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Characterization of *LEAFY* in *Rubus Coreanus* and Its Phylogenetic Application in *Rubus* Species

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Abstract. *Rubus coreanus* is endemically distributed in Southwest China and has great potential for *Rubus* breeding due to its nutritional values and insects and pests' resistance. *LEAFY*, as a single copy nuclear gene in diploid plants, plays an important role in the taxonomy and phylogeny, containing three exons and two introns. Here we designed new primers to amplify *LEAFY* gene fragment based on gene sequences from apple, strawberry, pear and peach. The new designed primers were demonstrated to be applicable for several other *Rubus* species in *Rubus* genus. The full length of *LEAFY* fragment in *R. coreanus* was 1127 bp, including 319 bp at the 3' end of the second exon, 729 bp of the second intron, and 39 bp from the 5' end of the third exon. Its GC content was 30% while AT content counted for 70%. We further reconstructed the phylogenetic tree using several *LEAFY* gene from Rosaceae species. These results could provide effective tool to study the phylogenetic relationships within the *Rubus* genus.

Introduction

The genus *Rubus* is one of the largest genera in the Rosaceae family, consisting of 750-1000 species in many parts of the world [1, 2]. Those *Rubus* plants used for horticultural plantations are generally named bramble or edible *Rubus*. Due to its nutritional values and medical uses, *Rubus* fruits are named Fruits of Life by Americans brambles were recommeded as the third generation fruits by FAO [3]. Until today, brambles have been widely planted in many parts of the world and have increasingly become the most rapidly developing small fruit tree just next to strawberry, blueberries and currants [4].

Rubus coreanus is endemically distributed in Southwest China and holds great potential for *Rubus* breeding [5, 6]. There existed divergences between taxonomy and phylogeny of *Rubus* [7], which affected the utilization of these excellent wild resources in breeding. DNA sequence is an ideal method for phylogeny and classification of various taxa in plants [8]. Compared with other sequences, low-copy nuclear genes (LCNG) can provide more informative sites, especially for lower taxonomic levels [9]. *LEAFY* plays an important role in the formation of floral meristem [10, 11], containing three exons and two introns. Previous study suggested that the second intron of the *LEAFY* gene is useful for phylogenetic reconstructions of closely related species [10, 12].

At present, the research on *LEAFY* in *Rubus*, including the function to regulate flowering and the application in phylogeny, are very limited. Yang et al. [13] used the *LEAFY* sequence to study the phylogenetic relationship among Korean *Rubus*. However, when we further verified the sequence, we found that all the reported 'LEAFY' did not match any sequences by the functional detection of *LEAFY* nucleic acid sequence from the GenBank database. Thus, we speculated that they did not obtain *Rubus LEAFY* gene. In this study, we designed new primers to amplify the *LEAFY* gene, and focused on the second intron to reconstructed phylogenetic tree. This study will provide the effective basis for the taxonomy and phylogeny of *R. coreanus*.

Materials and Methods

Materials

The genomic DNA of *R. coreanus*, *E. coli* JM109 competent cells were stored in laboratory and the cloning vector pMD19-T was purchased from Takara Company.

Methods

Based on the exon sequences of *LFY* second intron in apple, strawberry, peach and plum, primers LFY2F: 5'-GGCTGTCGGAGGAGCCRGTG-3', and LFY2R: 5'-CAATGTCCCARCCTTGGCSTGC-3' were designed for amplification. Polymerase chain reaction (PCR) amplification was performed in a total volume of 20 μL, containing 2 μL of template DNA (10-50 ng), 10 μL of Taq Mix, 1 μL of each primer (5 μM). Conditions for amplification consisted of an initial denaturation at 94 ^{o}C for 3 min, followed by 35 cycles at 94 ^{o}C for 45s, then at 66 ^{o}C to 58 ^{o}C with 0.5 ^{o}C decreasing per cycle for 1 min and 72 ^{o}C for 1 min, with a final extension at 10 min.

PCR products were verified by 1% agarose gel electrophoresis and photographed by EB staining, and purified by TaKaRa MiniBEST Agarose Gel DNA Extraction Kit. The amplicons were further ligated to pMD19-T vector and transformed into JM109 competent cells. A single clone was picked to expand cultivation, and identified by PCR amplification. The positive clones were sequenced by Sangon Biotech company.

The boundaries between exons and introns were determined by alignment *Rubus LEAFY* sequence and preservations of the 'GT' and 'AG' at two ends of introns. Sequences were aligned with Muscle, and manually adjusted in the Molecular Evolutionary Genetics Analysis sofaware (MEGA 7.0) with gaps treated as missing data. Some *LEAFY* sequences of other Rosaceae species were obtained from GenBank. The minimum evolution tree was reconstructed by MEGA 7.0.

Results

PCR Amplification

The newly designed primer successfully amplified a fragment of about 1200 bp (Fig. 1) without any non-specific products. PCR products were purified, cloned and ligated by vector pMD19-T. Then we obtained the true *LEAFY* gene sequences after sequencing. The fragment length of the gene was 1127 bp in length, including a fragment of 319 bp at the 3' end of the second exon, 729 bp of the second intron and a 39 bp fragment from the 5' end of the third exon. The sequence was characterized by high AT content (70%), but has high level of similarity to those of reported *LEAFY* sequence in other species (Fig. 2).

