Molecular systematic study of Cardamine glechomifolia Levl. (Brassicaceae) using internal transcribed spacer sequence of nuclear ribosomal DNA (ITS) and chloroplast trnL and trnL-F sequences

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Abstract The internal transcribed spacer (ITS) region of nuclear ribosomal DNA, trnL and trnL-F genes of Cardamine glechomifolia Levl. (family Brassicaceae) were sequenced and analyzed with the sequence of related Cardamine species retrieved from NCBI GenBank to detect pattern of evolutionary differentiation. All trees resulting from combined sequence analyses data of ITS, trnL and trnL-F gene resolve that C. glechomifolia – an endemic species to South Korea clade with Cardamine microzyga (100% bootstrap support). The evolutionary history was inferred using the Maximum Parsimony method. The consistency index is (0.588235), the retention index is (0.687500), and the composite index is 0.519622 (0.404412) for all sites and parsimony-informative sites (in parentheses). The result of the analysis using Maximum Parsimony was found congruence with Maximum Likelihood method and in Baseyan analysis.

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1. Introduction

The genus Cardamine L. (Brassicaceae) comprises about 200 species with indigenous taxa on all continents except Antarctica (Al-Shehbaz, 1988). The genus shows great morphological and karyological diversity and complex evolutionary history strongly affected by both historical and more recent reticulation events (Lihova and Marhold, 2006), providing an opportunity to study mechanisms of plant diversification. Major centers of diversity, assessed by species richness and endemism, occur in the Far East and the Himalayas, with about 70 Cardamine taxa reported (Al-Shehbaz, 1988). Several species have been reported to have spread as weeds beyond their natural...
### Table 1  Voucher information, GenBank Accession Number and Sequence characteristics of *Cardamine glechomifolia*.

| Geographical location | South Korea |
|-----------------------|-------------|
| **Voucher number**    | Changyoung (KRIB0001142) |
| **ITS**               | Total length | 622 |
|                       | GC contents  | 51  |
|                       | GenBank Accession Number | HM449939 |
| **trnL**              | Total length | 402 |
|                       | GC contents  | 34  |
|                       | GenBank Accession Number | HM449940 |
| **trnL-F**            | Total length | 160 |
|                       | GC contents  | 24  |
|                       | GenBank Accession Number | HM449941 |

