ORIGINAL ARTICLE

Phylogenetic inferences using nuclear ribosomal ITS and chloroplast sequences provide insights into the biogeographic origins, diversification timescales and trait evolution of *Rubus* in the Japanese Archipelago

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Abstract

This study aimed to reveal the evolutionary timescale and processes underlying the diversity of *Rubus* in the Japanese Archipelago. We conducted molecular phylogenetic analyses of most native species (35 species), along with previously published data from 116 foreign species, based on nuclear ribosomal internal transcribed spacer (ITS) and chloroplast DNA sequences. Most of the northern species of Japan, that is, *Rubus chamaemorus*, *R. pedatus*, *R. vernus*, *R. pseudojaponicus*, and *R. ikenoensis,* were found to belong to anciently diverged lineages; in particular, *R. ikenoensis* formed a unique lineage distinct from other species. The other species diverged into two evolutionary groups. One included subg. *Malachobatus*, *Chamaebatus,* and sects. *Pungentes*, *Idaeanthi*, and *Parvifolii* (subg. *Idaeobatus*), which was further divided into two clades in the chloroplast phylogenies. Although the phylogenetic structures within this group were unresolved, *R. sieboldii* has been proven to be recently derived. The second group represented a well-supported clade, comprising sects. *Microphylli*, *Corchorifolii*, *Peltati*, and *Rosifolii* (subg. *Idaeobatus*) and suggested early Miocene diversification of this Asian lineage associated with character specialization in vegetative reproduction and leaf shape. This clade was further resolved into lower clades primarily representing the sectional classifications, although the placement of the earliest diverged species, *R. suma-tranus*, *R. peltatus*, *R. corchorifolius*, and *R. chingii*, was incongruent among gene trees. At the lower taxonomic levels, *R. illecebrosus, R. grayanus,* and the thornless species of sect. *Microphylli* showed earlier divergence.

Keywords Ancestral trait · Chloroplast gene · Japanese Archipelago · Phylogeny · Ribosomal internal transcribed spacer · *Rubus*

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Introduction

Rubus is a large and taxonomically complex genus, comprising more than 740 described species worldwide (Hummer 2019). Focke's initial global classification (Focke 1910, 1911, 1914) of *Rubus* recognized 12 subgenera, although the vast majority belonged to the three largest subgenera: *Idaeobatus*, *Rubus* (formerly *Eubatus*), and *Malachobatus*. However, revision of the classic infrageneric classification has been recommended, as an increasing number of molecular phylogenetic studies have revealed the phylogenetic complexity of the genus (Alice and Campbell 1999; Carter et al. 2019; Eriksson et al. 2003; Howarth et al. 1997; Morden et al. 2003; Wang et al. 2016). Moreover, the taxonomic circumscription of some species is complicated because of their inclination toward agamospermy,

polyploidy, and frequent hybridization (Alice et al. 2001; Šarhanová et al. 2017; Sochor et al. 2015).

East Asia has been recognized as a diversity center for *Rubus*, where most species of subg. *Idaeobatus* and *Malachobatus* are distributed (Lu 1983, 1985; Lu and Boufford 2003). The Japanese Archipelago, located off the eastern coast of East Asia, is also characterized by a high diversity of *Rubus*. The current classification (Naruhashi 2001) recognizes 38 native species that are distributed across a variety of climate types, from cold-temperate to subtropical zones (Fig. 1, Table 1). The majority belong to subg. *Idaeobatus* (28 species) and *Malachobatus* (6 species), with some species found in the minor subgenera *Dalibarda* (*R. pedatus*), *Cylactis* (*R. pseudojaponicus*), *Chamaebatus* (*R. pectinellus*), and *Chamaemorus* (*R. chamaemorus*). Japanese *Rubus* has biogeographic affinities with continental East Asia; although it harbors 10 (> 25%) endemic species, all the other species have taxonomically identical species in the adjacent Asiatic mainland, with a few species, including *R. chamaemorus* and *R. idaeus*, extending to Europe and North America (Naruhashi and Satomi 1972).

Currently, the evolutionary relationships of *Rubus* in the Japanese Archipelago are poorly understood. Despite the abundance of taxonomic literature (Iwatsubo et al. 1996; Iwatsubo and Naruhashi 1991; Jinno 1958; Naruhashi 1971, 1968), the species circumscription and phylogenetic relationships have not been evaluated using molecular data, except for a few studies focusing on species from subg. *Idaeobatus* (Miyashita et al. 2015; Okada et al. 2020). However, advances in molecular phylogenetics have facilitated the accumulation of genetic information on *Rubus* worldwide, which could serve as a reference for understanding the evolution of Japanese *Rubus* in a biogeographic context (e.g., Alice and Campbell 1999; Wang et al. 2016). Moreover, analytical frameworks of divergence time and ancestral state estimation in previous studies (Alice and Campbell 1999; Carter et al. 2019; Okada et al. 2020) can be employed to better understand the evolutionary histories of Japanese *Rubus*.

This study aimed to reveal the evolutionary timescale and processes underlying the diversity of *Rubus* in Japan. To achieve this, we reconstructed the phylogeny of *Rubus* by sampling available native species and using previously published sequences, focusing on species monophyly, diversification, and phylogenetic origins. Moreover, the divergence time of major evolutionary/biogeographic events was estimated using molecular dating, and ancestral character states were reconstructed to infer their evolutionary history.

