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Chloroplast DNA Spacers로 분석한 국내 *Rubus* 재배종의 계통학적 유연관계

유기석* · 박명렬* · 백소현** · 윤성중***†

*전북대학교 농업생명과학대학 농업과학기술연구소, **농촌진흥청 국립식량 과학원,
***전북대학교 농업생명과학대학 작물생명과학과

Phylogenic Relationship of *Rubus* Cultivated in Korea Revealed by Chloroplast DNA Spacers

Gee Suck Eu*, Myoung Ryoul Park*, So Hyeon Baek and Song Joong Yun***†**

**Institute of Agricultural Science and Technology, Chonbuk National University, Jeonju 561-756, Korea.*
***National Institute of Crop Science, RDA, Suwon 441-857, Korea*
****Department of Crop Science, Institute of Agricultural Science and Technology, Chonbuk National University, Jeonju 561-756, Korea.*

ABSTRACT : There is a considerable difference in morphological traits between Bokbunja cultivated in Korea (KCB) and Korea native *Rubus coreanus*, contrary to the conviction that the cultivated Bokbunja is the domestication of *R. coreanus*. To infer the phylogenetic relationship of KCB with other *Rubus* species, we compared the chloroplast DNA spacers of KCB with those of several *Rubus* species including black raspberry, *R. occidentalis*. The three chloroplast DNA spacers, *atpB~rbcL*, *trnL~trnF*, and *trnT~trnL*, were amplified using the specific primer pairs and converted to Single Strand Conformational Polymorphism (SSCP) markers. The SSCP makers of the chloroplast DNA spacers showed a considerable variation both within and among *Rubus* species. In the phylogenetic tree generated by the SSCP markers, KCB accessions were located in the same clade with *R. occidentalis*, but *R. coreanus* accessions in the different clade. Also, in the phylogenetic tree by the nucleotide sequences of the chloroplast DNA spacer *trnL~trnF*, KCB located in the same clade with *R. occidentalis* but not with *R. coreanus*. These results suggest that the three KCB accessions share higher similarity with *R. occidentalis* than with *R. coreanus* in the three chloroplast DNA spacers.

Key Words **:** *Rubus* Species, Phylogenetic Relationship, Single Stranded Conformational Polymorphism (SSCP)

INTRODUCTION

Rubus belongs to the family Rosaceae, and there are over 250 species of *Rubus* in the world. Among them, most frequently found species are black (*R. occidentalis*) and red (*R. idaeus* L.) raspberries (Jennings, 1988). Blackberries belong to the subgenus Eubatus, which is the different subgenus from Idaeobatus where red and black raspberries belongs to subgenus Idaeobatus (Jennings, 1988).

Fresh or dried fruit of Korea native *Rubus coreanus* Miq. has been known as Bokbunja and used as a traditional herbal medicine for mental, kidney, liver, face, muscle and sexual disorders (Chang, 2003). Increasing attention has been given to Bokbunja with recent scientific findings supporting its beneficial effects on human health (Jeong *et al*., 2009; Jeong and Sin, 1996; Park *et al*., 2003). Timely meeting of the new findings with interests of food industry in natural functional substances triggered increased demand for 'Bokbunja' leading transition from gathering to cultivation of Bokbunja.

Cultivation of Bokbunja was initiated in Gochang-Gun, Jellabuk-do in 1980's and has been expanded to the whole country. Though the history of Bokbunja cultivation is not longer than 50 years, little scientific documentation is available on its process of cultivation. Most farmers cultivating Bokbunja recognize it as a domesticated *R. coreanus*. However, leaf shape and flower color of cultivated Bokbunja are different from those of *R. coreanus* and rather similar to those of black raspberry, *R. occidentalis* L. Thus, it is

†Corresponding author: (Phone) +82-63-270-2508 (E-mail) sjyun@chonbuk.ac.kr
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important to get scientific evidences explaining whether the cultivated Bokbunka is a domestication of wild *R. coreanus* or unintentional introduction of cultivated *Rubus* species such as *R. occidentalis*.

In the previous study we compared the nuclear genomic background of cultivated Bokbunka with that of several *Rubus* species including *R. coreanus* and *R. occidentalis* using Random Amplified Polymorphic DNA (RAPD) and the Internal Transcribed Spacer (ITS) markers. The genetic background of cultivated Bokbunja inferred from the RAPD and ITS markers was more similar to that of *R. occidentalis* than to that of *R. coreanus* (Eu *et al*., 2008; Eu *et al*., 2009). These findings are in line with the speculation based on the morphological similarities that the cultivated Bokbunja shares more close similarity to *R. occidentalis* than to *R. coreanus*.