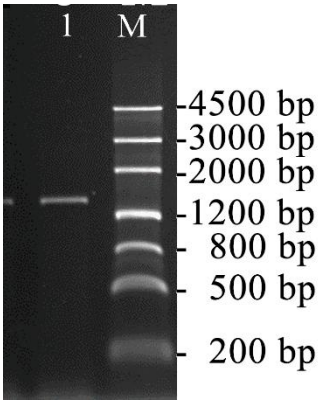

Figure 1. PCR amplification of *LEAFY* in *Rubus coreanus*

Figure 2. Blast hit results using *LEAFY* sequences in the GenBank database

Phylogeny Analysis Using *LEAFY*

We downloaded five *LEAFY* sequences of *Malus*, *Pryus*, and *Prunus* species from NCBI. Addtionally, three deposited *Rubus LEAFY* genes (*R. croceacanthus, R. corchorifolius, R. parvifolius*) from the Korean group were also included. The phylogenetic tree was reconstructed by MEGA 7.0 (Fig. 3). The cloned *LEAFY* gene of *R. coreanus* could form a normal evolution tree with other sequence of Rosaceae family. The topology for the other three *Rubus* species is distantly relate to that of *Rubus coreanus,* which clearly indicated that our *LEAFY* sequence is a completely new sequence.

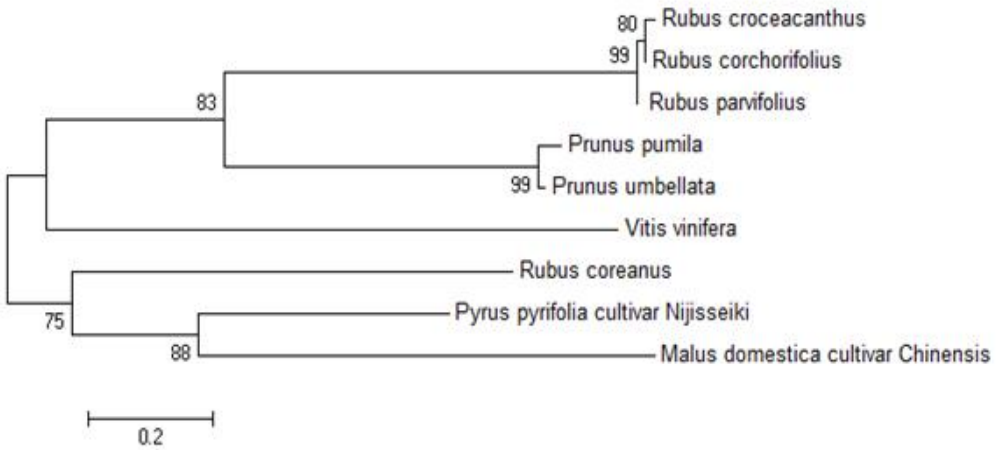

Figure 3. The minimal evolution tree reconstructed by the second intron of *LEAFY* from several Rosaceae species

Application of This *LEAFY* Primers in other *Rubus* Species

To verify the applicability of the designed primers on the *Rubus* genus, we used seven additional *Rubus* species for further amplification. As shown in Fig. 4, we obtained the same length *LEAFY* gene among different *Rubus* species. This indicated that the designed primers were feasible in the genus *Rubus*.

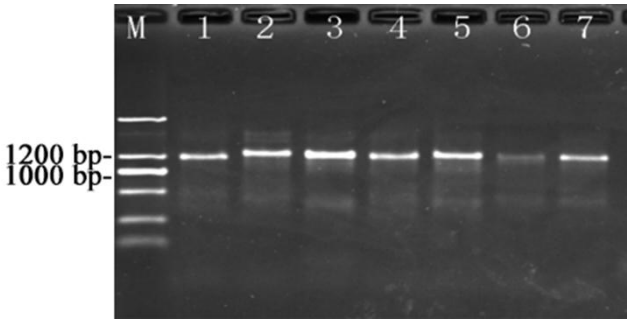

Figure 4. Amplified electrophoresis of *LEAFY* among different *Rubus* species
1: *Rubus lambertianus*, 2: *R. lambertianus* var. *glandulosus*, 3: *R. stimulans*, 4: *R. pacificus*, 5: *R. tephrodes*, 6: *R. alceaefolius*, 7: *R. amphidasys*.

Discussion

In this study, we designed new primers for low-copy nuclear *LEAFY* gene amplification in *R. coreanus*. We further applied the new designed primers in more *Rubus* species, indicating that they were feasible within the genus. By cloning and sequence analysis, we obtained the *LEAFY* gene fragment with full-length of 1127 bp, including 319 bp at the 3' end of the second exon, 729 bp of the second intron, and 39 bp from the 5' end of the third exon. Its GC content was 30%, while the AT content counted for 70%. Further reconstruction of the phylogenetic tree using several *LEAFY* gene from Rosaceae species clearly indicate the fake '*LEAFY*' genes deposited in NR database. These results could provide effective tool to study the phylogenetic relationships within the *Rubus* genus.

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