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Phylogenic Relationships of *Rubus* Species Revealed by Randomly Amplified Polymorphic DNA Markers

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Abstract

Korean cultivated bramble, which is known as Bokbunja-ddal-gi is regarded as having originated from Korean native *Rubus coreanus*. However, little scientific evidence and significant morphological differences between Korean cultivated bramble (KCB) and *R. coreanus* throws doubt on the ancestry of KCB. This study was carried out to obtain phylogenetic information on KCB by comparing its nuclear genomic background with those of *R. coreanus*, black (*R. occidentalis*) and red (*R. idaeus*) raspberry, blackberry (*R. lanciniatus*), and *R. crataegifolius*. A total of 99 random amplified polymorphic DNA (RAPD) markers were generated and used for phylogenetic analysis of 76 *Rubus* accessions. Accessions of each species were grouped into each distinct subclade by the RAPD markers at a similarity coefficient of about 0.59. The KCB subclade formed a clade with *R. occidentalis* and *R. crataegifolius* subclades at a similarity coefficient of 0.47. The *R. coreanus* subclade formed a clade with *R. idaeus*, *R. lanciniatus*, and *R. crataegifolius* subclades at a similar level of genetic similarity. Only one KCB accession from Hoengsung was included in the *R. coreanus* subclade. The accession shows leaf and flower characteristics different from the rest of the KCB accessions. The phylogenetic relationship inferred from the RAPD markers suggests that the nuclear genomic background of KCB accessions which show morphological similarity to black raspberry is more closely related to black raspberry than to *R. coreanus*. This brings about the need for close scientific evaluations on the ancestry of KCB at both morphological and molecular levels.

Key words: Random amplified polymorphic DNA (RAPD), phylogenetic relationship, *Rubus* species

Introduction

Bramble refers to thorny plants of the genus *Rubus*, such as raspberry, blackberry, and loganberries. Wild brambles first attracted the attention of herbalists due to their medicinal properties. *R. coreanus*, the *Rubus* species native to Korea, has been used for various medicinal purposes. Well-known medicinal properties of *R. coreanus* fruit include remedies for palsy, imbecility, arthritis, and stomach diseases. It also has beneficial effects for sexual disorders and cancer treatments (Jeong and Sin 1996; Park et al. 2003). The use of bramble fruit has been diversified as a beneficial food resource as customers' interest in healthier food has grown with economic development and changes in food industry. The demand for bramble fruit has increased as a variety of new foods and beverages containing bramble fruit or its extract as a major ingredient or supplements have been developed. The increased demand was followed by the cultivation of bramble plants in farm fields in South Korea. As the cultivation was initiated in Gochang-gun area in North Jeolla province, the cultivated bramble was collectively called Gochang Bokbunja-ddal-gi, or simply Bokbunja. The production of Bokbunja has increased drastically in the last five years and the cultivation area was estimated to be over 2,500 ha in 2006 (Korean Black Raspberry Experiment Station).

Rubus is one of the most diverse genera in the plant kingdom, and it contains 12 subgenera (Jennings 1988). With the efforts of plant breeders, species bearing edible fruits have been selected, and cultivars producing larger and more flavorsome fruits have been developed. Raspberries and blackberries are domesticated