Uncertainty about the identity of cultivated Bokbunja is also reflected in the use of its scientific name in the papers and literature. Among over 80 publications on Bokbunja in the recent two decades, considerable inconsistency is found in the use of scientific name for Bokbunja. In 53 cases, *R. coreanus* or *R. occidentalis* (Kee and Lim, 2007; Jung *et al*., 2009) was used without a clear indication whether the materials used were wild or cultivated Bokbunja. This inconsistency in the use of scientific name of cultivated Bokbunja mostly stems from the ambiguity regarding the original plants used for the cultivation of Bokbunja.

One simple way to investigate the identity of cultivated Bokbunja is to compare its genetic background with that of other *Rubus* species. Chloroplast genome is often used for phylogenetic analysis due to its structural and sequence stability (Markowicz and Loiseaux-de Goer, 1991; Waugh *et al*., 1990). Conversely, variations in nucleotide sequences of chloroplast genome have been used as valuable markers for phylogenetic analysis. Frequently targeted genes for sequence analysis include *rbcL* (Chase *et al*., 1993), *ndhF* (Kim and Jansen, 1995), *atpB* (Wolf, 1997) and *matK* (Steele and Vilgalys, 1994). Additionally, noncoding regions of the chloroplast genome have been explored as the regions providing greater levels of variation for phylogenetic analyses (Gielly and Taberlet, 1994). The regions frequently explored include the *trnT~trnL* and *trnL~trnF* region (Taberlet *et al*., 1991), the *atpB~rbcL* intergenic spacer (Golenberg *et al*., 1993), and the noncoding intron portions of the *trnK/matK* region (Johnson and Soltis, 1994; Steele and Vilgalys, 1994).

In this study we compared the three chloroplast DNA spacers among the cultivated Bokbunja, *R. coreanus*, black and red raspberries and blackberries to obtain phylogenetic information about the origin of the cultivated Bokbunja.

MATERIALS AND METHODS

1. Plant materials

A total of thirteen accessions of *Rubus* species were used in this study. Three accessions of cultivated Bokbunja were from the major cultivation areas in South Korea. Black raspberry (Roc, *R. occidentalis*, two cultivars) was obtained from National Clonal Germplasm Repository, Corvallis, Oregon, USA. *R. coreanus* (Rco, three species), red raspberry (Rid, *R. idaeus*, two species) and blackberry (Rla, *R. lanciniatus*, three species) were obtained from the Korean Black Raspberry Experiment Station, Gochang, Korea (Table 1).

Table 1. *Rubus* accessions used for the analysis of the three chloroplast DNA spacer regions, *atp*B-*rbc*L, *trn*L-*trn*F and *trn*T-*trn*L.

Entry Number	Common Name	Taxon	Collection location	Note (Variety)
KCB 1	Korea Cultivated Bokbunja (KCB)	Unknown	South Korea	Gochang, Jeonbuk
KCB 8				Sanchung, Gyeongnam
KCB 11				Jeongeup, Jeonbuk
Rco 25	Korean native Bokbunja (Rco)	*R. coreanus* Miq.		Danyang, Chungnam
Rco 26				Okcheon, Chungbuk
Rco 23				Gochang, Jeonbuk
Rid 52	Red raspberry (Rid)	*R. idaeus* L.	USA	Golden Harvest
Rid 54				Canby
Rla 56	Blackberry (Rla)	*R. lanciniatus*		Thorny
Rla 57				Creeping
Rla 58				Ebano
Roc 39	Black raspberry (Roc)	*R. occidentalis* L.		Shuttleworth
Roc 50				NC 98-8-1

2. Chemicals

DNA extraction kits were from Gentra Systems (Minneapolis, MN, USA). The DNA plasmid vector pGEM-T-Easy was purchased from Promega (Madison, WI, USA). All chemicals and enzymes were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated.