Materials and methods

Taxon sampling and DNA extraction

This study targeted 35 Japanese *Rubus* species, encompassing all of the native species listed in the Flora of Japan (Naruhashi 2001), except for *R. amamianus*, *R. hakonensis, and R. nesiotes*. We included *R. × utchinensis* (a putative hybrid between *R. nesiotes* and *R. sieboldii*) to obtain some genetic information regarding *R. nesiotes*, along with three other hybrid species, *R. × nakaii*, *R. × inaequiaculeatus* (formerly *R. × ribifolius*), and *R. × medius*. We incorporated multiple accessions (an average of 3.9 accessions per species) for each species in order to test for the monophyly of the species, except for *R. okinawensis*, *R. pedatus*, *R. lambertianus*, *R. nishimuranus*, *R. pseudojaponicus*, and *R. pseudoacer*, for which only one sample was available. We also included two foreign species (North American *R. spectabilis* and Korean *R. coreanus*) and four cultivars that were available. Samples of these species were acquired either through original collections or a DNA-barcoding project conducted by the Forestry and Forest Products Research Institute (Setsuko et al. unpublished; Table 1, Online Resource 1). For the samples from the original collections, we extracted genomic DNA using the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany), and voucher specimens were deposited in the herbarium at the FFPRI (Online Resource 1).

PCR amplification and sequencing

Sequences were determined for the nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast nucleotide sequences, including the *rbc*L, *mat*K, and three intergenic regions: *trn*L–*trn*F, *trn*S–*trn*G, and *rpl*20-*rps*12. The PCR reaction of all these regions was conducted in a 10 μL reaction mixture, containing template DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5–2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, and 0.25 U Ex *Taq* polymerase (TaKaRa, Otsu, Japan). A PerkinElmer 9700 thermocycler (PerkinElmer, Warrington, UK) was used with an initial denaturation step for 3 min at 94 °C, followed by 35–40 cycles of 1 min at 94 °C, 1 min at the annealing temperature (55 °C for each primer set), followed by 2 min at 72 °C, and a final extension step of 5 min at 72 °C. The amplicons were purified with EXOSAP-IT (Affymetrix, CA, USA) and subjected to direct sequencing using an ABI3130 Genetic Analyzer and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequences were read in both directions using forward and reverse amplifying primers for all the
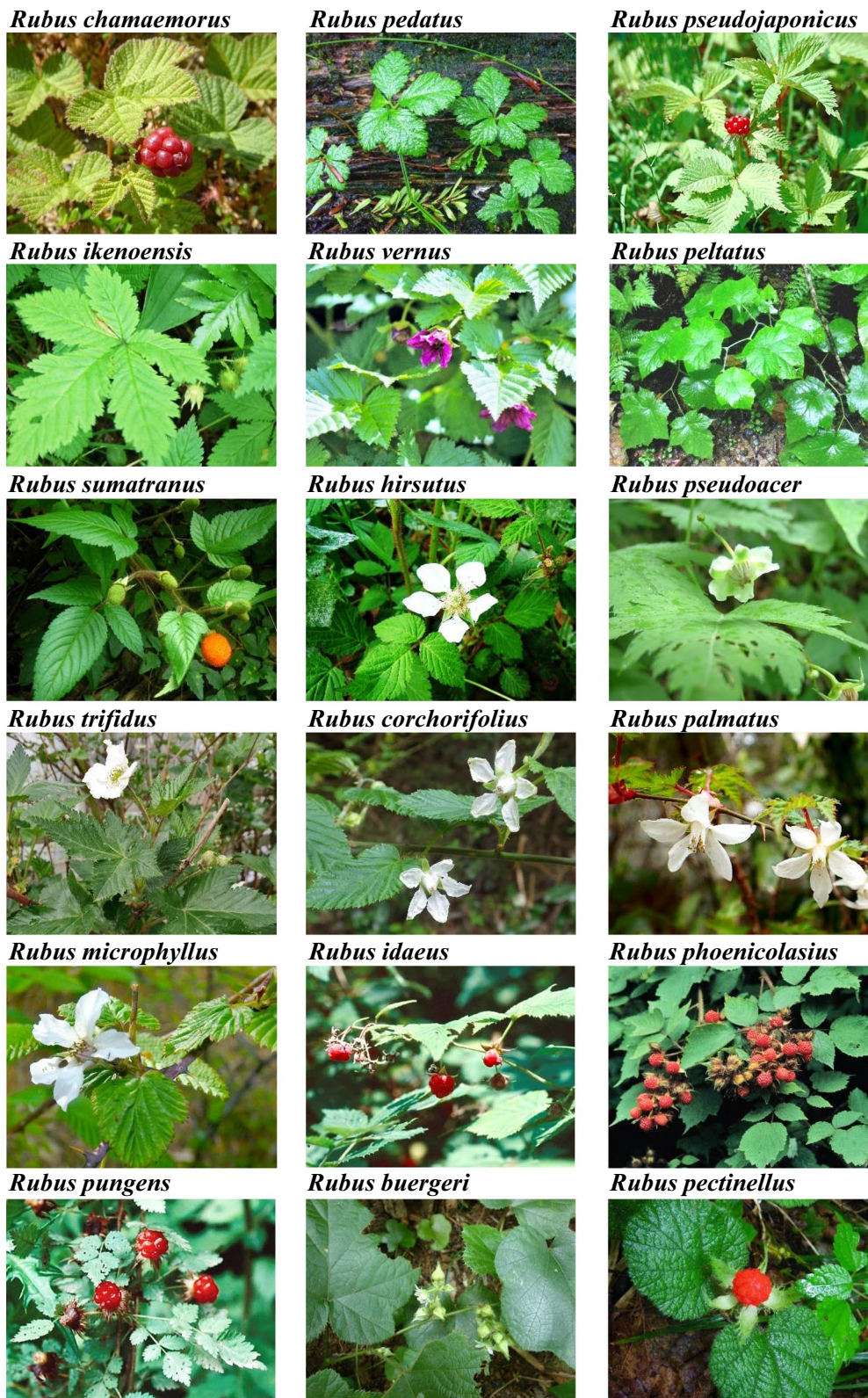
Rubus chamaemorus　　**Rubus pedatus**　　**Rubus pseudojaponicus**

