Title

Assessment of genetic relationships among cultivated and wild *Rubus* accessions using AFLP markers

Authors

Tomoya Miyashita[1], Hisato Kunitake[2], Norishige Yotsukura[3], and Yoichiro Hoshino[1, 3]*

Affiliations and addresses

[1] Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan

[2] Faculty of Agriculture, Miyazaki University, Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

[3] Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan

*Corresponding author: Yoichiro Hoshino

Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan

Telephone: +81-11-706-2857 FAX: +81-11-706-2857

E-mail: hoshino@fsc.hokudai.ac.jp

ABSTRACT

The genus *Rubus* comprises more than 740 species divided into 12 subgenera and contains the raspberries, blackberries, arctic fruits, and flowering raspberries, all of which have been utilized in breeding programs. The objective of this study was to evaluate the phylogenetic relationships among wild and cultivated *Rubus* species mainly collected in Japan. To evaluate genetic resources in *Rubus*, 81 accessions were analyzed with three amplified fragment length polymorphism (AFLP) primer pairs and data were analyzed with the neighbor-joining and unweighted pair group methods with arithmetic mean. Two of the generated phylogenetic trees grouped subgenera *Anoplobatus*, *Eubatus*, *Idaeobatus*, and *Malachobatus* into different clusters. Accessions of *Rubus idaeus* L. var. *aculeatissimus* collected from four regions in Hokkaido formed distinct clusters reflecting sampling sites. Four hybrid accessions between raspberry cultivars (*R. idaeus* L.) and *R. idaeus* L. var. *aculeatissimus*, and one hybrid accession between a raspberry cultivar and *R. spectabilis* Pursh were clearly distinguished from parental accessions. These results indicated that AFLP markers are a reliable technique for assessing genetic diversity and studying phylogenetic relationships in *Rubus*. Data from diversity and phylogenetic studies revealed valuable information on the availability of unique fragments in different accessions that would be useful for the development of improved genotypes through conventional breeding and marker-assisted selection.

Keywords: AFLP; blackberry; phylogenetic analysis; raspberry; *Rubus*

Abbreviations: AFLP, amplified fragment length polymorphism; NJ method, neighbor-joining method; PCR, polymerase chain reaction; UPGMA, unweighted pair group method with arithmetic mean

1. Introduction

The genus *Rubus* belongs to Rosaceae and comprises more than 740 species (Gu et al., 1990) divided into 12 subgenera (Jennings, 1988) and is distributed across all continents (Gustafsson, 1942). Most *Rubus* species are perennial plants with perennial crowns and biennial canes (Hummer, 1996). Ploidy level ranges from diploid to tetradecaploid, including odd-ploids and aneuploids (Thompson, 1995). The genus *Rubus* contains the raspberries, blackberries, arctic fruits, and flowering raspberries, all of which have been utilized in breeding programs (Graham and Jennings, 2009). The most economically important species are the raspberries and the blackberries that belong to the subgenera *Idaeobatus* and *Rubus*, respectively (Hummer, 1996). Hybridization between *Idaeobatus* and *Rubus* species has generated commercially important hybrid cultivars, such as 'Boysenberry' and 'Loganberry' (Clark and Finn, 2011). Approximately 142 raspberry and 50 blackberry/hybrid berry cultivars have been released since 1980 (Knight et al., 2004).

Wild *Rubus* species have been utilized in breeding programs as valuable sources of desirable horticultural traits (Knight et al., 2004). For instance, *R. parviflorus* and *R. odoratus* have been used as sources of resistance to cane midges (Graham and Jennings, 2009); *R. idaeus* and *R. crataegifolius* as sources of resistance to raspberry beetle (Briggs et al., 1982); and *R. crataegifolius*, *R. palmatus*, and *R. lambertianus* for the higher berry polyphenol content and antioxidant activity than raspberry cultivars (Shigyo et al., 2013). Attempts have been made to select raspberry cultivars that are resistant to Phytophthora root (Moore and Hoashi-Erhardt, 2012a) and raspberry bushy dwarf virus (Moore and Hoashi-Erhardt, 2012b). Graham et al. (2003) reported that wild *Rubus idaeus* populations are more diverse than cultivars; therefore, wild *Rubus* species are expected to provide novel traits in raspberry and blackberry breeding.

Molecular markers have been successfully used for assessing genetic diversity, allelic richness, and genetic relationships in several fruit genera such as *Vitis* (Upadhyay et al., 2007), *Citrus* (Biswas et al., 2011), and *Prunus* (Zeinalabedini et al., 2014). Phylogenetic relationships among 88 genera of Rosaceae family, including genus *Rubus*, have also been investigated using polymerase chain reaction (PCR) markers (Potter et al., 2007). In addition, *Rubus* species have been extensively studied with different types of molecular makers such as amplified fragment length polymorphism (AFLP) markers (Amsellem et al., 2000; Lpek et al., 2009; Agar et al., 2011), randomly amplified polymorphic DNA (RAPD) markers (Graham and McNicol, 1995; Graham et al., 1997; Weber, 2003), simple sequence repeat (SSR) markers (Graham et al., 2002; 2009a), and the combination of AFLP and SSR markers

(Graham et al., 2004; Marulanda et al., 2007); however, limited information is available on genetic relationships among cultivated and wild *Rubus* accessions in Japan. These wild *Rubus* species have been utilized as local fruits and are expected to be candidate parents for raspberry breeding. The objective of this study was to evaluate the phylogenetic relationships among raspberries, blackberries, Boysenberry, hybrid accessions, and wild *Rubus* species mainly collected in Japan.

