東京都立大学理学部、(2)〒263 千葉市稲毛区弥生町1-33 千葉大学理学部)