

Lack of allozyme and ISSR variation in the Rare endemic tree species, *Berchemia berchemiaefolia* (Rhamnaceae) in Korea

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Abstract – Rare plant species are commonly hypothesized to have little genetic variation because of genetic drift, strong and directional selection toward genetic uniformity in a limited number of environments, inbreeding depression and/or other factors. We investigated genetic variation in *Berchemia berchemiaefolia*, a rare and endangered tree species worldwide, by examining 14 allozyme loci and 28 I-SSR amplicons in 111 individuals distributed among four populations in Korea. No allozyme and I-SSR variation were detected with the exception of one variant from one individual at *Pgi-2* locus. A substantial genetic bottleneck accompanying the fluctuation of local population size caused by repeated human activities and inbreeding could account for this species' lack of genetic variation.

Berchemia berchemiaefolia / rare tree / no variation / allozyme / I-SSR

Résumé – Absence de variabilité d'allozyme et ISSR chez les espèces d'arbres rares et endémiques, *Berchemia berchemiaefolia* (Rhamnaceae) en Corée. On fait en général l'hypothèse que les espèces rares ne disposent que d'une faible variabilité génétique pour différentes raisons : dérive génétique, forte sélection dans le sens de l'uniformité dans un nombre limité de milieux, dépression due à la consanguinité et divers autres facteurs. Nous avons étudié la variabilité génétique de *Berchemia berchemiaefolia*, espèce rare et en voie de disparition au niveau mondial, en examinant 14 loci d'allozyme et 28 I-SSR amplicon pour 111 individus provenant de quatre populations coréennes. Aucune variabilité d'allozyme et d'ISSR n'a pu être détectée à l'exception d'un variant chez un individu au locus *Pgi-2*. Ces modifications de taille des populations dues à des activités répétées se traduisent par un « goulot d'étranglement génétique » qui explique l'absence de variabilité génétique de l'espèce.

Berchemia berchemiaefolia / espèce ligneuse rare / absence de variabilité / allozyme ISSR (Inter simple sequence repeat)

1. INTRODUCTION

The genus of *Berchemia* (the family Rhamnaceae) includes 12–22 deciduous woody plants distributed in Asia, East Africa, and South America [10]. They are usually climbing or scandent plants, but rarely trees or shrubs growing as high as 6 m. They have petiolated and pinnately many-veined leaves with small and caducous stipules. Small flowers have five sepals and five petals with fascicular inflorescence. Fruit is an elongate drupe and has leathery fleshy with one stone [10]. In Korea, the genus of *Berchemia* has only two native species [22]: *B. racemosa* Sieb. et Zucc., and *B. berchemiaefolia* (Makino) Koidz. The former one is a deciduous climber while *B. berchemiaefolia* is a deciduous small tree. The distribution of the two *Berchemia* species is quite limited. *B. racemosa* is known from only one population and *B. berchemiaefolia* is limited to 5–6 populations in Korea [22, 23].

B. berchemiaefolia was found in Korea in 1935 for the first time and since then it has been classified as a plant species endemic to Korea [12]. However, it also grows in the southern

part of Japan and the middle part of China with very restricted distribution ranges [4, 11, 14]. In China, it is only found in Xingshan County, western Hubei, Shexian County (the Baizi Mountain) and Huoshan County, Anhui [4], while it occurs in Honshu, Shikoku, and Kyushu in Japan [11, 14]. In both countries, *B. berchemiaefolia* is designated as a rare and endangered tree species [4, 11] and is consequently considered endangered worldwide. On the other hand, some authorities classified *B. berchemiaefolia* as *Rhamnella berchemiaefolia* Makino, *Chaydaia berchemiaefolia* (Makino) Koidz, *Berchemiella berchemiaefolia* (Makino) Nakai, or *Berchemiella wilsoni* (Shneid.) Nakai [4, 11, 14]. Therefore, the species is of interest in studies of relationships between some genera of the tribe Zizipheae in the Rhamnaceae.

In Korea, *B. berchemiaefolia* usually grows mostly on the rocks in open forests at lower altitude, sometimes along the river valley [12, 22]. It strongly demands light for early establishment. Bisexual flowers are yellow to greenish yellow in color and are produced from late June to early September. They are visited by insects, but there has been no study on their

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reproductive biology to determine whether *B. berchemiaefolia* is self-compatible or not. The fruit is found in small drupes about 7–8 mm long, turning from yellow to red in color when ripe. The fleshy pulp surrounds a kernel with one hard seed [12, 23]. Seeds appear to be dispersed primarily by gravity and occasionally by floating [22].