### Table 2  Taxon and GenBank Accession Number used for the molecular systematic study of *Cardamine glechomifolia*.

| Species                      | ITS Accession Number | trnL Accession Number | trnL-F Accession Number |
|------------------------------|----------------------|-----------------------|-------------------------|
| **Ingroup**                  |                      |                       |                         |
| *Cardamine trifolia*         | DQ209114             | FJ464526              | FJ464548                |
| *Cardamine enneaphylllos*    | EF136405             | FJ464515              | FJ464537                |
| *Cardamine microphylla*      | EU819347             | EU819173              | EU819432                |
| *Cardamine resedifolia*      | EU819364             | FJ464510              | FJ464530                |
| *Cardamine alpine*           | AM905716             | FJ464509              | FJ464529                |
| *Cardamine graeca*           | FJ384197             | EU819164              | FJ384356                |
| *Cardamine maritima*         | FJ384224             | FJ384295              | FJ384355                |
| *Cardamine rupestris*        | FJ384200             | FJ384283              | FJ384343                |
| *Cardamine falae*            | FJ384230             | FJ384279              | FJ384341                |
| *Cardamine serbica*          | FJ384212             | FJ384274              | FJ384337                |
| *Cardamine carnosa*          | FJ384181             | FJ384269              | FJ384333                |
| *Cardamine pancicii*         | FJ384179             | FJ384263              | FJ384328                |
| *Cardamine glauca*           | FJ384178             | FJ384262              | FJ384327                |
| *Cardamine monteluccii*      | FJ384208             | FJ384175              | FJ384319                |
| *Cardamine blaisdelli*       | EU819313             | EU819152              | EU819303                |
| *Cardamine victoria*         | EU819383             | EU819195              | EU819300                |
| *Cardamine umbellata*        | EU819380             | EU819191              | EU819297                |
| *Cardamine tangutorum*       | EU819376             | EU819234              | EU819282                |
| *Cardamine tanakae*          | EU819375             | EU819233              | EU819281                |
| *Cardamine scutata*          | EU819372             | EU819227              | EU819279                |
| *Cardamine rapicola*         | EU819368             | EU819232              | EU819278                |
| *Cardamine pedata*           | EU819356             | EU819176              | EU819277                |
| *Cardamine ovata*            | EU819353             | EU819225              | EU819270                |
| *Cardamine nutallii*         | EU819350             | FJ464523              | EU819267                |
| *Cardamine microzyga*        | EU819348             | EU819221              | EU819266                |
| *Cardamine douglassii*       | EU819332             | EU819209              | EU819247                |
| *Cardamine constancei*       | EU819322             | EU819205              | EU819244                |
| *Cardamine bonariensis*      | EU819314             | EU819200              | EU819241                |
| *Cardamine impatiens*        | AM905720             | DQ268171              | DQ268339                |
| *Cardamine pectinata*        | DQ268502             | DQ268175              | DQ268338                |
| *Cardamine niagatensis*      | DQ266493             | DQ268165              | DQ268332                |
| *Cardamine longifructus*     | DQ268498             | DQ268155              | DQ268322                |
| *Cardamine pensylvanica*     | DQ268649             | DQ268136              | DQ268304                |
| *Cardamine paucijuga*        | DQ268655             | AY047640              | DQ268294                |
| *Cardamine fallax*           | DQ268464             | DQ268123              | DQ268288                |
| *Cardamine parviflora*       | DQ209133             | DQ268070              | DQ268237                |
| *Cardamine debilis*          | DQ268392             | DQ268059              | DQ268226                |
| *Cardamine raphanifolia*     | AY260612             | AF079335              | EF067933                |
| **Outgroup**                 |                      |                       |                         |
| *Rorippa divaricata*         | AF100693             | AF361900              | AY030247                |
| *Barbarea vulgaris*          | AJ232915             | DQ479855              | DQ518352                |
ranges following introduction to distant areas and even different continents. Apart from the European species, most of which have been thoroughly investigated taxonomically (Libova and Marhold, 2006), the taxonomy of species from other continents, e.g., South America (Sjostedt, 1975), Australia and New Zealand (Hewson, 1982; Webb et al., 1988) or Eastern Asia (Ohwi, 1984; Zhou et al., 2001), is very complex and remains in many cases controversial and unresolved. In South Korea, the genus *Cardamine* is represented by 16 species i.e. *Cardamine parviflora*, *Cardamine impatiens*, *Cardamine fallax*, *Cardamine amaraeformis*, *Cardamine flexuosa*, *Cardamine scutata*, *Cardamine komarovi*, *Cardamine bellidifolia*, *Cardamine changbaiana*, *Cardamine pratensis*, *Cardamine glechomifolia*, *Cardamine leucantha*, *Cardamine koreana*, *Cardamine prorepens*, *Cardamine yeozensis* and *Cardamine lyrata* (Park, 2007). *C. glechomifolia* (Korean name Beol-kkae-naeng-i) is endemic to South Korea and it was described by Lévl in 1913. (Park, 2007). This species has been morphologically character-

Figure 1 50% Majority rule tree inferred from combined sequence data analysis of internal transcribed spacer region of nuclear ribosomal DNA, *trnL* and *trnL-F* from *Cardamine*. The tree constructed in MEGA4 after multiple alignment in ClustalX.
ized as: plants with rhizomes and tubers; leaflets of cauline leaves only three, lyrate, petiolulate. The perusal of literature reveals that the taxonomic relationship of *C. glechomifolia* within the genus has not been established so far (pers. obs.). Hence this study was taken to compare the sequences of the internal transcribed spacer (ITS) sequence of nuclear ribosomal DNA and chloroplast *trnL*-F sequence among the genus to detect pattern of evolutionary differentiation.

2. Materials and methods

The fresh leaf material of *C. glechomifolia* was collected from nature during plant exploration in South Korea. Total DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Amsterdam, Netherlands). Internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA and *trnL* and *trnL*-F were amplified using primers ITS1, ITS4 (White et al., 1990) and *trnL* and *trnL*-F, respectively, via the polymerase chain reaction (PCR) using AccuPower HF PCR PreMix (Bioneer, Daejeon, South Korea) in 20 μL volumes containing 2 μL of 10X buffer, 300 μM dNTPs, 1 μL of a 10 pM solution of each primer, 1 unit of HF DNA polymerase. One round of amplification consisting of denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min and extension at 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The PCR products were ligated into the pT7Blue cloning vector using Perfectly Blunt Cloning Kit (Novagen, Inc.) according to the manufacturer’s instructions. Resulting recombinant plasmids were used to transform competent cells included in the kit. The transformation mix was incubated in 250 μL SOC medium for 1 hour at 37 °C on a rotary shaker, then plated on LB agar with