2. Materials and methods

2.1. Plant material

A total of 81 different *Rubus* accessions were analyzed in this study, corresponding to 15 wild species (*R. idaeus* var. *aculeatissimus, R. parvifolius, R. vernus, R. crataegifolius, R. phoenicolasius, R. pseudojaponicus, R. mesogaeus, R. buergeri, R. sieboldii, R. hirsutus, R. croceacanthus, R. palmatus* var. *coptophyllus, R. palmatus* var. *palmatus, R. trifidus, R. spectabilis,* and *R. parviflorus*), 6 red raspberry cultivars ('Chilcotin', 'Heritage', 'Indian Summer', 'Leon', 'Nootka', and 'Skeena'), 2 blackberry cultivars ('Black Satin' and 'Thorn-free'), 1 Boysenberry, and 5 hybrid accessions (ChId_1, ChId_2, SfId, SkId, and ChSp). The five hybrid accessions were produced by crossing red raspberry cultivars and wild *Rubus* species cultivated in the experimental station of Hokkaido University, Sapporo, Japan. Parental accessions used in phylogenetic study are described in Table 1. All accession names are also presented in Table 1. Sampling sites of wild *Rubus* species are shown in Fig. 1. *Malus domestica* cv. 'Fuji' that is a major apple variety was selected from the Rosaceae family and included in the analysis as an out-group.

2.2. Molecular techniques

DNA was isolated from young leaves of all accessions using ISOPLANTII kit (Nippon Gene, Japan) following manufacturer's instructions. DNA concentration was quantified using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, USA) and diluted to 100 $ng \cdot \mu L^{-1}$ for AFLP analysis.

AFLP analysis was performed according to the AFLP® Plant Mapping Protocol (Applied Biosystems, USA) with some minor modifications. Approximately 5 μg of genomic DNA were digested with 0.4 U *Mse*I and 5 U *Eco*RI restriction enzymes. The restricted DNA fragments were ligated to *Mse*I and *Eco*RI barcoded adapters for 2 h at 37 °C. Ligation products of each sample were diluted 20-fold with TE buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and used for pre-selective amplification. Pre-selective PCR reaction was

performed using the following profile: initial denaturation at 72 °C for 2 min, followed by 20 cycles of denaturation at 94 °C at 20 sec, annealing at 56 °C for 30 sec, and extension at 72 °C for 2 min, and a final extension at 60 °C for 30 min.

Pre-selective amplification products of each sample were diluted 20-fold with TE buffer and used for selective PCR with three selective primer pairs: E-ACA/M-CAG, E-ACG/M-CTA, and E-AAC/M-CAT. E-AAC/M-CAT was prepared based on previous reports (Amsellem et al., 2000; Marulanda et al., 2007), while E-ACA/M-CAG and E-ACG/M-CTA were selected by preliminary studies. Selective PCR was performed using the following touch-down profile by lowering annealing temperature through PCR cycles: an initial denaturation at 94 °C for 2 min, followed by 11 cycles of denaturation at 94 °C for 20 sec, annealing at 65 °C (lowering the temperature by 1 °C over the next cycles) for 30 sec, extension at 72 °C for 2 min, and 25 cycles of denaturation at 94 °C for 2 min, annealing at 56 °C for 30 sec, and extension at 72 °C for 2 min, and a final extension at 60 °C for 30 min.

For analysis, 1 μL of three differently labeled fluorescent AFLP products (6-FAM, JOE, and NED) were mixed with 0.5 μL GeneScan-500 ROX size standard (Applied Biosystems, USA) in 11.5 μL Hi-Di formamide (Applied Biosystems, USA). This mixture was denatured at 94 °C for 3 min and cooled on ice. AFLP analysis was performed on a ABI 3130 Genetic Analyzer (Applied Biosystems, USA) and data were analyzed by GeneScan v3.0 software (Applied Biosystems, USA).

2.3. Data analysis

Fragment analysis was performed using GeneMapper v4.0 software (Applied Biosystems, USA). AFLP profiles were converted into a presence/absence (1/0) character matrix using a minimum detection threshold of 300 relative fluorescent units (RFU) to avoid artifacts i.e., fragments with an intensity above 300 RFU were scored as “1” and below 300 RFU were scored as “0.” The 1/0 character matrix was converted to a pairwise distance matrix (Table S1), which was used to construct two phylogenetic trees with the neighbor-joining (NJ) method and unweighted pair group method with arithmetic mean (UPGMA) using MEGA6 software (Tamura et al., 2013). Bootstrap analysis (1,000 replicates) was performed on the data set. The evolutionary distances were computed using the pair-distance method (Nei and Kumar, 2000) and were expressed as the number of base-pair differences per site.

3. Results

Analysis of 81 *Rubus* accessions with three AFLP primer pairs identified a total of 786 bands. Of those, only one band was common across accessions, while the rest were polymorphic. Across 21 different species and subspecies, a total of 243 bands were identified as species-specific bands, ranging from 1 in ChSp hybrid to 31 in *R. parvifolius* (Table 2). Within each species, the number of AFLP bands ranged from 72 in *Rubus palmatus* var. *coptophyllus* to 317 in *R. idaeus* var. *aculeatissimus*, and the number of polymorphic bands ranged from 39 (41.5% of total number of bands) in *R. buergeri* to 301 (95.0% of total number of bands) in *R. idaeus* var. *aculeatissimus* (Table 2). The pairwise distance of accessions ranged from 0.027 (between *R. phoenicolasius*_1 and *R. phoenicolasius*_2) to 0.220 (between *R. idaeus*_1 and *R. sieboldii*) with a mean value of 0.154 (Table S1).