After many years of allozyme surveys, some general patterns of genetic diversity in plants are beginning to emerge [6, 7]. However, so far, relatively few studies have examined patterns of genetic variation in rare plants compared to those for the plants with wide distribution ranges [13]. We might expect such species to maintain lower levels of variation than common plants do because of their more restricted population sizes and consequently their reduced opportunities for gene flow. They may also have experienced genetic drift because of founder effects and/or an enforced population bottleneck [13, 28].

The inter-simple sequence repeat (I-SSR) markers have recently become a popular tool in plant genetic studies [5, 9, 25, 27]. The I-SSR technique can yield a large number of loci, thereby providing a more representative sample of the genome than is possible with allozymes. However, I-SSR also has some significant limitations. One of the most critical limitations for its use in genetic studies may be its dominant allelic expression. This characteristic precludes direct estimates of allelic frequencies from diploid materials and thus biases the estimates of genetic diversity and genetic differentiation as is in the case of RAPDs (Random Amplified Polymorphic DNAs) [21, 24].

The objectives of this study were (1) to examine genetic variation in *B. berchemiaefolia* throughout its range in Korea employing isozyme and I-SSR markers; and (2) to compare the results with previous reports for other rare plant species.

2. MATERIALS AND METHODS

2.1. Plant materials

From the late June to the mid-July of 2001, foliage tissues were collected from four natural stands located throughout the native range of *B. berchemiaefolia* in Korea (Fig. 1). Within each stand, over 30 (31–36) trees were selected for foliage collection with a minimum distance of 20 m in order to decrease the risk of relatedness. However, within a couple of stands, some trees were sampled in close proximity (within 20 m) until the goal of 30 trees was reached. The leaves were placed in ice chests, and transported to the laboratory within 48 h, where they were stored at 4 °C until needed.

2.2. Enzyme extraction and allozyme procedure

Enzymes were extracted between 1 and 7 d after collection. Leaves were cut finely, and crushed with a mortar and pestle in an extraction buffer. In preliminary trials, enzyme activity showed the best results in the Cheliak and Pitel [1] extraction buffer with some modifications. Then, enzyme extract was absorbed onto 4 mm × 10 mm wicks cut from Whatmann 3MM chromatography paper, which were stored at –70 °C until needed for analysis.

Using techniques of starch-gel electrophoresis based on Conkle et al. [2], 20 enzyme systems were surveyed in a preliminary test, and ten enzyme systems showing consistent and clear banding patterns were finally chosen: aspartate aminotransferase (AAT, E.C.2.6.1.1), glutamate dehydrogenase (GDH, E.C.1.4.1.2), glucose 6-phosphate

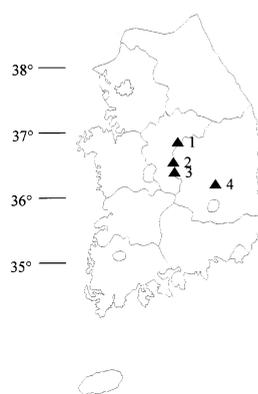


Figure 1. Locations of 4 sampled sites for *Berchemia berchemiaefolia* in Korea. 1: Wolak; 2: Sadam; 3: Seowon; 4: Juwang.

dehydrogenase (G6PD, E.C.1.1.1.49), isocitrate dehydrogenase (IDH, E.C.1.1.1.42), leucine aminopeptidase (LAP, E.C.3.4.11.1), malate dehydrogenase (MDH, E.C.1.1.1.37), phosphoglucose isomerase (PGI, E.C.5.3.1.9), phosphoglucomutase (PGM, E.C.2.7.5.1), 6 phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44) and shikimate dehydrogenase (SDH, E.C.1.1.1.25).

2.3. DNA extraction and PCR amplification

Total genomic DNA was extracted from foliages by a modified CTAB method [8]. PCRs (polymerase chain reactions) were carried out in a volume of 25 µL with final concentrations of 5 ng of template DNA; 0.2 mM each of the four dNTPs; 0.025% BSA (Boehringer Mannheim, Germany); 5 µL of 1.5 µM primer; 1.2 µL of 25 mM MgCl₂ and 1 unit of Taq DNA polymerase (Advanced Biotechnology, UK). Amplifications were performed in a PTC-200 thermocycler (MJR Resaerch, USA) using a period of 5 min of initial denaturation at 94 °C, followed by 45 cycles of 30 s of denaturation at 94 °C, 30 s annealing at 52 °C, 1 min of extension at 72 °C, and a final extension step of 10 min at 72 °C. Subsequent amplification products were electrophoresed using 2% agarose gels containing ethidium bromide fluorescence with a 1× TBE (tris-boric acid-ethylendiamine tetraacetic acid) buffer at pH 8.0 for 3.5 h and then photographed under UV light.

