Genetic Characterization of *Epimedium* Species Using Random Amplified Polymorphic DNA (RAPD) and PCR-Restriction Fragment Length Polymorphism (RFLP) Diagnosis

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Total DNA was extracted from the leaves of seven *Epimedium* species grown in different places in Japan. Their genetic characterization was performed by DNA analyses of random amplified polymorphic DNA (RAPD) using 32 random primers having 10 base sequences, and by restriction fragment length polymorphism (RFLP). *E. sagittatum* and *E. koreanum* were easily distinguished by a representative amplified band pattern. It became evident that *E. sagittatum* had extremely different genetic composition compared to the other species. A dendrogram obtained from the similarity matrix by cluster analysis indicates that *E. sagittatum* can be completely isolated from the other species. Moreover, it became evident that *E. grandiflorum var. higoense*, *E. trifoliatobinatum* and *E. koreanum* are independent species, contrary to the previous assumption that they are subspecies or a variety. The geographical variation of *E. sempervirens* was confirmed by cluster analysis. *E. diphylum* showed wide genetic variations, in spite of sampling from the same area.

Key words *Epimedium* species; Berberidaceae; Chinese medicine; chemotaxonomy; RAPD; PCR-RFLP

The aerial parts of *Epimedium* species (Inyokaku in Japanese, Yin-yang-huo in Chinese) are widely used as a tonic in China and Japan. Many flavonol glycosides have been isolated from a number of *Epimedium* plants, 1–7 although the relation between these flavonol glycosides and the pharmacological activities of the drug is still obscure. Japanese *Epimedium* species are classified into three types according to the spur length of flower, which can be used as a significant characteristic for species diagnosis. 8 Histological characteristics and various ramifications of their leaves have also been previously investigated. 8–10 From these results, it is suggested that at least seven species of *Epimedium* occur in Japan. 11 However, it is known that hybridization between species occurs, resulting in the diversification of populations of *Epimedium* species. 11 Morphological and chemotaxonomic classification of *Epimedium* species is therefore still somewhat obscure.

Biochemical markers, such as some isoenzymes, are considered to be appropriate tools. 12,13 The complexity of the necessary identification procedures was, however, required. Recently, molecular markers, such as restriction fragment length polymorphism (RFLP) 14 and random amplified polymorphic DNA (RAPD) 15 appear to be good candidates for the identification of plant species. Therefore, we have planned to perform a combination of RAPD and PCR-RFLP to classify the important medicinal plant, *Epimedium* species, and to confirm the geographical variations of these strains. RAPD analysis is more appropriate than RFLP analysis for the determination of species and crude drugs. First, since RAPD analysis can be simply conducted by an automated procedure, as compared to RFLP analysis, the screening of polymorphisms can be carried out rapidly. Second, no digestion by a restriction enzyme is needed in RAPD analysis, resulting in direct diagnosis; thus, less plant material is required than is required for RFLP analysis.

Previously we presented plant regeneration by shoot tip culture and organogenesis of *Epimedium* species, 16 and investigated the taxonomy of *Epimedium* species using a combination of the chemical analysis of flavonol glycosides and several isoenzymes. 17 In this study we wish to further discuss the characterization of *Epimedium* species using RAPD and PCR-RFLP diagnosis.

MATERIALS AND METHODS

**Plant Materials** *E. diphylum*, *E. sempervirens* and *E. setosum* were collected in Hiroshima prefecture. *E. grandiflorum var. higoense* was collected in Oita prefecture. *E. trifoliatobinatum* came from Oshima Island, Oita prefecture. *E. koreanum* was donated from Dr. M. Mikage, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan. *E. sagittatum* and *E. grandiflorum* were donated from the herbal garden of Takeda Chemical Industries, Ltd. They are listed in Table 1. All species were identified morphologically, 11 and by electrophoretic analysis of isoenzymes, 17 and they were cultivated in the herbal garden of the Faculty of Pharmaceutical Sciences, Kyushu University. Three plants of individual *Epimedium* species were used for assay, except

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name of species</th>
<th>Collection place</th>
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<tbody>
<tr>
<td>A</td>
<td><em>E. sagittatum</em></td>
<td>From China</td>
</tr>
<tr>
<td>B</td>
<td><em>E. koreanum</em></td>
<td>Kanazawa city</td>
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</tbody>
</table>
| C      | *E. sempervirens*| a: Shimane prefecture  
|        |                | b, c: Kanazawa city |
| D      | *E. setosum*   | Futami-gun, Hiroshima prefecture |
| E      | *E. diphylum*  | Futami-gun, Hiroshima prefecture |
| F      | *E. trifoliatobinatum*| Oshima Island, Oita prefecture |
| G      | *E. grandiflorum var. higoense*| Kuiya, Oita prefecture |
| H      | *E. grandiflorum*| From Takeda Pharmaceutical Industry Co., Ltd. |

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for *E. sagittatum*, of which a single plant was analyzed.