Both NJ method (Fig. 2) and UPGMA (Fig. 3) yielded 8 major clusters (clusters A–H). Cluster A included *R. idaeus* cultivars (6 accessions), *R. idaeus* var. *aculeatissimus* (27 accessions), and all the hybrids (5 accessions). Cluster B included *R. phoenicolasius* (4 accessions), *R. mesogaeus* (1 accession), and *R. parvifolius* (9 accessions). Cluster C included *R. fruticosus* (2 accessions) and Boysenberry. Cluster D included *R. spectabilis* (1 accession) and *R. vernus* (8 accessions). Cluster E included *R. parviflorus* (1 accession) and *R. pseudojaponicus* (2 accessions). Cluster F included *R. buergeri* (2 accessions) and *R. sieboldii* (1 accession). Cluster G included *R. palmatus* var. *coptophyllus* (1 accession), *R. palmatus* var. *palmatus* (1 accession), *R. croceacanthus* (1 accession), *R. hirsutus* (1 accession), *R. trifidus* (1 accession), and *R. crataegifolius* (6 accessions). Cluster H was a single-branch cluster that included the out-group, *Malus domestica* cv. ‘Fuji.’ All accessions of subgenus *Idaeobatus* were grouped together in clusters A, B, D, and G, while accessions of subgenus *Rubus* and *Malachobatus* were grouped in clusters C and F. The subgenus *Anoplobatus*, which includes *R. parviflorus*, was grouped in cluster E along with *R. pseudojaponicus*. In NJ tree, *R. idaeus* cultivars (6 accessions) were distinguished from *R. idaeus* var. *aculeatissimus* (27 accessions) in cluster A.

4. Discussion

In this study, 81 accessions were analyzed with three AFLP primer pairs, and 786 bands were obtained. Graham et al. (2004) genotyped two phenotypically different cultivars in red raspberry with 17 *Pst*I/*Mse*I and 14 *Eco*RI/*Mse*I markers and a total of 358 bands were obtained. Agar et al. (2011) analyzed wild and cultivated blackberry (*R. caucasicus*) accessions with three AFLP primer pairs, producing a total of 223 bands. The total number of AFLP bands in this study was higher than that in previous studies. Out of 786 bands, one

band was common across accessions, while the rest were polymorphic. The AFLP markers used in this study revealed a considerable amount of variation across the accessions, which indicated suitability for diversity studies in *Rubus*.

Previous phylogenetic studies in wild and cultivated *Rubus* species using AFLP markers reported a percentage of polymorphic bands that ranged from 70.3% (Ercisli et al., 2008) to 91.3% (Marulanda et al., 2007). In the present study, AFLP markers were used to assess diversity within each species and the percentage of polymorphic bands ranged from 41.5% (*R. buergeri*) to 95.0% (*R. idaeus* var. *aculeatissimus*). The high levels of polymorphism observed in *Rubus* suggested a high intra- and inter-specific polymorphic potential and subsequently a broad genetic base, probably due to the accumulation of diverse gene combinations in response to environmental stresses and natural selection. The ability for successful cross-species/ecotype hybridization might also contribute to the broad genetic base, which could be the material for natural selection and genetic diversity across environments.

The distribution of species has been affected by past climate changes, and the present genetic structure of species was formed during the Quaternary Period (Hewitt, 2000). Mimura et al. (2014) reported that the response of *Rubus* species to the climate changes during the Quaternary Period led to repeated hybridization with ecologically distinct relatives, and these interactions between different gene pools increased genetic variation. Amsellem et al. (2000) suggested that *R. alceifolius* was introduced to Madagascar and other Indian Ocean islands by birds or humans. Thus, these factors might also influence the genetic diversity of *Rubus* accessions in our study, which derived from several locations throughout Japan, including across oceanic channels.

Although *R. idaeus* var. *aculeatissimus* was only collected from a single site (Hokkaido, Japan), its intraspecific polymorphic ratio was higher than the interspecific polymorphic ratio of accessions collected from multiple sites (Honshu, Kyushu, and Hokkaido) such as *R. crataegifolius*, *R. parvifolius*, and *R. phoenicolasius*. Graham et al. (1997) reported that wild *R. idaeus* accessions collected from sites within a 20-m radius showed identical molecular patterns, while plants collected from distant sites showed diverse molecular profiles. In this study, two accessions of *R. idaeus* var. *aculeatissimus* accessions (*R. idaeus* 8 and 9) were collected from sites within a 70-m radius, yet their molecular profiles were diverse. Kollmann et al. (2000) suggested that the level of genetic variability in *Rubus* is determined by the plant propagation system. Ercisli et al. (2008) suggested that wild *Rubus* plants in northeast Turkey probably reproduced more sexually (outcrossing and selfing) than

asexually through suckers or adventitious roots. The high intraspecific variation observed in this study also supports the increased chance of sexual seed production in *Rubus*.

AFLP analysis of 100 *Pyrus* accessions grouped *P. ussuriensis*, *P. betulaefolia*, and *P. communis* into independent clusters (Bao et al., 2008). Cluster analysis of *Glycine* accessions based on AFLP data separated *Glycine max* from *G. soja* accessions (Maughan et al., 1996). Ercisli et al. (2008) reported that raspberry cv. 'Heritage' was clearly distinguished from wild accessions. In this study, wild and cultivated *Rubus* accessions were also grouped into independent clusters, indicating that AFLP markers are a very valuable technique for future diversity and phylogenetic studies.

Four subgenera *Idaeobatus*, *Rubus*, *Anoplobatus*, and *Malachobatus* grouped into distinct clusters; however, accessions of subgenus *Idaeobatus* were not grouped into a single cluster, but segregated into 4 clusters (cluster A, B, D, and G). These results suggested that subgenus *Idaeobatus* might be highly diverse. Analysis of internal transcribed spacers in *Rubus* showed that subgenera *Idaeobatus*, *R. trifidus*, and *R. crataegifolius* were grouped together in separate clusters from *R. idaeus* and *R. phoenicolasius* (Alice and Campbell, 1999). The results of this study were similar, since *R. trifidus* and *R. crataegifolius* were grouped into cluster G, while *R. idaeus* and *R. phoenicolasius* were grouped into cluster A and B, respectively. Alice and Campbell (1999) reported that *R. idaeus* and *R. phoenicolasius* were located in same cluster, while in our study they were located into different clusters. This may be affected by differences in *R. idaeus* and *R. phoenicolasius*, due to genetic diversity, or in experimental procedures. Additional studies will be needed to further analyze the relationship between *R. idaeus* and *R. phoenicolasius*.