3. Extraction of chloroplast DNA

DNA was isolated from fresh young leaves using the Puregene DNA purification kit following the instruction provided by the manufacturer. Fresh leaf samples (30 ㎎) were ground with a mortar and pestle in liquid nitrogen. Cells of the ground leaf tissue were lysed by incubating in lysis solution at 65℃ for 60 min. Cell lysate was collected by centrifugation and treated with proteinase K (6 ㎎/㎖) for 60 min at 55℃. RNA was degraded by adding RNase A (1.5 ㎎/㎖) in the cell lysate and incubating the lysate at 37 ℃ for 15 min. Proteins were precipitated by adding the protein precipitation solution to the cell lysate followed by inverting racks containing the samples for about 2 min and centrifuging at 13,000-16,000 × g for 3 min. DNA in the supernatant was precipitated using isopropanol. Precipitated DNA was collected by centrifugation and DNA pellet was washed with 70% ethanol. DNA pellet was dried and hydrated in 50 ㎕ DNA hydration solution by incubating the DNA sample overnight at room temperature. DNA concentration was measured by both spectrophotometric assay and gel electrophoresis. The resulting DNA sample contained chloroplast and nuclear DNAs and were successfully used for PCR for chloroplast genome.

4. Analysis of the spacer regions of chloroplast DNA

The three chloroplast DNA spacers (*atpB*~*rbcL*; Hodges and Arnold, 1994; *trnL*~*trnF* and *trnT*~*trnL*; Taberler *et al*., 1991) were amplified using the specific primer pairs. The orientation and approximate positions of the primers are indicated in Fig. 1A, and the primer name and sequences are as in Table 2. Chloroplast DNA amplification and Single Strand Conformational Polymorphism (SSCP) analysis of the PCR product were performed as described previously (Kong *et al*., 2003).

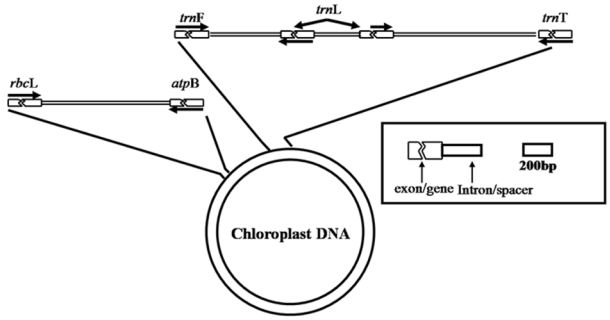
Polymerase Chain Reaction (PCR) was performed in a total volume of 25 ㎕. Each reaction was consisted of 0.01-0.1 ng of template DNA, 10 pmol each primer for either *atpB* (F) and *rbcL* (R), *trnL* (F) and *trnF* (R), or *trnT* (F) and *trnL* (R) pair, dNTPs (0.2 mM each), and *Taq* polymerase (1 unit, Ex Taq PCR, TaKaRa). PCR reactions were conducted essentially the same as in ITS amplification reactions (Eu *et al*., 2009). For SSCP analysis, the PCR reaction products were separated on non-denaturing polyacrylamide gels. 1 ㎕ of individual PCR products was mixed with 9 ㎕ of the denaturing buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue). After a brief spin, mixtures were heated at 96℃ for 10 min then chilled on ice. Five micro liter of each mixture was loaded on 8% acrylamide : Bis (29 : 1) nondenaturing gel cast using a Hoefer SE 600 Series (Amersham Pharmacia Biotech, USA).

Denatured PCR products were separated in prechilled 1X TBE buffer (Tris borate 89 mM, 2 mM EDTA, pH 8.0) at 200 V for 10 h (*atpB*(F)~*rbcL*(R) and *trnT*(F)~*trnL*(R) regions) and 6 h (*trnL*(F)~*trnF*(R) region) at room temperature. After electrophoresis, polyacrylamide gels were peeled from glass plates and soaked in 150 ㎖ (per gel) of Sol A (mixture of 135 ㎖ ddH_2O, 15 ㎖ ethyl alcohol and 810 ㎕ glacial acetic acid) for 5 min, and gels were stained in the same amount of Sol B (mixture of 135 ㎖ ddH_2O, 15 ㎖ ethyl alcohol, 810 ㎕ glacial acetic acid and 0.3 g silver nitrate) for 10 min. Then rinsed three times in 200 ㎖ ddH_2O, gels were developed by briefly rinsing in 150 ㎖ Sol C (mixture of 135 ㎖ ddH_2O, 410 ㎕ formaldehyde and 4 g NaOH) until desired band intensity was reached. Gel images were captured using a camera (Nikon COOLPIX 995, Japan).