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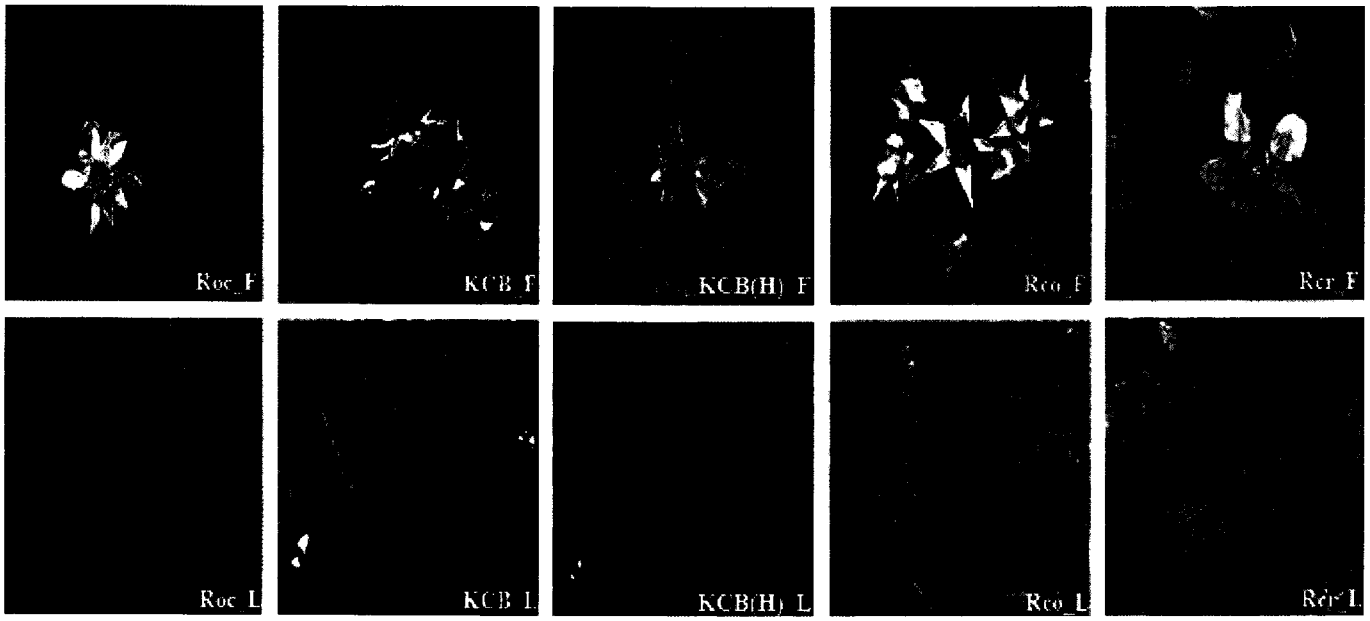

Fig. 1. Morphological characteristics of leaf (L) and flower (F) of *Rubus* species used in this study. KCB: Korean cultivated bramble, KCB(H): Korean cultivated bramble in Hoengsung, Rco: bramble native to Korea (*R. coreanus* Mique), Roc: black raspberry (*R. occidentalis* L.), Rcr: *R. crataegifolius*.

forms of the wild *Rubus* species. Raspberries belong to the subgenus Idaeobatus, which contains about 200 species. Black, red, and purple raspberries are major domesticated species belonging to Idaeobatus. Black raspberries include the two main subspecies, *R. occidentalis* L. and *R. leucodermis* Dougl., which are indigenous to North America. *R. occidentalis* L. is more common in the eastern part of North America, and *R. leucodermis* Dougl. in the western part of North America. Black raspberry cultivars commercially cultivated in the USA originated from *R. occidentalis*, but not from *R. leucodermis* Dougl. (Jennings 1988). Red raspberries are widely distributed in the temperate regions of Europe, Asia, and North America. The two major subspecies in red raspberries are *R. idaeus* and *R. strigosus*. Blackberries belong to the subgenus Eubatus, which contains very large number of species (Jennings 1988).