Previous studies using nuclear ribosomal DNA internal transcribed spacer region sequences and RAPD markers showed that the subgenera *Anoplobatus* and *Idaeobatus* were grouped into genetically distinct clusters (Graham and McNicol, 1995; Alice and Campbell, 1999). In the present study, cluster G that included subgenus *Idaeobatus* accessions was more closely related to cluster E that included subgenus *Anoplobatus* accessions than to cluster A, which also included *Idaeobatus* accessions. These results indicated that the genetic analysis of *Rubus* using multiple molecular marker systems would provide more reliable data for the phylogenetic study of *Rubus* subgenera.

In this study, the hybrid accessions ChSp, ChId_1, ChId _2, SfId, and SkId were clearly distinguished from parental species (Figs. 2 and 3). In addition, ChId_1 and ChId _2 that were derived from the same parental combination were the most closely related

accessions in cluster A. The AFLP analysis revealed novel bands in the molecular profile of hybrid accessions, suggesting genetic recombination.

In summary, molecular analysis of 81 wild and cultivated *Rubus* accessions revealed considerable genetic diversity and suggested that AFLP markers are a reliable technique for assessing genetic diversity and studying phylogenetic relationships in *Rubus*. Overall, diversity and phylogenetic studies reveal valuable information on the availability of unique fragments in different accessions that are useful for the development of improved genotypes through conventional breeding and marker-assisted selection.

Acknowledgements

This study was supported by grants from a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports Science, and Technology (MEXT), Japan. The authors would like to thank I. Asakura for his assistance in plant collection. We are grateful to Editage (www.editage.jp) for English language editing.

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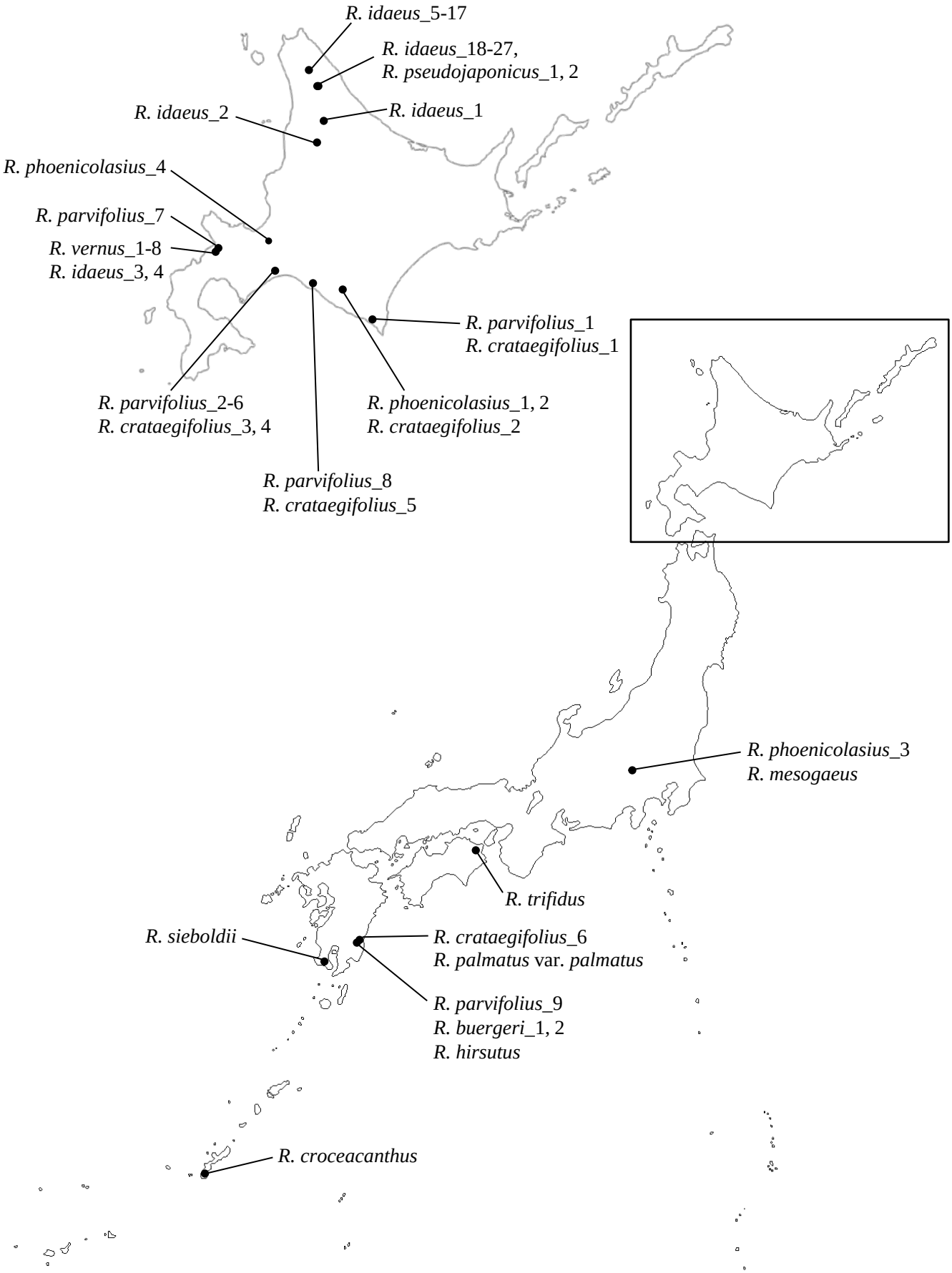
Figure legends

Fig. 1. Sampling locations of wild *Rubus* species accessions. A list of accession names is presented in Table 1.