**Figure 2** A Neighbor-Joining tree inferred from combined sequence data analysis of internal transcribed spacer region of nuclear ribosomal DNA, *trnL* and *trnL*-F from *Cardamine*. The tree constructed in SeaView after multiple alignment in MUSCLE. The scale bar indicates relative length of the branch.
50 μg/mL ampicillin. Colonies were randomly selected and were put into PCR buffer. The PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. The purified fragments were directly sequenced using dye terminator chemistry following the manufacturer’s protocol. Cycle sequencing was conducted using same primers used in amplification and BigDye version 3 reagents and an ABI PRISM 3730XL DNA Analyzer (Perkin-Elmer, Applied Biosystems) by following the manufacturer’s instructions. Cycling conditions included an initial denaturing set at 94 °C for 5 min., followed by 30 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Each sample was sequenced in the sense and antisense direction. The sequences were analyzed with ABI Sequence Analysis and ABI Sequence Navigator software (Perkin-Elmer, Applied Biosystems). Nucleotide sequences of both DNA strands were obtained and compared to ensure accuracy.

Phylogenetic analysis of ITS and trnL and trnL-F sequences of related 38 species of Cardamine and (outgroups Barbarea vulgaris, Rorippa divaricata) were retrieved from GenBank database (Table 1). Initially the sequence alignments were performed using ClustalX version 1.81 (Thompson et al., 1997) with gap opening penalty = 10 and gap extension penalty = 3.0. Sequence alignments were subsequently adjusted manually using BioEdit (Hall, 1999) and SeaView (Gouy et al., 2010). Insertion-deletions (Indels) were scored as single characters when we had confidence in positional homology (Appendix A). The boundaries between the ITS1, 5.8S, and ITS2 and trnL and trnL-F were determined by comparisons with earlier published angiosperm sequences (Baldwin, 1992; Baldwin and Markos, 1998). Gaps were treated as missing data in phylogenetic analyses. All sequences generated in the present study were deposited in GenBank and GenBank accession number included in Table 1. Parsimony analyses were

Figure 3  Bayesian phylogeny (consensus tree) based on combined sequence data analysis of internal transcribed spacer region of nuclear ribosomal DNA, trnL and trnL-F from Cardamine.
performed Using MEGA4. Molecular evolutionary analyses were conducted using MEGA version 4 (Nei and Gojobori, 1986; Kumar and Gadagkar, 2001; Tamura et al., 2004, 2007) and the result were verified with Maximum Likelihood method (using SeaView) and Baseyan analysis (MrBayes). For Bayesian analysis, the best-fit model of nucleotide evolution was found using jModelTest v1.0.1 (Posada, 2008). Bayesian posterior probabilities for the clades were obtained using Metropolis-coupled Markov chain Monte Carlo analysis as implemented in MrBayes. Two simultaneous independent runs with four Markov chains were done for 5 million generations, and trees were sampled every 100th generation, resulting in 50,000 trees. The first 10,000 trees were considered as the burn-in phase and discarded. A majority-rule consensus tree based on the remaining 40,000 trees was computed (see Table 2).

Figure 4  Bootstrap strict consensus tree based on combined sequence data analysis of internal transcribed spacer region of nuclear ribosomal DNA, trnL and trnL-F from Cardamine. The tree constructed using Maximum Parsimony method in SeaView after multiple alignment in MUSCLE. Bootstrap values greater than 50% in 1000 replicates are shown.
3. Results and discussion

The sequence characteristics (total length and GC content) of *C. glechomifolia* along with voucher information, GenBank Accession Number are presented in Table 1. All trees (Figs. 1–6) resulting from combined sequence data analyses (Under MEGA, SeaView and MrBayes) of ITS, *trnL* and *trnL-F* gene resolve that *C. glechomifolia* clade with *Cardamine microzygya* (100 bootstrap) The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). The consensus tree under MEGA4 inferred from 13 most parsimonious trees is shown in Fig. 1. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is (0.588235), the retention index is (0.687500), and the composite index is 0.519622 (0.404412) for all sites and parsimony-informative sites (in parentheses). The percentage of parsimonious trees in which the associated taxa clustered together was shown above the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 (Nei and Kumar, 2000; Tamura et al., 2007) in which the initial trees were obtained with the random addition of sequences (100 replicates). The codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 997 positions in the final dataset, out of which 104 were parsimony informative. The number of base substitutions per site from averaging over all sequence pairs is 0.030. All results are based on the pairwise analysis of 41 sequences. Analyses were conducted using the Maximum Composite Likelihood method in MEGA4 (Tamura et al., 2004, 2007). The evolutionary history was inferred in MEGA4 (Tamura et al., 2007) using the Neighbor-Joining method in SeaView after multiple alignment in MUSCLE. Bootstrap values greater than 50% in 1000 replicates are shown above lines.
The optimal tree with the sum of branch length = 0.42296005 is shown in Fig. 2. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site.

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Appendix A

Molecular systematic study of *Cardamine glechomifolia* Levl. (Brassicaceae) using internal transcribed...
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