A total of 20 primers (UBC, Canada) were screened using three representatives from each of the four populations. Four primers that gave clear and reproducible fragment patterns over multiple (at least four) amplifications were selected for final analysis: UBC#808 (AGAGA-GAGAGAGAGAGC), UBC#826 (ACACACACACACACC), UBC#829 (TGTGTGTGTGTGTGTGC), and UBC#834 (AGAGA-GAGAGAGAGAG(CT)T).

3. RESULTS

3.1. Allozymes

We detected no allozyme variation among any of the plants or populations with the exception of one individual from the Seowon population at *Pgi-2* locus. The leaves of the 111 plants were analyzed and all isozymes except PGI were monomorphic. One *Pgi-2* variant appeared to be a heterozygote (Fig. 2). It was not possible to confirm patterns of inheritance for the enzymes studied owing to the lack of controlled-cross of full-sib progenies as well as to the lack of enzyme variability. Consequently, the number of loci and alleles were interpreted by drawing on the experience gained in our laboratory from

studies of other angiosperm tree species and on the known sub-unit structures and cellular compartmentalization of the enzyme [29]. We conservatively estimated the number of genes encoding the 10 enzymes screened to be 14.

3.2. I-SSRs

A total of 28 I-SSR amplicons, amplified with 4 I-SSR primers [UBC#808 (6 amplicons), UBC#826 (6 amplicons), UBC#829 (6 amplicons), and UBC#834 (10 amplicons)], were scored. As in the case of allozymes, none of the amplicons showed polymorphism (Fig. 3).

4. DISCUSSION

Rare endemic plant species are commonly hypothesized to have little genetic variation because of changes in allelic frequencies caused by chance events (small population size, founder effect or bottleneck effect), strong and directional selection toward genetic uniformity in a limited number of environments, inbreeding and/or other factors [13, 28].

In fact, according to Karron [13], most of the 24 rare plant species reviewed revealed low to moderate levels of genetic diversity. Likewise, Hamrick and Godt [6] reported that, of the four geographic range categories (endemic, narrow, regional, and widespread), endemic species had the lowest levels of genetic variation: endemic species (100 endemic taxa among the 480 species reviewed) had less than 50% of the genetic diversity of widespread species and 70 and 64% of the genetic diversity of narrowly and regionally distributed species. This trend has been confirmed in other studies [16, 18, 20], although there are exceptions [13, 17, 19]. On the other hand, only a few studies have reported a complete absence of genetic variability for rare and/or very locally distributed plant species. The narrow endemic Torrey pine (*Pinus torreyana*) displayed no variation among 59 loci within each of two populations, and alleles at only five loci differed between the populations [15], despite the fact that pines generally show high levels of isozyme variation. In contrast to *Pinus torreyana*, red pine (*P. resinosa*) is widely distributed throughout much of the northeastern United States and adjacent regions in Canada, but is also remarkably uniform with respect to both allozymes and RAPDs [3, 26]. This situation is attributed to Pleistocene glaciation, which appears to have reduced red pine to a small area and eliminated variation. Another rare plant species with no genetic polymorphism is *Pedicularis furbishiae*, which is restricted to the St. John River valley in northern Maine of the United States [28]. No allozyme variation appeared at 22 loci in 28 individuals.

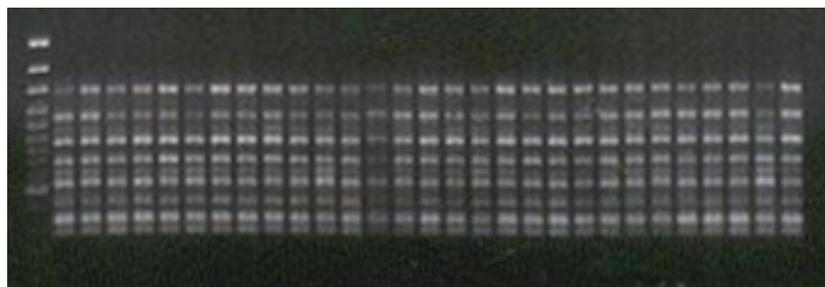


Figure 3. Example of I-SSR profiles (UBC#834 primer) of *Berchemia berchemiaefolia*. Size markers (left-hand lanes) are fragments of 100-bp ladder (MBI Fermentas).