Rubus ikenoensis　　**Rubus vernus**　　**Rubus peltatus**

Rubus sumatranus　　**Rubus hirsutus**　　**Rubus pseudoacer**

Rubus trifidus　　**Rubus corchorifolius**　　**Rubus palmatus**

Rubus microphyllus　　**Rubus idaeus**　　**Rubus phoenicolasius**

Rubus pungens　　**Rubus buergeri**　　**Rubus pectinellus**

Fig. 1 Representative *Rubus* species in the Japanese Archipelago

Table 1 List of Japanese native *Rubus* species and hybrids included in this study

Subgenus	Section	Species	*N*	Chromosome	Distribution
Chamaemorus		*Rubus chamaemorus* L.	2	2*n* = 56	North America, Europe, Amur, Primorye, Sakhalin, Okhotsk, Kamchatka, the Komander Islands, the Aleutian, the Kuriles, Japan, North Korea and NE. China
Dalibarda[a]		*Rubus pedatus* Sm.	1	2*n* = 14	N. Sakhalin, the Kuriles, Japan and North America
Cylactis	*Saxatiles*	*Rubus pseudojaponicus* Koidz.	1	2*n* = 14	The Kuriles and Japan
Chamaebatus		*Rubus pectinellus* Maxim.	2	2*n* = 42	Japan, China, Taiwan and the Philippines
Malachobatus	*Sozostyli*	*Rubus nesiotes* Focke	NC	2*n* = 28	Endemic
	Moluccani	*Rubus amamianus* Hatus. & Ohwi	NC	ND	Endemic
		Rubus sieboldii Blume	8	2*n* = 28	Endemic
		Rubus buergeri Miq.	3	2*n* = 42, 56	Japan, Taiwan, South Korea and China
		Rubus hakonensis Franch. & Sav.	NC	2*n* = 42, 56	Japan and China
		Rubus lambertianus Ser.	1	2*n* = 28	Japan, Taiwan and China
Idaeobatus	*Peltati*	*Rubus peltatus* Maxim.	3	2*n* = 14	Japan and China
	Corchorifolii	*Rubus corchorifolius* L.f.	5	2*n* = 14	Japan, Taiwan, Korea, China and Vietnam
		Rubus chingii Hu	1	2*n* = 14	Japan and China
		Rubus kisoensis Nakai	2	2*n* = 14	Endemic
		Rubus palmatus Thunb.	16	2*n* = 14, 21	Japan and Korea
		Rubus grayanus Maxim.	4	2*n* = 14	Japan and China(?)
		Rubus ribisoideus Matsum.	3	2*n* = 14	Japan and South Korea
	Microphylli	*Rubus trifidus* Thunb.	5	2*n* = 14	Japan and Korea
		Rubus boninensis Koidz.	2	ND	Endemic
		Rubus microphyllus L.f.	7	2*n* = 14	Japan and China
		Rubus subcrataegifolius (H.Lév. & Vaniot) H.Lév.	4	2*n* = 14	Japan, Taiwan, Korea and China
		Rubus crataegifolius Bunge	18	2*n* = 14	E. Siberia, Amur, Primorye, Japan, Korea, and China
		Rubus pseudoacer Makino	1	2*n* = 14	Endemic
	Spectabiles	*Rubus vernus* Focke	3	2*n* = 14	Endemic
	Idaeanthi	*Rubus idaeus* L.	6	2*n* = 14, 21, 28, 35, 42	Europe, the Caucasus, Siberia, Amur, Primorye, Sakhalin, Okhotsk, Kamchatka, the Kuriles, Japan, Korea, China and North America
	Rosifolii	*Rubus illecebrosus* Focke	7	2*n* = 14	Endemic
		Rubus minusculus H.Lév. & Vaniot	3	2*n* = 14	Japan and China
		Rubus sumatranus Miq.	1	2*n* = 14	Japan, Taiwan, South Korea, W. & C. China, Indochina, Thailand, Sumatra, Assam and the E. Himalaya
		Rubus hirsutus Thunb.	9	2*n* = 14	Japan, Korea and China
		Rubus okinawensis Koidz.	1	2*n* = 14	Endemic
		Rubus croceacanthus H.Lév. & Vaniot	5	2*n* = 14	Japan, Taiwan and Korea
	Nishimurani	*Rubus nishimuranus* Koidz.	2	2*n* = 28	Endemic
	Parvifolii	*Rubus mesogaeus* Focke	3	2*n* = 14	The Kuriles (kunashiri), Japan, Taiwan, E. & C. China and the Himalaya (Nepal to Bhutan)
		Rubus phoenicolasius Maxim.	8	2*n* = 14	Japan, Korea and China
		Rubus parvifolius L.	4	2*n* = 14, 21	The Kuriles (Kunashiri), Japan, Taiwan, Korea and China
		Rubus yoshinoi Koidz.	3	2*n* = 14, 21	Japan and China

Table 1 (continued)