Fig. 2. Dendrogram constructed using the neighbor-joining (NJ) method based on the pairwise distance generated from three AFLP primer pairs. Main clusters (A-H) are labeled on root branches. Bootstrap percentages obtained after 1,000 replicates are shown. The evolutionary distances were computed using the pair-distance method (Nei and Kumar, 2000), and are expressed as the number of base-pair differences per site.

Fig. 3. Dendrogram constructed using the unweighted pair group method with arithmetic mean (UPGMA) based on the pairwise distance generated from three AFLP primer pairs. Main clusters (A-H) are labeled on root branches. Bootstrap percentages obtained after 1000 replicates are shown. The evolutionary distances were computed using the pair-distance method (Nei and Kumar, 2000), and are expressed as the number of base-pair differences per site.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*R. idaeus*_18-27,
*R. pseudojaponicus*_1, 2
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*R. idaeus*_1
*R. phoenicolasius*_4
*R. parvifolius*_7
*R. vernus*_1-8
*R. idaeus*_3, 4
*R. parvifolius*_1
*R. crataegifolius*_1
*R. parvifolius*_2-6
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*R. crataegifolius*_2
*R. parvifolius*_8
*R. crataegifolius*_5
*R. phoenicolasius*_3
R. mesogaeus
R. trifidus
R. sieboldii
*R. crataegifolius*_6
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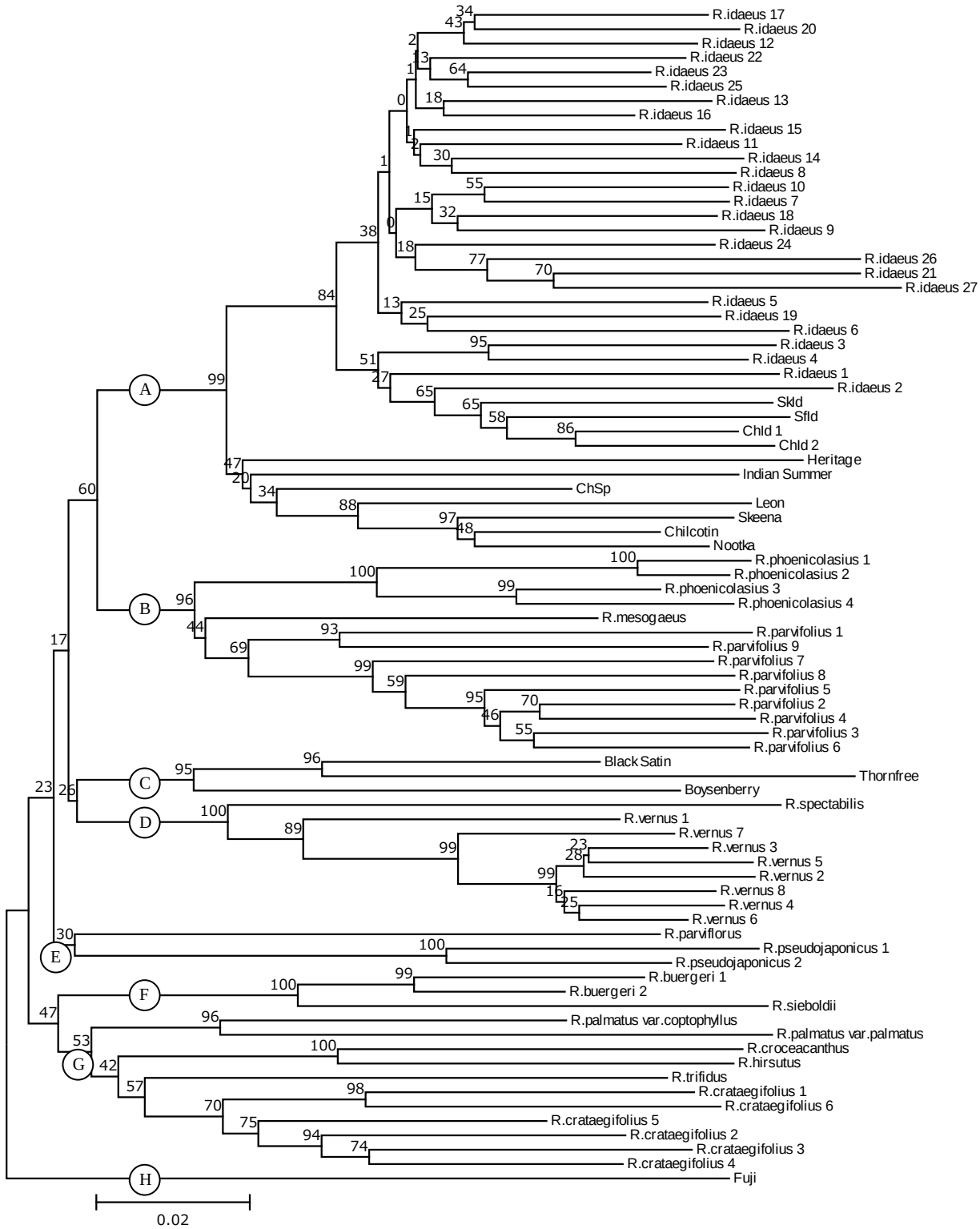
*R. buergeri*_1, 2
R. hirsutus
R. croceacanthus