5. Sequence and data analysis

SSCP markers were analyzed by UPGMA (Unweighted Pair-Group Method with Arithmetic Average) method using NTSYS (Numerical Taxonomy and Multi-Variate Analysis System) program (Sneath and Sokal, 1973). The nucleotide sequences were blasted against the sequences in GenBank and annotated based on the sequence similarity. Sequence homology searches were performed using the program BLAST (Altschul *et al*., 1997) against DNA and protein sequences in GenBank. Nucleotide and deduced amino acid sequence analyses were performed using DNASIS (Hitachi, USA), and the programs and databases offered by the National Center for Biotechnology Information (NIH, USA) and European Bioinformatics Institute (EBI, UK). Multiple sequence analysis was performed using the program AliBee (Brodsky *et al*., 1995). The GC content of sequence was analyzed using the SeqApp program (version 1.9a169, Gilbert, 1994).

A



B

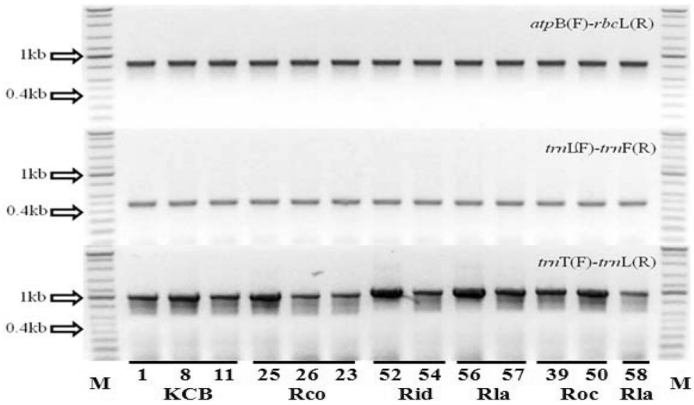


Fig. 1. A. The three chloroplast DNA noncoding regions sampled. B. DNA fragments amplified with the primers specifically designed for the three targeted regions, *atp*B(F)-*rbc*L(R), *trn*L(F)-*trn*F(R) and *trn*T(F)-*trn*L(R). F and R in parenthesis indicate forward and reverse primers, respectively. M; marker, KCB; Korea cultivated Bokbunja, Rco; Bokbunja native to Korea (*R. coreanus*), Rid; red raspberry (*R. idaeus*), Rla; blackberry (*R. lanciniatus*), Roc; black raspberry (*R. occidentalis*).

Table 2. The primer sequences targeted at the three chloroplast DNA spacer regions.

Region	Primer sequence (5' → 3')	Reference	GenBank accession number
atpB-rbcL spacer	*atpB* : GTGGAAACCCCGGGACGAGAAGTAGT *rbcL* : ACTTGCTTTAGTTTCTGTTTGTGGTGA	Hodges and Arnold, 1994	AF031445 AF031450
trnL-trnF spacer	*trnL E* : GGTTCAAGTCCCTCTATCCC *trnL F* : ATTTGAACTGGTGACACGAG	Taberlet et al., 1991	AF031439 AF031444
trnT-trnL spacer	*trnT A* : CATTACAAATGCGATGCTCT *trnT B* : TCTACCGATTTCGCCATATC		AF031433 AF031438

RESULTS AND DISCUSSION

A total of thirteen accessions were selected from each *Rubus* species which formed a separate subclade by the RAPD markers (Eu *et al*., 2008) for the analysis of the chloroplast DNA spacers, *atpB~rbcL*, *trnL~trnF*, and *trnT~trnL* (Table 2 and Fig. 1A). The size of the amplified fragments for *atpB~rbcL*, *trnL~trnF*, and *trnT~trnL* spacers were about 0.9 kb, 0.5 kb and 1.0 kb, respectively. There was little variation in the size of the amplified fragments among all *Rubus* accessions tested for all the three spacers (Fig. 1B). Therefore, SSCP analysis was conducted. All the spacers showed two to four SSCP markers among the *Rubus* accessions (Fig. 2).