The bramble cultivated widely in South Korea has been known as a domesticated form of the Korean native *R. coreanus*, which has been the traditional source of Bokbunja used for various medicinal purposes. Even though the cultivation history of the bramble in Korea is relatively new, as recent as the last five decades, little scientific documentation is available on the domestication or cultivation history. Therefore, the claim that KCB is a domesticated form of *R. coreanus* suffers from lack of supporting scientific evidence and documentation. Though *R. coreanus* bears black berries as KBC and black raspberry, flower color, and leaf shapes of KCB are rather more similar to those of black raspberry than to those of Korea native *R. coreanus*. KCB has white flowers and trifoliate leaves, whereas *R. coreanus* has purple flowers and penta- or nona-foliate leaves (Fig. 1). These morphological similarities between KCB and black raspberry have been the ground of speculation that KCB is a derivative of black raspberry. Furthermore, cultivars of black raspberry and red raspberry were introduced to Korea from Canada and the USA in the 1960s (Ham et al. 1997). The random amplified polymorphic DNA (RAPD) markers generated using random primers and polymerase chain reaction (PCR) have been widely used for various polymorphism analyses including identification of cultivars, differentiation of plant species, and genetic analysis of phylogenetic relationships among strains or populations (Antonius and Klemola 1999; Charcosset and Moreau 2004; Mohan et al. 1997; Rafalski and Tingey 1993). Therefore, the objective of this study was to obtain basic phylogenetic information about the ancestry of KCB by comparing genomic background of KCB with those of *R. coreanus*, *R. crataegifolius*, black raspberry, red raspberry, and blackberry.

Materials and Methods

Plant materials

A total of 76 accessions of *Rubus* species were used in this study. Fourteen accessions of KCB were collected from the major cultivation areas in South Korea (Table 1). Black raspberry (Roc, *R. occidentalis* L., 15 cultivars) was obtained from National Clonal Germplasm Repository, Corvallis, Oregon, USA. *R. coreanus* (Rco, 21 species), red raspberry (Rid, *R. idaeus*, four species), blackberry (Rla, *R. lanciniatus*, four species), and *R. crataegifolius* (Rcr, 18 species) were obtained from the Korean Black Raspberry Experiment Station, Gochang, Korea.

Table 1. A total 76 *Rubus* accessions of Korean cultivated bramble, *R. coreanus*, *R. crataegifolius*, *R. occidentalis*, *R. idaeus*, and *R. lanciniatus*.

Entry Number	Common Name (Taxon)	Collection location	Note (Variety)
1	Korean cultivated bramble (Unknown) -KCB-	South Korea	Gochang, Jeonbuk
2			Pyeongchang, Kangwon
3			Hoengsung, Kangwon
4			Sanchung, Gyeongnam
5			Yeonggwang, Jeonnam
6			Jangsung, Jeonnam
7			Kimje, Jeonbuk
8			Sunchang, Jeonbuk
9			Wanju, Jeonbuk
10			Jangsu, Jeonbuk
11			Jeongeup, Jeonbuk
12			Jinan, Jeonbuk
13			Taean, Chungnam
14			Cheongwon, Chungbuk
15	Korean native bramble (*R. coreanus* Mique) -Rco-	South Korea	Taebak, Kangwon
16			Hongcheon, Kangwon
17			Hoengsung, Kangwon
18			Yangpyeong, Gyeonggi
19			Hamyang, Gyeongnam
20			Sangju, Gyeongbuk
21			Muan, Jeonnam
22			Bosung, Jeonnam
23			Wanju, Jeonbuk
24			Danyang, Chungnam
25			Okcheon, Chungbuk
26			Jecheon, Chungbuk
27			Cheongwon, Chungbuk
28			Cheongju, Chungbuk
29			Gosung, Gyeongnam
30			Goheung, Jeonnam
31			Gokseong, Jeonnam
32			Gurye, Jeonnam
33			Youngam, Jeonnam
34			Gongju, Chungnam
35			Taean, Chungnam
36	Black raspberry (*R. occidentalis* L.) -Roc-	USA	Black Hawk
37			Bristol
38			Cumberland
39			Plum Farmer
40			Shuttleworth
41			New Logan
42			Black Knight
43			Red fruit
44			NC 84-10-3
45			NC 84-10-7
46			NC 84-10-2
47			Haut
48			Dundee
49			John Robertson
50			Mac Black
51	*R. crataegifolius* -Rcr-	South Korea	Gangneung, Kangwon
52			Pyeongchang, Kangwon
53			Gosung, Gyeongnam
54			Kimhae, Gyeongnam
55			Ulryungdo, Gyeongbuk
56			Goheung, Jeonnam
57			Gokseong, Jeonnam
58			Gurye, Jeonnam
59			Youngam, Jeonnam
60			Jangheung, Jeonnam
61			Gongju, Chungnam
62			Asan, Chungnam
63			Taean, Chungnam
64			Yeongwol, Kangwon
65			Gochang, Jeonbuk
66			Okcheon, Chungbuk
67			Yeongwol, Kangwon
68	*R. crataegifolius* -Rcr-	North Korea	Mt Paekdu, North Korea
69	Red raspberry (*R. idaeus*) -Rid-	USA	Autum Bliss
70			Golden Harvest
71			NOVA
72			Canby
73	Blackberry (*R. lanciniatus*) -Rla-	USA	Thorny
74			Thornless
75			Creeping
76			Ebano