Figure 1


R.idaeus 17
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R.idaeus 22
R.idaeus 23
R.idaeus 25
R.idaeus 13
R.idaeus 16
R.idaeus 15
R.idaeus 11
R.idaeus 14
R.idaeus 8
R.idaeus 10
R.idaeus 7
R.idaeus 18
R.idaeus 9
R.idaeus 24
R.idaeus 26
R.idaeus 21
R.idaeus 27
R.idaeus 5
R.idaeus 19
R.idaeus 6
R.idaeus 3
R.idaeus 4
R.idaeus 1
R.idaeus 2
Skld
Sfld
Chld 1
Chld 2
Heritage
Indian Summer
ChSp
Leon
Skeena
Chilcotin
Nootka
R.phoenicolasius 1
R.phoenicolasius 2
R.phoenicolasius 3
R.phoenicolasius 4
R.mesogaeus
R.parvifolius 1
R.parvifolius 9
R.parvifolius 7
R.parvifolius 8
R.parvifolius 5
R.parvifolius 2
R.parvifolius 4
R.parvifolius 3
R.parvifolius 6
Black Satin
Thornfree
Boysenberry
R.spectabilis
R.vernus 1
R.vernus 7
R.vernus 3
R.vernus 5
R.vernus 2
R.vernus 8
R.vernus 4
R.vernus 6
R.parviflorus
R.pseudojaponicus 1
R.pseudojaponicus 2
R.buergeri 1
R.buergeri 2
R.sieboldii
R.palmatus var.coptophyllus
R.palmatus var.palmatus
R.croceacanthus
R.hirsutus
R.trifidus
R.crataegifolius 1
R.crataegifolius 6
R.crataegifolius 5
R.crataegifolius 2
R.crataegifolius 3
R.crataegifolius 4
Fuji
A
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D
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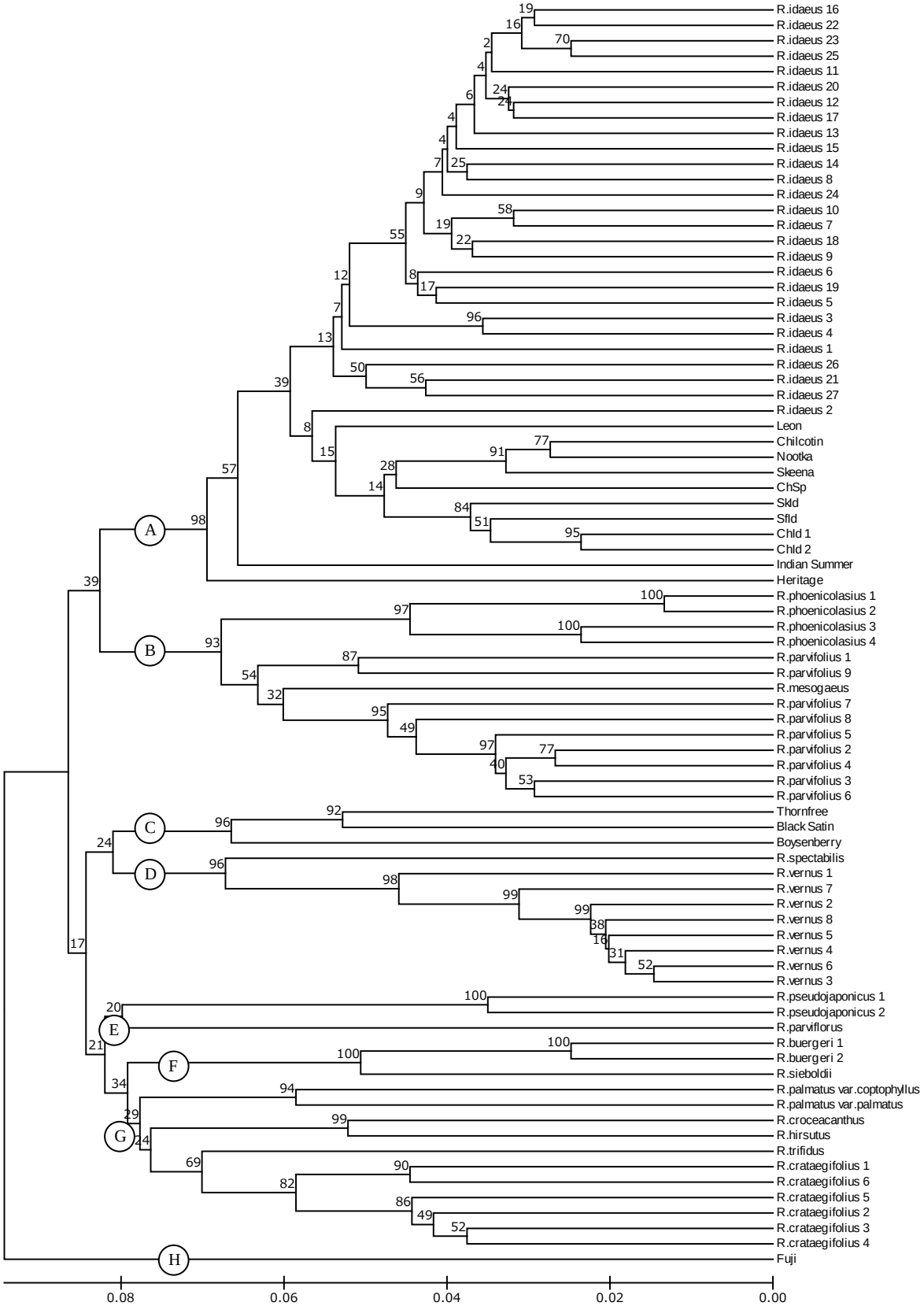
G
H
0.02