Subgenus	Section	Species	N	Chromosome	Distribution
	Pungentes	*Rubus pungens* Cambess.	3	$2n=14$	Japan, Taiwan, Korea, China and Himalaya
	Ikenoenses	*Rubus ikenoensis* H.Lév. & Vaniot	1	$2n=14$	Japan and Korea
Hybrids		*R.* × *nakaii* Tsuyama	1	NI	Endemic
		R. × *medius* Kuntze	1	$2n=14$	Endemic
		R. × *inaequiaculeatus* Kuntze	1	$2n=14$	Endemic
		R. × *utchinensis* Koidz.	1	$2n=14$	Endemic

NC not collected in this study, *NI* not investigated

[a]Although *R. pedatus* is classified as subg. *Cylactis* by Naruhashi (2001), here we accepted the classification by Focke (1910), which places it in subg. *Dalibarda*

regions, and internal sequencing primers designed for this study for the ITS region (Online Resource 2), and were assembled using CodonCode Aligner v7.1.2 (CodonCode Corporation, MA, USA) to generate consensus sequences. Heterozygous peaks at a single position were found in the ITS region of 72 individuals and were coded using IUPAC ambiguity codes. Heterozygous indels found in a few species (*R. vernus* and *R. ikenoensis*) were encoded as Ns. All newly generated sequences were registered in the DNA Data Bank of Japan (Online Resource 1), which has been partially reported in other studies (Okada et al. 2000; Setsuko et al. unpublished).

Phylogenetic reconstruction

In addition, sequences of corresponding genes were obtained from 132 *Rubus* species (including 116 foreign species) from previous studies (Alice and Campbell 1999; Eriksson et al. 1998; Wang et al. 2016) through GenBank (Online Resource 3). Sequences were aligned using the MUSCLE algorithm and manually refined using SeaView v4.3.3 (Gouy et al. 2010), and heterozygous sequences in ITS regions were imported into DnaSP v5 (Librado and Rozas 2009) to generate phased haplotypes. Therefore, the chloroplast dataset comprised the sequences of three regions (*rbc*L, *trn*S–*trn*G and *rpl*20–*rps*12) from 303 individuals of 107 *Rubus* species, and the ITS dataset contained 467 phased sequences from 371 individuals of 157 *Rubus* species. Moreover, we prepared another dataset sourced only from our original data (162 individuals), covering all five chloroplast regions, to construct a more-resolved chloroplast phylogenetic tree, which is referred to as "partial phylogeny." Sequence alignments for these data sets are available in Dryad (doi https://doi.org/10.5061/dryad.bzkh189bp). For each dataset, the optimal substitution model of nucleotide substitution for each locus was selected from 88 models using the corrected Akaike

information criterion in jModelTest v0.1.1 (Posada 2008). The data matrices for each chloroplast gene were concatenated for phylogenetic reconstruction.

Phylogenetic reconstruction was performed using the maximum likelihood (ML) and Bayesian inference (BI) methods. The ML method was conducted with RAxML v8.2.10 (Stamatakis 2014) using raxmlGUI 2 (Edler et al. 2021), applying a $GTR + I + \Gamma$ substitution model. One thousand bootstrap replicates were performed to estimate the confidence values of the nodes. The BI method was executed using MrBayes v3.2.1 (Ronquist et al. 2012). Two independent runs containing four Markov chain Monte Carlo (MCMC) chains (one hot and three cold) were run over one million generations under the best-fitting substitution models (Online Resource 4), until the average standard deviation of split frequencies fell below 0.01, saving trees every 500 generations and discarding the first 10% as burn-ins. For both analyses, partitions of the concatenated chloroplast sequences were unlinked, allowing the model parameters for each partition to be estimated independently.