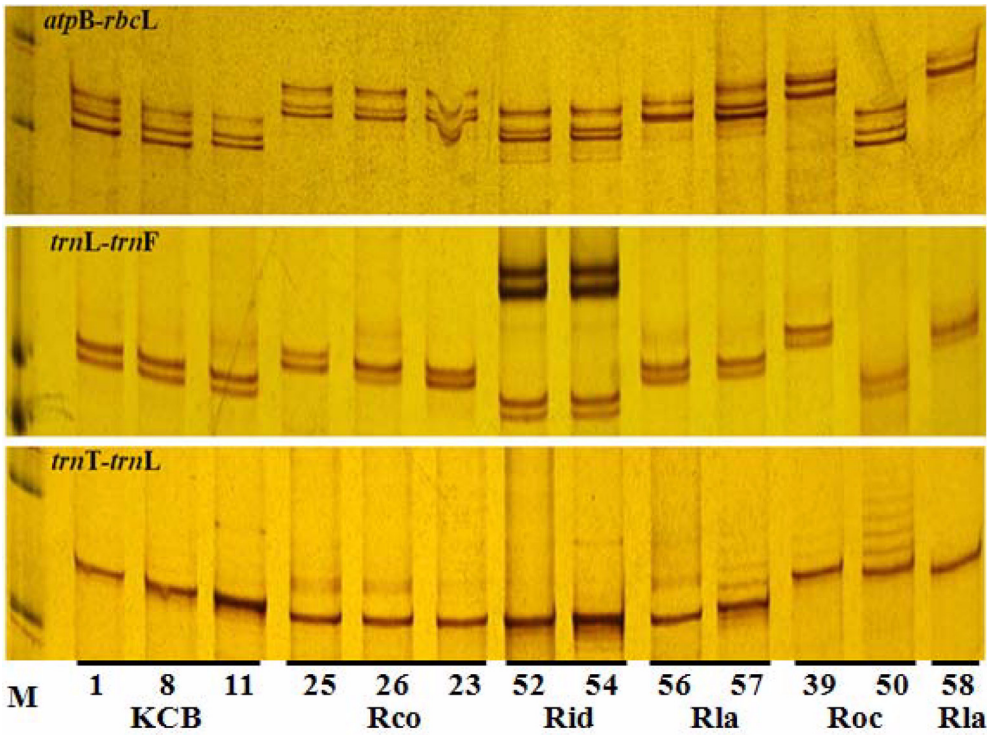


Fig. 2. Single Strand Conformational Polymorphism (SSCP) analyses for the amplified DNA fragments of the three chloroplastid regions. M; marker, KCB; Korea cultivated Bokbunja, Rco; Bokbunja native to Korea (*R. coreanus*), Rid; red raspberry (*R. idaeus*), Rla; blackberry (*R. lanciniatus*), Roc; black raspberry (*R. occidentalis*).

In the *atpB~rbcL* spacer, KCB, *R. occidentalis*, and black raspberry had three, two, and two or three SSCP bands, respectively. One of the black raspberry accessions Roc 50 shared all three bands, but the other accession Roc 39 shared no bands in common with the three KCB accessions. *R. coreanus* accessions shared only one band in common with KCB accessions. In the *trnL~trnF* spacer, all *Rubus* species had two SSCP bands except *R. idaeus*, which had two additional distinct bands. KCB shared one of the bands with *R. coreanus* but none with black raspberry. In the *trnT~trnL* spacer, all *Rubus* species had one SSCP band which was shared among all *Rubus* species except black raspberry which had unique SSCP band (Fig. 2).

A phylogenetic tree was constructed using the SSCP markers. A clade was formed at the genetic distance of 0.55 with four subclades. Subclade A contained the three KCB, subclade B the two black raspberry and two red raspberry accessions. Subclade C contained three *R. coreanus* accessions and subclade D three blackberry accessions (Fig. 3).

SSCP markers for the three chloroplast DNA spacers

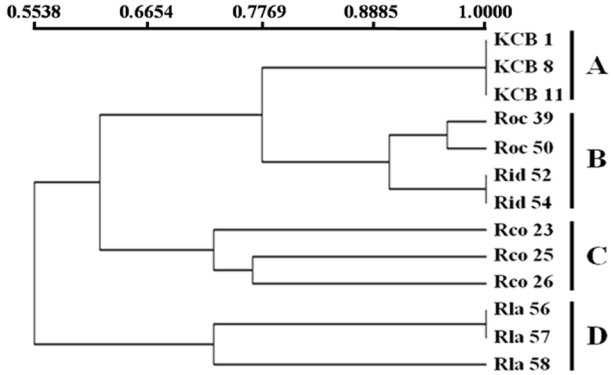


Fig. 3. Phylogenetic relationships of 13 *Rubus* species based on the 11 SSCP plastome markers. KCB; Korea cultivated Bokbunja, Rco; Bokbunja native to Korea (*R. coreanus*), Rid; red raspberry (*R. idaeus*), Rla; blackberry (*R. lanciniatus*), Roc; black raspberry (*R. occidentalis*).

suggest a similar phylogenetic relationship as revealed by RAPD and ITS markers (Eu *et al.*, 2008; Eu *et al.*, 2009). The SSCP markers of the three KCB accessions, KCB 1, 8 and 11, clustered more closely to two black raspberry accessions, Roc 39 and Roc 50 than to those of *R. coreanus*. Accessions of KCB were remotely related to *R.*

Table 3. Size and base composition of the chloroplast *trnL~trnF* spacer sequences of *Rubus* species.