**RAPD Analysis** DNA for RAPD analysis was extracted from young leaves from field grown plants by the method of Murray and Thompson[18] with some modification. The obtained crude DNA was further purified by a Geneclean II kit (Bio 101, U.S.A.) as follows: To the DNA solution, NaCl at three times the volume of the DNA solution, plus a Glass milk (5 μl) were added and mixed using a vortex mixer. The mixture was incubated at 0 °C for 10 min, with mixing every 1 min. After 10 min incubation, the mixture was centrifuged at 6000 rpm at 2 °C for 30 s. The supernatant was removed and then washed with New Wash 3 times. Finally, the DNA fraction was centrifuged at 14000 rpm at 2 °C for 30 s. The supernatant was removed. After the addition of 50 μl of sterile pure water, the DNA solution was incubated at 55 °C for 5 min. The mixture was again centrifuged at 15000 rpm at r.t. for 20 min. The supernatant of 45 μl was recovered and its DNA concentration was determined using a U-3210 Spectrophotometer (Hitachi). Amplifications were carried out in 10 μl reaction volumes composed of 50 mM Tris–HCl (pH 8.5), 5 mM MgCl₂, 500 μg/ml BSA, 2.0% Ficol, 4 mM tartrazine, 10 μM EDTA, 15 ng of genomic DNA, 0.25 μM of primer, 500 μM dNTPs and 0.32 units of *Tth* DNA polymerase (Toyobo). [19]

The 1605 Air Thermo-Cycler (Idaho Technology) was programmed for 60 s at 94 °C, as well as 60 cycles of 10 s at 94 °C, 30 s at 36 °C, and 60 s at 72 °C, followed by 120 s at 72 °C, to carry out the amplifications. Amplification products were separated in 1% agarose gels in 0.5× Tris–borate–EDTA (TBE) buffer. Gels containing ethidium bromide (5 μl/100 ml) were developed at 120 V for 3 h. Gels were detected and photographed by a DNA transilluminator (UVP Inc., model NTM-20) at 302 nm. The sizes of the amplification products were estimated using a 100 bp (100 bp-2.0 kb) ladder (Pharmacia). The random primers consisted of ten base sequences from a kit purchased from Operon Technologies, Inc. The KFB series, having ten base sequences synthesized previously, [49] were shown as follows: KFB-01 (5’-CCACCGGCCAG-3’), KFB-02 (5’-CAGTTCGGCC-3’), KFB-05 (5’-ACTCGTAGCC-3’), KFB-07 (5’-ACGTAGCCGC-3’), KFB-09 (5’-CTCGGTGATC-3’) and KFB-10 (5’-ATCCTCCGC-3’).

The genetic similarity (F) values were determined by the Nei and Li’s method[20]:\[ F = 2M_{xy}/M_x + M_y \]
in which \( M_{xy} \) is the number of shared bands, and \( M_x \) and \( M_y \) are the number of bands of populations x and y between pairs of species. Genetic distances (1 - F) were calculated from the F values as indicated in Table 1. A dendrogram was produced by the unweighted pair-group method with arithmetic mean (UPGMA), as shown in Figs. 2 and 4.

**PCR-RFLP Analysis** Total DNA was extracted and purified as already mentioned in RAPD analysis. Amplifications were carried out in 100 μl reaction volumes composed of 10 mM Tris–HCl (pH 8.4), 1.5 mM MgCl₂, 50 mM KCl, 10 μg/ml gelatin, 200 μM dNTPs, 0.25 units/10 μl *Tth* DNA polymerase (Toyobo), 0.5 μM of primer (for *rbcL*: 5’-GTCCGATTCAAACGCTGGTGTG-3’ and 5’-TCACAGACGACGACAGTCTC-3’), 22 and 10 ng/100 μl of genomic DNA. The Astec Program Temp Control System PC-700 (Astec) was programmed for 60 s at 94 °C, as well as 30 cycles of 30 s at 94 °C, 30 s at 54 °C and 90 s at 72 °C, followed by 60 s at 72 °C, to carry out the amplifications. Amplification products were purified by a Geneclean II kit as indicated in RAPD analysis. To 150 ng of amplified and purified DNA, 8 μl sterile H₂O, 2 μl of 50 mM Tris–HCl (pH 7.5) containing 10 mM MgCl₂, 100 mM NaCl and 1 mM dithiothreitol[23] and 10 units/ml of restriction enzyme (ScrF 1) solution were added, and the mixture was incubated at 37 °C for 4 h. The reaction mixtures were treated at 65 °C for 10 min, then cooled. Amplification products were separated in 2% agarose gels in a 0.5× TBE buffer. Gels containing ethidium bromide (5 μl/100 ml) were developed at 70 V for 1 h 45 min. Gels were detected and photographed using the same RAPD analysis indicated above.