Figure 2


R.idaeus 16
R.idaeus 22
R.idaeus 23
R.idaeus 25
R.idaeus 11
R.idaeus 20
R.idaeus 12
R.idaeus 17
R.idaeus 13
R.idaeus 15
R.idaeus 14
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R.idaeus 6
R.idaeus 19
R.idaeus 5
R.idaeus 3
R.idaeus 4
R.idaeus 1
R.idaeus 26
R.idaeus 21
R.idaeus 27
R.idaeus 2
Leon
Chilcotin
Nootka
Skeena
ChSp
SkId
SfId
ChId 1
ChId 2
Indian Summer
Heritage
R.phoenicolasius 1
R.phoenicolasius 2
R.phoenicolasius 3
R.phoenicolasius 4
R.parvifolius 1
R.parvifolius 9
R.mesogaeus
R.parvifolius 7
R.parvifolius 8
R.parvifolius 5
R.parvifolius 2
R.parvifolius 4
R.parvifolius 3
R.parvifolius 6
Thornfree
Black Satin
Boysenberry
R.spectabilis
R.vernus 1
R.vernus 7
R.vernus 2
R.vernus 8
R.vernus 5
R.vernus 4
R.vernus 6
R.vernus 3
R.pseudojaponicus 1
R.pseudojaponicus 2
R.parviflorus
R.buergeri 1
R.buergeri 2
R.sieboldii
R.palmatus var.coptophyllus
R.palmatus var.palmatus
R.croceacanthus
R.hirsutus
R.trifidus
R.crataegifolius 1
R.crataegifolius 6
R.crataegifolius 5
R.crataegifolius 2
R.crataegifolius 3
R.crataegifolius 4
Fuji
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0.02
0.00

Figure 3

Table 1. *Rubus* accessions used in the molecular phylogenetic study.

Accession no.	Accession name	Species / Cultivar	Subgenus	Location	Latitude	Longitude
	Wild species					
1	*R. idaeus*_1	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Uryu Experimental Forest, Horokanai, Hokkaido	N44 24.402	E142 14.768
2	*R. idaeus*_2	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Horokanai, Hokkaido	N44 08.758	E142 08.497
3	*R. idaeus*_3	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi-cho, Hokkaido	N42 54.011	E140 32.386
4	*R. idaeus*_4	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.106	E140 32.122
5	*R. idaeus*_5	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N45 04.341	E142 05.643
6	*R. idaeus*_6	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N45 03.584	E142 05.504
7	*R. idaeus*_7	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N45 02.766	E142 06.445
8	*R. idaeus*_8	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N45 02.743	E142 06.491
9	*R. idaeus*_9	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 53.752	E142 03.183
10	*R. idaeus*_10	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 55.573	E142 04.740
11	*R. idaeus*_11	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 58.986	E142 00.488
12	*R. idaeus*_12	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 59.317	E142 00.454
13	*R. idaeus*_13	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N45 01.109	E142 02.910
14	*R. idaeus*_14	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 57.950	E141 59.871
15	*R. idaeus*_15	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 57.672	E141 59.844
16	*R. idaeus*_16	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 56.737	E141 58.223
17	*R. idaeus*_17	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Teshio Experimental Forest, Teshio, Hokkaido	N44 55.676	E141 59.697
18	*R. idaeus*_18	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 48.039	E142 05.211
19	*R. idaeus*_19	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 47.211	E142 06.231
20	*R. idaeus*_20	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 45.857	E142 09.375
21	*R. idaeus*_21	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 46.303	E142 09.769
22	*R. idaeus*_22	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 46.750	E142 09.637
23	*R. idaeus*_23	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 47.347	E142 10.220
24	*R. idaeus*_24	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 47.712	E142 10.995
25	*R. idaeus*_25	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 46.620	E142 12.832
26	*R. idaeus*_26	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 46.428	E142 12.881
27	*R. idaeus*_27	*R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 45.985	E142 13.301
28	*R. parvifolius*_1	*R. parvifolius*	*Idaeobatus*	Mt. Apoi, Samani, Hokkaido	N42 06.839	E142 59.546
29	*R. parvifolius*_2	*R. parvifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 40.943	E141 35.480
30	*R. parvifolius*_3	*R. parvifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 41.601	E141 34.388
31	*R. parvifolius*_4	*R. parvifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 41.609	E141 34.386
32	*R. parvifolius*_5	*R. parvifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 42.057	E141 34.179
33	*R. parvifolius*_6	*R. parvifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 42.474	E141 34.148
34	*R. parvifolius*_7	*R. parvifolius*	*Idaeobatus*	Kyowa, Hokkaido	N42 57.307	E140 35.518
35	*R. parvifolius*_8	*R. parvifolius*	*Idaeobatus*	Monbetsu, Hokkaido	N42 28.352	E142 05.486
36	*R. parvifolius*_9	*R. parvifolius*	*Idaeobatus*	Miyazaki, Miyazaki	N31 47.088	E131 22.412
37	*R. vernus*_1	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.011	E140 32.386
38	*R. vernus*_2	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.032	E140 32.369