Entry Number*	Length	G + C (%)	Homology	Homology Related to KCB
KCB 1	479	34		
KCB 8	479	34	99.8	–
KCB 11	479	35		
Rco 25	491	35		
Rco 26	491	35	98.5	96.6
Rco 23	491	35		
Rid 52	484	35	92.6	95.6
Rid 54	477	34		
Rla 56	477	34		
Rla 57	477	34	100	96.6
Rla 58	477	34		
Roc 39	478	34	98.1	99.1
Roc 50	479	34		

* KCB; Korea cultivated Bokbunja, Rco; Bokbunja native to Korea (*R. coreanus*), Rid; red raspberry (*R. idaeus*), Rla; blackberry (*R. lanciniatus*), Roc; black raspberry (*R. occidentalis*).

coreanus. This result implies that there is a considerable variation in the spacer sequences among *Rubus* species. The result also suggests that some KCB accessions share a significant similarity with black raspberry in the three chloroplast spacer regions.

To conform this result in nucleotide sequence level, we analyzed nucleotide sequences for the *trnL~trnF* region. Sequences of the *trnL~trnF* region of each *Rubus* species were determined and their similarity to the known *trnL-trnF* region sequences were calculated. The nucleotide of the *trnL~trnF* region ranged from 479~491 bp in *Rubus* species tested and its average G+C content ranged from 34% to 35%. The homology between each accessions in each taxon ranged from 96.2% to 99.6%. The homology of Roc accessions related to KCB accessions was higher than that of Rco assession (Table 3).

A phylogenetic tree based on the sequences of the *trnL~trnF* region contained two strongly supported clade and three subclades. All KCB accessions were found nested within subclade A containing the two black raspberry accessions, Roc 39 and Roc 50. However, three *R. coreanus* were found included in subclade B (Fig. 4). Phylogenetic relationships inferred from the *trnL~trnF* region sequences share a significant similarity with those from SSCP, RAPD and ITS analysis (Eu *et al*., 2008; Eu *et al*., 2009).

In summary, the SSCP markers for the three chloroplast DNA spacers, *atpB~rbcL*, *trnL~trnF* and *trnT~trnL*, and the nucleotide sequences of *trnL~trnF* region imply that some

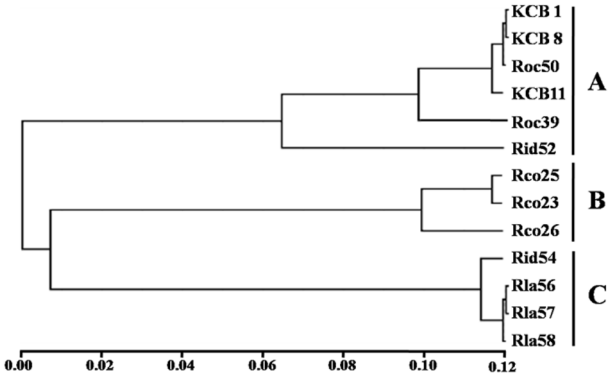


Fig. 4. Phylogenetic relationships of 13 *Rubus* species based on the sequence of the *trnL~trnF* spacer region. KCB; Korea cultivated Bokbunja, Rco; Bokbunja native to Korea (*R. coreanus*), Rid; red raspberry (*R. idaeus*), Rla; blackberry (*R. lanciniatus*), Roc; black raspberry (*R. occidentalis*).

KCB accessions share a significantly higher similarity with *R. occidentals* than with *R. coreanus* in the chloroplast genome. This result does not support the common conviction that KCB is a domesticated *R. coreanus*. Thus, the result also brings up the need for close and systematic investigations on the genetic identity of KCB. Clarification of the identity of KCB might be a prerequisite for accurate communications on scientific findings on KCB and also for proper advertisement and business on KCB products.

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