Subsequently, to visualize the evolutionary history of Japanese *Rubus* and to identify the origin of the hybrid species, we conducted a consensus network analysis using the program SplitsTree4 (Huson and Bryant 2006). Here, we combined one chloroplast and two ITS trees, all pruned to retain target species using the *keep.tip* function implemented in the package *ape* v5.4 (Paradis et al. 2004) in *R* (R Core Team 2020), and each ITS tree contained a different set of phased haplotypes from heterozygous sequences. Here, we targeted 34 non-hybrid Japanese species, five putatively hybrid taxa (*R.* × *nakaii*, *R.* × *medius*, *R.* × *utchinensis*, *R.* × *inaequiaculeatus*, and *R. nishimuranus*). We also targeted 12 foreign Chinese species, which represented the lineages in which Japanese *Rubus* species were not involved, along with two outgroups published by Wang et al. (2016). *Rubus lambertianus* was represented by a Chinese accession because of suspected hybridization in the Japanese sample.
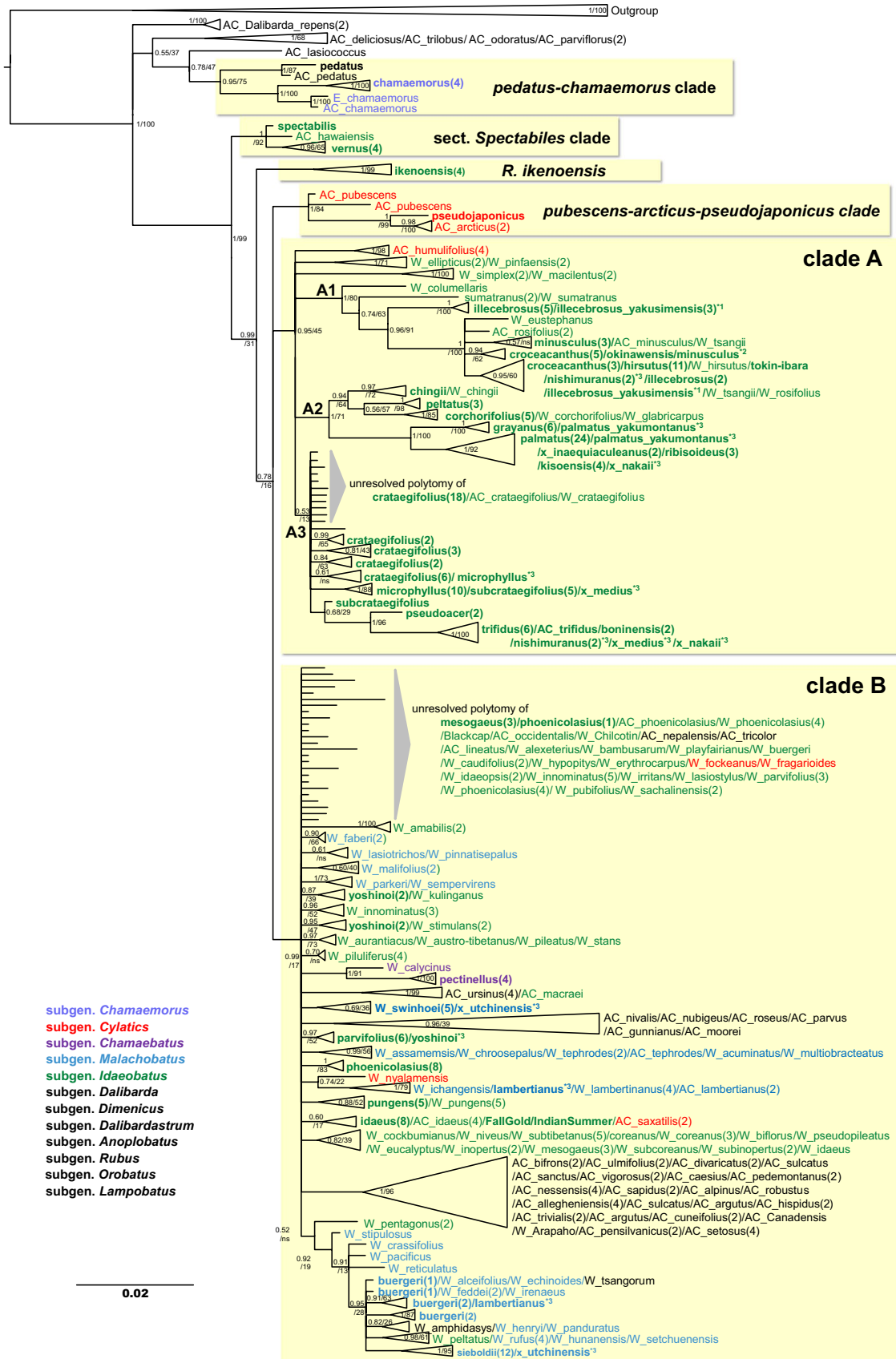
1/100 Outgroup

AC_Dalibarda_repens(2)

1/100

1/68 AC_deliciosus/AC_trilobus/ AC_odoratus/AC_parviflorus(2)

0.55/37 AC_lasiococcus

0.78/47

1/87 pedatus

0.95/75 AC_pedatus

1/100 chamaemorus(4)

1/100 E_chamaemorus

1/100 AC_chamaemorus

***pedatus-chamaemorus* clade**

1 spectabilis

AC_hawaiensis

0.96/65 vernus(4)

sect. *Spectabiles* clade

1/99 ikenoensis(4)

R. ikenoensis

AC_pubescens

1/84 AC_pubescens

1 pseudojaponicus

0.98

/99 /100 AC_arcticus(2)

***pubescens-arcticus-pseudojaponicus* clade**

1/98 AC_humulifolius(4)

1/71 W_ellipticus(2)/W_pinfaensis(2)

1/100 W_simplex(2)/W_macilentus(2)

clade A

A1 W_columellaris

1/80 sumatranus(2)/W_sumatranus

0.74/63 1 illecebrosus(5)/illecebrosus_yakusimensis(3)*1

/100

0.96/91 W_eustephanus

AC_rosifolius(2)

1 1/57/ns minusculus(3)/AC_minusculus/W_tsangii

/100 0.94 croceacanthus(5)/okinawensis/minusculus*2

/62 croceacanthus(3)/hirsutus(11)/W_hirsutus/tokin-ibara

0.95/60 /nishimuranus(2)*3 /illecebrosus(2)

/illecebrosus_yakusimensis*1 /W_tsangii/W_rosifolius

A2 0.94 0.97 chingii/W_chingii

/64 /72 1 peltatus(3)

0.56/57 /98

1/71 1/85 corchorifolius(5)/W_corchorifolius/W_glabricarpus

1/100 grayanus(6)/palmatus_yakumontanus*3

palmatus(24)/palmatus_yakumontanus*3

1/92 /x_inaequiaculeanus(2)/ribisoideus(3)

/kisoensis(4)/x_nakaii*3

unresolved polytomy of

crataegifolius(18)/AC_crataegifolius/W_crataegifolius

A3 0.53

/13

0.99 crataegifolius(2)

/65

0.84 0.81/43 crataegifolius(3)

/63 crataegifolius(2)

0.61

/ns crataegifolius(6)/ microphyllus*3

1/85 microphyllus(10)/subcrataegifolius(5)/x_medius*3

subcrataegifolius

0.68/29 pseudoacer(2)