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.

Extraction of genomic DNA

DNA was isolated from fresh young leaves using the Puregene DNA purification kit (Gentra System, Minneapolis, MN, USA) following the instruction provided by the manufacturer. Fresh leaf samples (30 mg) 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°C for 60 min. Cell lysate was collected by centrifugation and treated with proteinase K (6 mg/ml) for 60 min at 55°C. RNA was degraded by adding RNase A (1.5 mg/ml) in the cell lysate and incubating the lysate at 37°C 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 x 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 μℓ DNA hydration solution by incubating the DNA sample overnight at room temperature. DNA concentration was measured by both spectrophotometric assay and gel electrophoresis. DNA samples were used for PCR for nuclear genomes.

Random amplified polymorphic DNA analysis

Primers for the analysis of RAPD were purchased from SeouLin Bioscience (SRILS uniprimer kit, Seoul, Korea). RAPD analysis of nuclear genomic DNA was carried out with the DNA template prepared from leaf samples. PCR amplification was carried out in a 30 μℓ reaction mixture, containing ~0.01-0.1 ng of template DNA, 10 pmol primer (SRILS uniprimer kit), dNTPs (0.2 mM each), and *Taq* polymerase (1 unit, Ex *Taq* PCR, TaKaRa). The thermal cycles consisted of an initial denaturation (5 min at 94 °C), 40 cycles of amplification (repeated cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C)), and a last extension (10 min at 72 °C). The amplified products were separated in 1% agarose gel at 100V for 30 min and visualized by staining the gel in ethidium bromide solution and photographed under UV light. RAPD markers were analyzed by UPGMA (unweighted pair-group method with arithmetic average) method using NTSYS (numerical taxonomy and multivariate analysis system) program (Sneath and Sokal 1973).

Results and Discussion

A total of 99 RAPD markers including 84 major bands and 15 minor bands were generated using the 12 SRILS primers. Typical markers are shown in Fig. 2.

Profiles of RAPD markers were scored and analyzed by the UPGMA method using a NTSYS program. Accessions of each species were grouped into a distinct separate subclade based on the RAPD markers (Fig. 3). Some of the accessions in a species were very similar to each other and the accessions of wild *R. crataegifolius* were clearly distinct both morphologically and phenogenetically.

Accessions of each species were grouped into a distinct separate subclade by the species at the similarity coefficient of about 0.59. The RAPD markers revealed that KCB forms a distinct subclade separately from that of each *Rubus* species. The KCB subclade formed a clade with *R. occidentalis* and *R. crataegifolius* subclade at the similarity coefficient of 0.47. The *R. coreanus* subclade formed a clade with *R. idaeus* and *R. lanciniatus* subclades at the similarity coefficient of 0.45. The clade consisting of *R. coreanus* and *R. lanciniatus* subclades is remotely but more closely related to *R. idaeus* subclade than the clade containing KCB and *R. occidentalis* (Fig. 3). This result indicates that KCB has a genetic constitution significantly different from other *Rubus* species. Among the *Rubus* species tested in this study, nuclear genomic background of KCB accessions shows relatively closer relatedness to that of *R. occidentalis* than to that of *R. coreanus*. This strongly suggests that the widely accepted ancestry of KCB to *R. coreanus* might have originated from legendary assumptions rather than sound scientific evidence.