39	*R. vernus*_3	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.104	E140 32.361
40	*R. vernus*_4	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 53.984	E140 31.712
41	*R. vernus*_5	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.038	E140 31.819
42	*R. vernus*_6	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.106	E140 32.122
43	*R. vernus*_7	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 54.106	E140 32.230
44	*R. vernus*_8	*R. vernus*	*Idaeobatus*	Mt. Mekun-nai, Rankoshi, Hokkaido	N42 53.882	E140 32.566
45	*R. crataegifolius*_1	*R. crataegifolius*	*Idaeobatus*	Mt. Apoi, Samani, Hokkaido	N42 06.562	E142 59.989
46	*R. crataegifolius*_2	*R. crataegifolius*	*Idaeobatus*	Shizunai Livestock Farm, Shizunai, Hokkaido	—	—
47	*R. crataegifolius*_3	*R. crataegifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 42.474	E141 34.148
48	*R. crataegifolius*_4	*R. crataegifolius*	*Idaeobatus*	Tomakomai Experimental Forest, Tomakomai, Hokkaido	N42 42.532	E141 34.159
49	*R. crataegifolius*_5	*R. crataegifolius*	*Idaeobatus*	Monbetsu, Hokkaido	N42 28.352	E142 05.486
50	*R. crataegifolius*_6	*R. crataegifolius*	*Idaeobatus*	Miyazaki, Miyazaki	N31 49.383	E131 24.357
51	*R. phoenicolasius*_1	*R. phoenicolasius*	*Idaeobatus*	Shizunai Livestock Farm, Shizunai, Hokkaido	—	—
52	*R. phoenicolasius*_2	*R. phoenicolasius*	*Idaeobatus*	Shizunai Livestock Farm, Shizunai, Hokkaido	—	—
53	*R. phoenicolasius*_3	*R. phoenicolasius*	*Idaeobatus*	Chichibu, Saitama	—	—
54	*R. phoenicolasius*_4	*R. phoenicolasius*	*Idaeobatus*	Nopporo Forest Park, Sapporo, Hokkaido	—	—
55	*R. pseudojaponicus*_1	*R. pseudojaponicus*	Unknown	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 45.881	E142 09.619
56	*R. pseudojaponicus*_2	*R. pseudojaponicus*	Unknown	Nakagawa Experimental Forest, Nakagawa, Hokkaido	N44 46.162	E142 09.913
57	*R. mesogaeus*	*R. mesogaeus*	*Idaeobatus*	Chichibu, Saitama	—	—
58	*R. buergeri*_1	*R. buergeri*	*Malachobatus*	Miyazaki, Miyazaki	N31 47.196	E131 22.397
59	*R. buergeri*_2	*R. buergeri*	*Malachobatus*	Miyazaki, Miyazaki	N31 46.339	E131 22.292
60	*R. sieboldii*	*R. sieboldii*	*Malachobatus*	Kagoshima, Kagoshima	N31 25.097	E130 29.272
61	*R. hirsutus*	*R. hirsutus*	*Idaeobatus*	Miyazaki, Miyazaki	N31 47.140	E131 22.385
62	*R. croceacanthus*	*R. croceacanthus*	*Idaeobatus*	Naha, Okinawa	N26 12.316	E127 43.184
63	*R. palmatus* var. *coptophyllus*	*R. palmatus* var. *coptophyllus*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
64	*R. palmatus* var. *palmatus*	*R. palmatus* var. *palmatus*	*Idaeobatus*	Miyazaki, Miyazaki	N31 49.383	E131 24.357
65	*R. trifidus*	*R. trifidus*	*Idaeobatus*	Tokushima, Tokushima	N34 05.162	E134 28.269
66	*R. spectabilis*	*R. spectabilis*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
67	*R. parviflorus*	*R. parviflorus*	*Anoplobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
	Cultivars and others					
68	Chilcotin	*R. idaeus* cv. ‘Chilcotin’	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
69	Heritage	*R. idaeus* cv. ‘Heritage’	*Idaeobatus*	Akita prefectural University	—	—
70	Indian Summer	*R. idaeus* cv. ‘Indian Summer’	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
71	Leon	*R. idaeus* cv. ‘Leon’	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
72	Nootka	*R. idaeus* cv. ‘Nootka’	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
73	Skeena	*R. idaeus* cv. ‘Skeena’	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
74	Black Satin	*R. fruticosus*	*Rubus*	Hokkaido University, Sapporo, Hokkaido	—	—
75	Thornfree	*R. fruticosus*	*Rubus*	Hokkaido University, Sapporo, Hokkaido	—	—
76	Boysenberry	*R. ursinus* × *R. idaeus*	*Rubus*	Hokkaido University, Sapporo, Hokkaido	—	—

	Hybrids					
77	ChId_1	*R. idaeus* cv. ‘Chilcotin’ × *R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
78	ChId_2	*R. idaeus* cv. ‘Chilcotin’ × *R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
79	SfId	*R. idaeus* cv. ‘Summer Festival’ × *R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
80	SkId	*R. idaeus* cv. ‘Skeena’ × *R. idaeus* var. *aculeatissimus*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—
81	ChSp	*R. idaeus* cv. ‘Chilcotin’ × *R. spectabilis*	*Idaeobatus*	Hokkaido University, Sapporo, Hokkaido	—	—

In latitude and longitude, ‘—’ indicates no data.

Table 2. Number of amplified fragments generated from AFLP analysis of 81 *Rubus* accessions.

	No. of accessions	Total no. of bands amplified	Total no. of polymorphic bands	Percentage of polymorphic bands (%)	Total no. of species-specific bands	Percentage of species-specific bands (%)
R. idaeus var. *aculeatissimus*	27	317	301	95.0	29	9.1
R. parvifolius	9	236	218	92.4	31	13.1
R. vernus	8	165	131	79.4	17	10.3
R. crataegifolius	6	202	187	92.6	24	11.9
R. phoenicolasius	4	145	98	67.6	9	6.2
R. pseudojaponicus	2	109	55	50.5	20	18.3
R. buergeri	2	94	39	41.5	7	7.4
R. trifidus	1	83	- [a)]	-	8	9.6
R. mesogaeus	1	74	-	-	2	2.7
R. hirsutus	1	80	-	-	4	5.0
R. croceacanthus	1	90	-	-	4	4.4
R. sieboldii	1	88	-	-	9	10.2
R. palmatus var. *coptophyllus*	1	72	-	-	6	8.3
R. palmatus var. *palmatus*	1	96	-	-	14	14.6
R. spectabilis	1	95	-	-	9	9.5
R. parviflorus	1	76	-	-	13	17.1
R. idaeus (hybrids)	4	151	96	63.6	5	3.3
R. idaeus (cultivars)	6	217	201	92.6	13	6.0
R. fruticosus	2	121	83	68.6	14	11.6
R. idaeus × *R. spectabilis*	1	87	-	-	1	1.1
R. ursinus × *R. idaeus*	1	92	-	-	4	4.3

a) '–' indicates no polymorphism, because only one accession was studied.