1/96 trifidus(6)/AC_trifidus/boninensis(2)

1/100 /nishimuranus(2)*3 /x_medius*3 /x_nakaii*3

0.78

/16

0.99

/31

0.95/45

1/99

1/100

clade B

unresolved polytomy of

mesogaeus(3)/phoenicolasius(1)/AC_phoenicolasius/W_phoenicolasius(4)

/Blackcap/AC_occidentalis/W_Chilcotin/AC_nepalensis/AC_tricolor

/AC_lineatus/W_alexeterius/W_bambusarum/W_playfairianus/W_buergeri

/W_caudifolius(2)/W_hypopitys/W_erythrocarpus/W_fockeanus/W_fragarioides

/W_idaeopsis(2)/W_innominatus(5)/W_irritans/W_lasiostylus/W_parvifolius(3)

/W_phoenicolasius(4)/ W_pubifolius/W_sachalinensis(2)

1/100 W_amabilis(2)

0.90

/68 W_faberi(2)

0.61

/ns W_lasiotrichos/W_pinnatisepalus

1/60/40 W_malifolius(2)

1/73 W_parkeri/W_sempervirens

0.87 yoshinoi(2)/W_kulinganus

/39

0.96 W_innominatus(3)

/52

0.95 yoshinoi(2)/W_stimulans(2)

0/47

0.97 W_aurantiacus/W_austro-tibetanus/W_pileatus/W_stans

/73

0.99 0.70 W_piluliferus(4)

/17 /ns W_calycinus

1/91 1 pectinellus(4)

/100

1/99 AC_ursinus(4)/AC_macraei

0.69/36 W_swinhoei(5)/x_utchinensis*3

0.96/39 AC_nivalis/AC_nubigeus/AC_roseus/AC_parvus

0.97 /AC_gunnianus/AC_moorei

/52 parvifolius(6)/yoshinoi*3

0.99/56 W_assamemsis/W_chroosepalus/W_tephrodes(2)/AC_tephrodes/W_acuminatus/W_multiobracteatus

/83 phoenicolasius(8)

0.74/22 W_nyalamensis

1/79 W_ichangensis/lambertianus*3/W_lambertinanus(4)/AC_lambertianus(2)

0.88/52 pungens(5)/W_pungens(5)

0.60

/17 idaeus(8)/AC_idaeus(4)/FallGold/IndianSummer/AC_saxatilis(2)

W_cockbumianus/W_niveus/W_subtibetanus(5)/coreanus/W_coreanus(3)/W_biflorus/W_pseudopileatus

0.82/39 /W_eucalyptus/W_inopertus(2)/W_mesogaeus(3)/W_subcoreanus/W_subinopertus(2)/W_idaeus

AC_bifrons(2)/AC_ulmifolius(2)/AC_divaricatus(2)/AC_sulcatus

/AC_sanctus/AC_vigorosus(2)/AC_caesius/AC_pedemontanus(2)

/AC_nessensis(4)/AC_sapidus(2)/AC_alpinus/AC_robustus

1/96 /AC_alleghenicnsis(4)/AC_sulcatus/AC_argutus/AC_hispidus(2)

/AC_trivialis(2)/AC_argutus/AC_cuneifolius(2)/AC_Canadensis

/W_Arapaho/AC_pensilvanicus(2)/AC_setosus(4)

0.52 W_pentagonus(2)

/ns W_stipulosus

0.92 0.91 W_crassifolius

/19 /13 W_pacificus

W_reticulatus

buergeri(1)/W_alceifolius/W_echinoides/W_tsangorum

0.91/63 buergeri(1)/W_feddei(2)/W_ireneaus

0.95 buergeri(2)/lambertianus*3

/28 1/87 buergeri(2)

0.82/26 W_amphidasys/W_henryi/W_panduratus

0.98/61 W_peltatus/W_rufus(4)/W_hunanensis/W_setchuenensis

1/95 sieboldii(12)/x_utchinensis*3

subgen. *Chamaemorus*
subgen. *Cylatics*
subgen. *Chamaebatus*
subgen. *Malachobatus*
subgen. *Idaeobatus*
subgen. *Dalibarda*
subgen. *Dimenicus*
subgen. *Dalibardastrum*
subgen. *Anoplobatus*
subgen. *Rubus*
subgen. *Orobatus*
subgen. *Lampobatus*

0.02

◄**Fig. 2** Summarized phylogenetic tree estimated with Bayesian inference (BI) and maximum likelihood (ML) methods, based on the internal transcribed spacer data. Sequences obtained from this study are shown in bold, and those from Wang et al. (2016), Alice and Campbell (1999), and Eriksson et al. (1998) are indicated by the prefix letters "W_," "AC_," and "E_," respectively. The numbers of conspecific sequences in the same (collapsed) branches are shown in parentheses. The numbers at each node indicate the posterior probabilities/bootstrap values. An original version of the ITS BI and ML phylogenies is given in Online Resource 4. [*1]This includes one of the phased haplotypes of a putative hybrid of distant lineages (illecebrosus_yakusimensis_YK637). [*2]This *R. minusculus* sample lacks glandular hairs and is identified as *R. minusculus* var. *hongnoensis* (Naruhashi, pers. comm.). [*3]All of these sequences were either one of the phased haplotypes with the other haplotypes belonging to distant lineages and were suspected to be hybrids