Nevertheless, scientific studies which used KCB as an experimental material referred to the scientific name of the material as *R. coreanus* rather than *R. occidentalis* (Cha et. at. 2007; Yoon et al. 2002). Furthermore, nearly all advertisements of the products made of KCB fruits refer KCB as *R. coreanus*. *R. occidentalis* is not included in the introduction to Bokbunja produced in

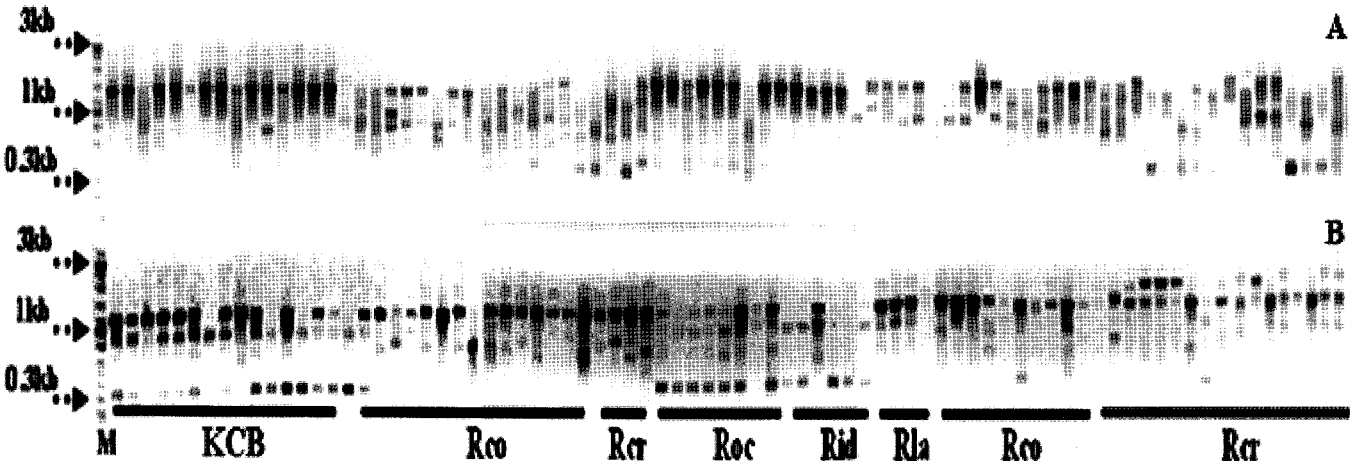

Fig. 2. Typical random amplified polymorphic DNAs generated for Rubus species. M: DNA ladder, KCB: Korean cultivated bramble, Rco: Korean native bramble (*R. coreanus* Mique), Roc: black raspberry (*R. occidentalis* L.), Rcr: *R. crataegifolius*, Rla: blackberry (*R. lanciniatus*), and Rid: red raspberry (*R. idaeus*). A (URP primer11), B (URP primer12).

Gochang-gun (http://www. gochang. jeonbuk.kr/farm/).

Only one KCB accession from Hoengsung was included in *R. coreanus* subclade. Hoengsung accession has unique morphological characteristics different from all the other KCB accessions. Its leaf and flower characteristics are similar to *R. coreanus* rather than to *R. occidentalis* to which all the rest of the KCB accessions share a close similarity. A few Korean black raspberry breeders have used wild *R. coreanus* germplasms collected from mountains of the southern part of the Korean peninsula (Kim et al. 2002a; Kim et al. 2002b). They assumed that KCB is *R. occidentalis* rather than *R. coreanus* (Kim et al. 2002a). Accessions in the KCB subclade show a diverse genetic distance. Especially, the KCB accession from Sunchang (accession #8) is included in the subclade at the similarity coefficient of 0.59. This result indicates that there is a considerable genetic variation among the local KCB accessions. Also, the KCB accession from Hoengsung is most likely domesticated from *R. coreanus*.