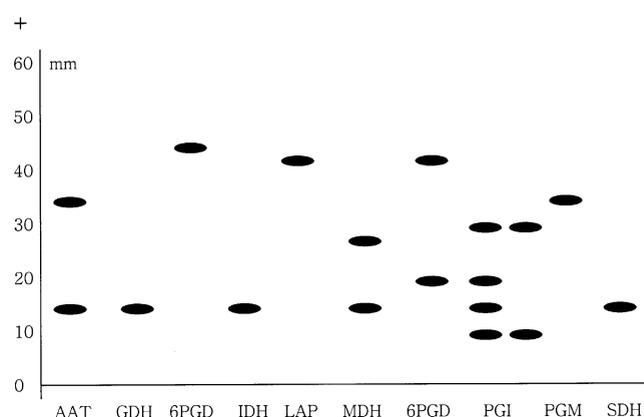


Figure 2. Phenotypes for 10 isozymes of *Berchemia berchemiaefolia*.

In Korea, *B. berchemiaefolia* has been severely disturbed by anthropogenic activities such as massive collection because it has been used as a traditional medicine, and its wood has been harvested to make furniture and handicraft or as fuel. Additionally, its distribution in farmland areas has promoted anthropogenic disturbances. These factors might have reduced *B. berchemiaefolia* to a small area and eliminated genetic variation through bottlenecks. Besides, some management activities might have negative impacts on *B. berchemiaefolia*. Foremost among these may be a high-grading cutting, in which the most valuable trees are removed and inferior trees are left to reproduce. Centuries of such dysgenic selection might reduce the gene pools of *B. berchemiaefolia* because whenever some trees are left after harvest to regenerate the stand, diversity of their offspring may be affected. In other words, if only a few trees are left to serve as seed parents, then inbreeding and its depression of viability are likely to take place. A study reported an evidence to support this hypothesis. According to Lee [22], most of *B. berchemiaefolia* trees in a natural stand produced empty seeds. Inbreeding depression reduces fitness and vigor in terms of survival, growth, and fertility by increased homozygosity of deleterious recessive allele as a result of inbreeding in a normally outbreeding population. More detailed studies on the reproductive biology and inbreeding depression in *B. berchemiaefolia* are needed, because a recent study showed that even self-fertile species can reveal dramatic levels of inbreeding depression [13].

We have no idea of whether the present range of *B. berchemiaefolia* corresponds to its past distribution. *B. berchemiaefolia* has difficulties in regeneration in a natural stand [12, 22, 23]. It requires light for its early establishment.

Consequently, seedlings can be found only in the margin of a forest and/or within a gap in a closed forest. Besides, most seedlings do not develop into mature trees. According to Kang et al. [12], of 8 655 000 seeds/ha/yr of *B. berchemiaefolia*, only 406 000 seeds developed into seedlings. Of these seedlings, 630 individuals grew into saplings and finally only 4 individuals developed into mature trees. These results may be, at least partially, connected with the inbreeding depression as discussed above. Accordingly, it is likely that *B. berchemiaefolia* has never occupied a large range owing to its ecological and reproductive traits. As a consequence, a substantial genetic bottleneck, combined with the fluctuation of local population sizes due to human activities, as well as local inbreeding could account for this species' lack of genetic variation. For a better understanding of the issue mentioned above, further studies are needed in the near future using highly variable molecular markers such as AFLPs.

In Korea, one natural population (Sadam population in the present study) and two old trees of *B. berchemiaefolia* are legally protected as natural living monuments. However, most populations including the legally protected area are not currently regenerated by seeds. Most individuals are regenerated by the sprouts from the trunk of logged trees. Accordingly sprouting appears to be a main factor in *B. berchemiaefolia*'s survival and maintenance in a natural habitat. So more active management such as partial clearing of vegetation to make gaps in a forest is needed to regenerate *B. berchemiaefolia* by seeds and to increase the population size in a more efficient way. Taking its rarity into account, we need to extend the legally protected areas, to give it legal protection against reckless collection, and/or to establish an ex situ conservation stand.

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