Estimation of divergence time

We estimated the divergence times of the representative nodes based on ITS and chloroplast sequences. We applied a Bayesian divergence-time estimation method implemented in BEAST 2.5.3 (Drummond and Rambaut 2007) using a calibrated Yule model of speciation and an HKY model of nucleotide substitution. Posterior mean values and 95% highest posterior density (HPD) intervals of divergence time were estimated with 10 million MCMC generations, sampling every 5000, and discarding the first 10% as burn-ins. For these analyses, we employed non-hybrid native species and 12 Chinese species, as stated above, and the ITS-based analysis further involved one Asian and 14 American species from this study (*R. spectabilis*) and Alice and Campbell (1999), so that major lineages of *Rubus* and intercontinental vicariant events (e.g., *R. pseudojaponicus* and *R. arcticus*) could be covered. We constrained monophyly of the clades that were supported (> 0.8) in the BI phylogenies. To select the best-fit molecular clock model, we compared the performance of different models, including the strict clock, uncorrelated lognormal relaxed clock (UCLN), uncorrelated exponential relaxed clock, and random local clock models, based on Bayes factor analysis. Marginal likelihoods of each model were evaluated using nested sampling with 10 particles, a subchain length of 10,000, and an epsilon value of 1×10^{-6}. The oldest fossil records of *Rubus* have been found in the lower Eocene deposits (54–48 million years ago [Mya]) in North America (DeVore and Pigg 2007). Previous molecular dating based on a plastid gene estimated the origin of this genus as far back as 56.93–65.66 Mya (Zhang et al. 2017), which is congruent with the fossil record. Carter et al. (2019) therefore conducted molecular dating of *Rubus*, constraining the age of the root node to 56.93–65.66 Mya. In this study, we followed the method of Carter et al. (2019) and constrained the node age with a log-normal distribution with parameters $M = 4.112$ Mya and $S = 0.043$ Mya, which generated a 95% confidence interval of ca. 56.9–65.5 Mya.

Phylogenetic signal and ancestral state reconstruction

Furthermore, we examined the trait evolution of Japanese *Rubus* using ancestral trait reconstruction and phylogenetic signal tests. Five morphological characteristics were examined: petal color (white–pink, light purplish red, dark purplish red, or petalless flowers), fruit color (red–purple, yellow–orange, purplish black, yellowish white), leaf shape (simple, ternate, pedate-digitate, or pinnately compound), stem prickle (present or absent), and vegetative reproduction (by subterranean runners or cane apices). Here, we targeted each accession from all the examined species for which trait information was available. The trait data, except for vegetative reproduction, were collected from Flora of Japan (Naruhashi 2001), Flora of China, Flora of North America (www.eFloras.org), and Howarth et al. (1997). Vegetative reproductive traits were only obtained for Japanese species and two foreign species (*R. saxatilis* and *R. arcticus*) based on previous life-history studies (Ryynänen 1972; Suzuki 1990, 1997, personal communications; Eriksson and Bremer 1993). The BI phylogenies were pruned to retain the focal species, and the phylogenetic signals were tested based on Blomberg's *K* (Blomberg et al. 2003) and Pagel's *λ* (Pagel 1999), using the package *phytools* v 0.7–47 in *R* (Revell 2012). Ancestral states for each node were inferred using likelihood under equal rates and symmetric models, employing the *rerootingMethod* function in *phytools*.

Results

Phylogeny of Japanese *Rubus*

The ITS phylogenies (Fig. 2; Online Resource 5) showed a basal divergence of a well-supported clade comprising *R. pedatus* (subg. *Dalibarda*) and *R. chamaemorus* (subg. *Chamaemorus*). This clade was sister to New World's *R. lasiococcus*, and was related to subg. *Anoplobatus*.

The core clade of *Rubus* was split into five lineages. The first diverging clade comprised all species of the sect. *Spectabiles* (*R. vernus, R. hawaiensis,* and *R. spectabilis*). The second was monotypic for *R. ikenoensis*. *R. pseudojaponicus* (subg. *Cylactis*) formed a third clade with *R. pubescens* and *R. arcticus*.

The fourth lineage was a large clade (clade A), which included sects. *Microphylli*, *Corchorifolii*, *Rosifolii*, and *Peltati* of subg. *Idaeobatus* within three subclades (A1–A3). Three other lineages of Asian species (*humulifolius*, *ellipticus/pinfaensis*, and *macilentus/simplex*) also joined the unresolved polytomy of this clade. Subclade A1 contained the species of sect. *Rosifolii* (*R. illecebrosus, R. minusculus, R. sumatranus, R. hirsutus, R. okinawensis,* and *R.*

croceacanthus), along with Chinese *R. columellaris* (sect. *Leucanthi*), as the most basally branching species. This clade also included one of the phased haplotypes of *R. nishimuranus* of the monotypic section *Nishimurani*, which is suspected to be of hybrid origin between sects. *Rosifolii* and *Microphylli*. *R. sumatranus* was the earliest diverged in this section, and the other species were split into two divergent lineages (*R. illecebrosus* vs. the rest). Subclade A2 was formed by sects. *Corchorifolii* (*R. corchorifolius, R. chingii, R. kisoensis, R. palmatus, R. grayanus,* and *R. ribisoideus*) and *Peltati* (*R. peltatus*). This subclade was subdivided into two divergent lower clades, with the first comprising *R. chingii*, *R. corchorifolius*, *R. peltatus*, and Chinese *R. glabricarpus*. There was no apparent phylogenetic structure within the second, except that southerly distributed *R. grayanus* formed a divergent lineage to the rest (*R. palmatus*, *R. kisoensis*, and *R. ribisoideus*) together with a haplotype of *R. palmatus* var. *yakumontanus*. Poorly supported subclade A3 comprised sect. *Microphylli* (*R. trifidus, R. boninensis, R. microphyllus, R. subcrataegifolius, R. crataegifolius* and *R. pseudoacer*) and *R. nishimuranus* as stated above. Within A3, three unarmed species, *R. pseudoacer, R. trifidus,* and *R. boninensis*, formed a distinct subclade, where *R. pseudoacer* was sister to the others. Phased haplotypes of *R. × medius* both occurred in this subclade, with one in the *trifidus-boninensis* lineage and the other in the *microphyllus-subcrataegifolius* lineage. The ITS phylogenies showed species monophyly only for three species, *R. chingii*, *R. peltatus,* and *R. pseudoacer*, in clade A.