In conclusion, the genetic background inferred from the RAPD fragments shows that all KCB accessions, except one from Hoengsung, exhibit the closest relatedness to *R. occidentalis* among the *Rubus* species used in this study such as *R. coreanus* Mique, *R. crataegifolius*, *R. occidentalis*, *R. idaeus*, and *R. lanciniatus*. This brings about the need for close scientific investigations on the ancestry of KCB at both morphological and molecular levels.

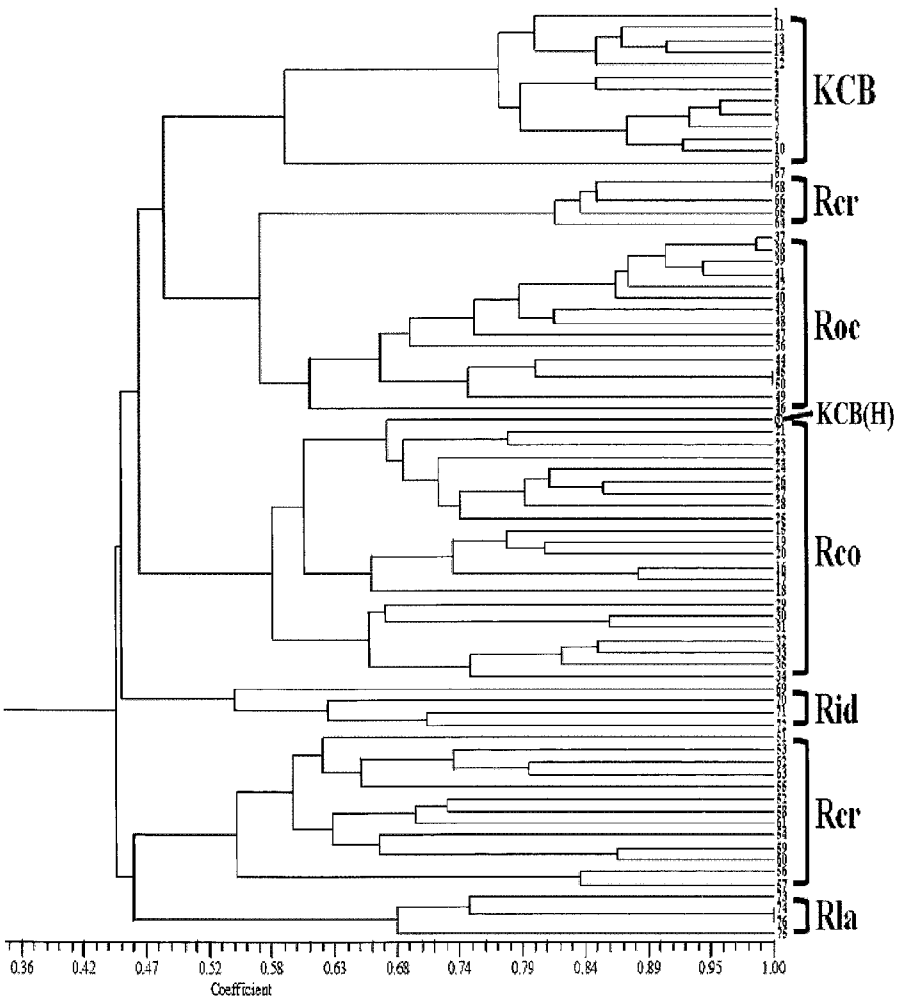

Fig. 3. A phylogram generated from the 99 nuclear RAPD markers for the *Rubus* accessions. KCB: Korean cultivated bramble, KCB (H): Korean cultivated bramble in Hoengsung, Rco: Korean native bramble (*R. coreanus* Mique), Roc: black raspberry (*R. occidentalis* L.), Rcr: *R. crataegifolius*, Rla: blackberry (*R. lanciniatus*), and Rid: red raspberry (*R. idaeus*).

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