**RESULTS AND DISCUSSION**

The total DNA extracted from the leaves of *Epimedium* species was analyzed by RAPD using 32 random primers resulting in 180 total bands. Figure 1 indicated a representative amplified band pattern diagnosed by primer KFB-10. Seven major bands (2, 4, 5, 7, 8, 11 and 13) and six minor bands (1, 3, 6, 9, 10 and 12) were detected (Fig. 1). Remarkable differences between *E. sagittatum* (lane A of Fig. 1), which grows in China (Table 1), *E. koreanum*...
(lane B of Fig. 1), and the other species (lanes C to H of Fig. 1) were found by this band pattern. Band 13 disappeared in *E. sagittatum*, and *E. koreamun* also had a unique pattern which shows only two major bands, 2 and 13. In the previous paper, *E. diphyllum* was easily distinguished from the other species by comparisons of isocitrate dehydrogenase (IDH), glutamate dehydrogenase (GDH), phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) in the isoenzyme analysis. However, polymorphisms in the amplified DNA of *E. diphyllum* (lane E of Fig. 1) were of two types: two plants had two clear bands (bands 5 and 13), and the other had three major bands (bands 2, 5 and 13), as indicated in Fig. 1.

Table 2 indicates the genetic distance matrix between individual species calculated according to the Nei and Li's method (see Materials and Methods section). It became evident that only *E. sagittatum* had extremely different genetic distance compared to the other species.

A dendrogram obtained from the similarity matrix by cluster analysis indicates that *E. sagittatum* (A in Fig. 2) can be completely isolated from the other species, as indicated in Fig. 2. This result is reasonable since *E. sagittatum* is indigenous in China. It has been judged that *E. trifoliatobinatum* (F in Fig. 2) is a hybrid of *E. grandiflorum* (H in Fig. 2) and *E. diphyllum* (E in Fig. 2), by morphological comparisons. However, since *E. trifoliatobinatum* formed a clear cluster, as indicated in Fig. 2, the above assumption is not correct.

It is known that *E. koreamun* (B in Fig. 2) and *E. grandiflorum var. higoense* (G in Fig. 2) are not independent species, the former having been reported to be a subspecies of *E. sempervirens* (C in Fig. 2). The results of cluster analysis, however, showed that *E. koreamun* is an independent species. This is supported by the amplified band pattern diagnosed by primer KFB-10, as already discussed in Fig. 1. Subsequent cluster analysis confirmed that *E. grandiflorum var. higoense* is an independent species, as indicated in Fig. 2, although this species was classified as a variety of *E. grandiflorum*. This finding was completely confirmed by RFLP analysis of *rbcL* using *SacF I* as a restriction enzyme, showing that a different band appeared from *E. grandiflorum var. higoense* compared to the other species, as shown in Fig. 3.

Previously, we reported that *E. diphyllum* was easily distinguished from the other species by comparisons of IDH, GDH, PGI and PGM. However, since the dendrogram of *E. diphyllum* did not form a clear cluster (Fig. 2), wide genetic variations are thought to have occurred. Figure 4 shows the dendrogram of six plants of this species, which were collected from the narrow part of Hiroshima prefecture in Japan (Table 1). Genetic

![Fig. 3. Restriction Fragment Length Polymorphism Diagnosis of *rbcL* by *SacF I* Digestion in Seven *Epimedium* Species](image)


![Fig. 4. A Dendrogram of Six Plants of *Epimedium diphyllum* Collected from the Narrow Part of Hiroshima Prefecture](image)
variations were observed, although six plants built up one cluster. Moreover, it became clear that geographical variations appeared (Fig. 2) in *E. sempervirens* when compared with three plants of *E. sempervirens* of which two plants (b and c) were collected from Kanazawa City in Japan and one (a) was from Shimane prefecture.

*E. setosum* seems to be a hybrid of *E. diphyllyum* and *E. sempervirens*, because *E. setosum* is sympatric with *E. diphyllyum* and *E. sempervirens* from the narrow location in Hiroshima prefecture (Table 1). This was supported by isoenzyme analysis, indicating that *E. setosum* is closely related to *E. diphyllyum* and *E. sempervirens*, in good agreement with the finding of Suzuki, who found that they crossed easily with each other. Therefore, in order to clarify the relations of the above three species, *E. setosum*, *E. diphyllyum* and *E. sempervirens*, cluster analysis was investigated. However, the cluster of *E. setosum* collapsed, although *E. setosum* is closely related to *E. sempervirens*. This may depend on geographical differences between *E. diphyllyum* and *E. sempervirens*, as already discussed. Moreover, *E. setosum* seems to be related to *E. grandiflorum*, judging from the cluster analysis in Fig. 2. However, more detail experiments are required to further investigate this suggestion.

Since the classification and identification of *Epimedium* species by flavonoid components and by electrophoretic analysis are unclear, RAPD analysis may be a useful methodology for the chemotaxonomical classification of *Epimedium* species. Actually, we confirmed that three species, *E. koreum*, *E. trifoliatobinatum* and *E. grandiflorum* var. *higoense* are independent species, contrary to previous classification of the Japanese *Epimedium* species. It is therefore suggested that this assay system may provide a useful method for determining the quality and homogeneity of the crude drug of the *Epimedium* species, because the flavonol glycosides are significantly different qualitatively and quantitatively, depending on the strain grown in Japan.

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REFERENCES