The fifth lineage (clade B) included all Japanese species from subg. *Chamaebatus*, *Malachobatu*s, and three section-sof subg. *Idaeobatus*, that is, sect. *Idaeanthi* (*R. idaeus*), *Parvifolii* (*R. mesogaeus, R. phoenicolasius, R. parvifolius* and *R. yoshinoi*), and *Pungentes* (*R. pungens*). This clade also included the accessions of foreign species of subg. *Dalibardastrum, Dimenicus, Lampobatus, Orobatus, Rubus,* and *Idaeobatus*. This clade is characterized by an unresolved basal polytomy without a clear phylogenetic structure in the BI tree, although the ML tree shows weak evidence of the divergence of subg. *Malachobatus* and *Chamaebatus*, along with some other species (Online Resource 5). Species monophyly was only evident in *R. pungens* and *R. pectinellus* in this clade. Monophyly of *R. sieboldii* was disrupted by a phased haplotype of *R. × utchinensis* (*R. sieboldii × R. nesiotes*), with the other haplotype being included in the *swinhoei* lineage. Similarly, *R. lambertianus* was not monophyletic only because a haplotype of a Japanese accession was related to *R. buergeri*. The ITS phylogenies also displayed close relationships between *R. pectinellus* and Chinese *R. calycinus*, between *R. idaeus* and *R. saxatilis* (widespread in Asia and Europe), and between a few samples of *R. yoshinoi* and Chinese *R. kulinganus*, which were regarded as identical by Naruhashi (2010). A haplotype of *R. yoshinoi* (K59) was closely related to *R. parvifolius* and could be regarded as a hybrid *R. × pseudoyoshinoi* (Naruhashi 2001).

Chloroplast phylogeny of Japanese *Rubus*

The chloroplast phylogenies (Fig. 3; Online Resoures 6 and 7) showed a basal divergence of *the pedatus-chamaemorus* clade. They also demonstrated the early divergence of the sect. *Spectabiles* clade, *R. pseudojaponicus*, and *R. ikenoensis.* These were resolved as a single clade in the partial phylogenies, whereas *R. pseudojaponicus* and *R. ikenoensis* belonged to separate lineages at the root of clade A (stated below) in the complete phylogenies.

The rest of the Japanese *Rubus* were resolved in three major clades, namely A, B1, and B2, for comparison with the ITS phylogenies. Clade A contained sects. *Peltati*, *Microphylli*, *Rosifolii,* and *Corchorifolii* and was rooted by ancient Asian lineages (*R. ellipticus*, *R. pinfaensis*, *R. columellaris*, *R. macilentus*, and *R. simplex*). *R. peltatus* was the earliest branching in this clade, and the other species were split into three subclades (A1–A3). Subclade A3 comprises sect. *Microphylli* and yielded patterns similar to those of the ITS phylogeny; *R. pseudoacer, R. trifidus,* and *R. boninensis* formed a distinct clade within which *R. pseudoacer* first diverged. Species monophyly was supported for *R. pseudoacer* and *R. boninensis*, whereas *R. nishimuranus* and *R. × medius* both joined the *trifidus* lineage. In contrast, the sister subclade was a mixture of sects. *Rosifolii* and *Corchorifolii*. In the complete phylogeny, it was split into two distant lower clades corresponding to sects. *Rosifolii* (A2) and *Corchorifolii* (A1), except for *R. sumatranus* (sect. *Rosifolii*) included in A1. In the partial phylogeny, *R. sumatranus*, *R. chingii,* and *R. corchorifolius* formed the first branching internal clade, and the rest were placed in subclades A1 and A2. Within A1, species monophyly was supported for *R. kisoensis* (locally endemic) and *R. grayanus*, whereas *R. palmatus*, *R. ribisoideus*, and their hybrid *R. × inaequiaculeatus* made an unresolved group. Within A2, *R. illecebrosus* was monophyletic, and the rest were split into two groups: one group included *R. croceacanthus*, *R. minusculus*, and *R. okinawensis*, and the other was formed by *R. hirsutus* and two Chinese species (*R. rosifolius* and *R. eustephanus*).

Clade B in the ITS phylogenies was divided into two separate clades (B1 and B2) in the chloroplast phylogenies. Subclade B1 included various subgenera (*Malachobatus, Chamaebatus, Dalibardastrum, Cylactis* and *Idaeobatus*), in which the Japanese species splitted into three lower clades: First, *R. pectinellus* formed a clade with *R. calycinus,* the other Asian *Chamaebatus* species. Second, *R. pungens* formed a clade with *R. leucodermis* and *R. idaeus* cultivars. Finally, all the Japanese *Malachobatus* species belonged to the *Malachobatus/Dalibardastrum* clade (Wang et al. 2016), in which *R. sieboldii* formed
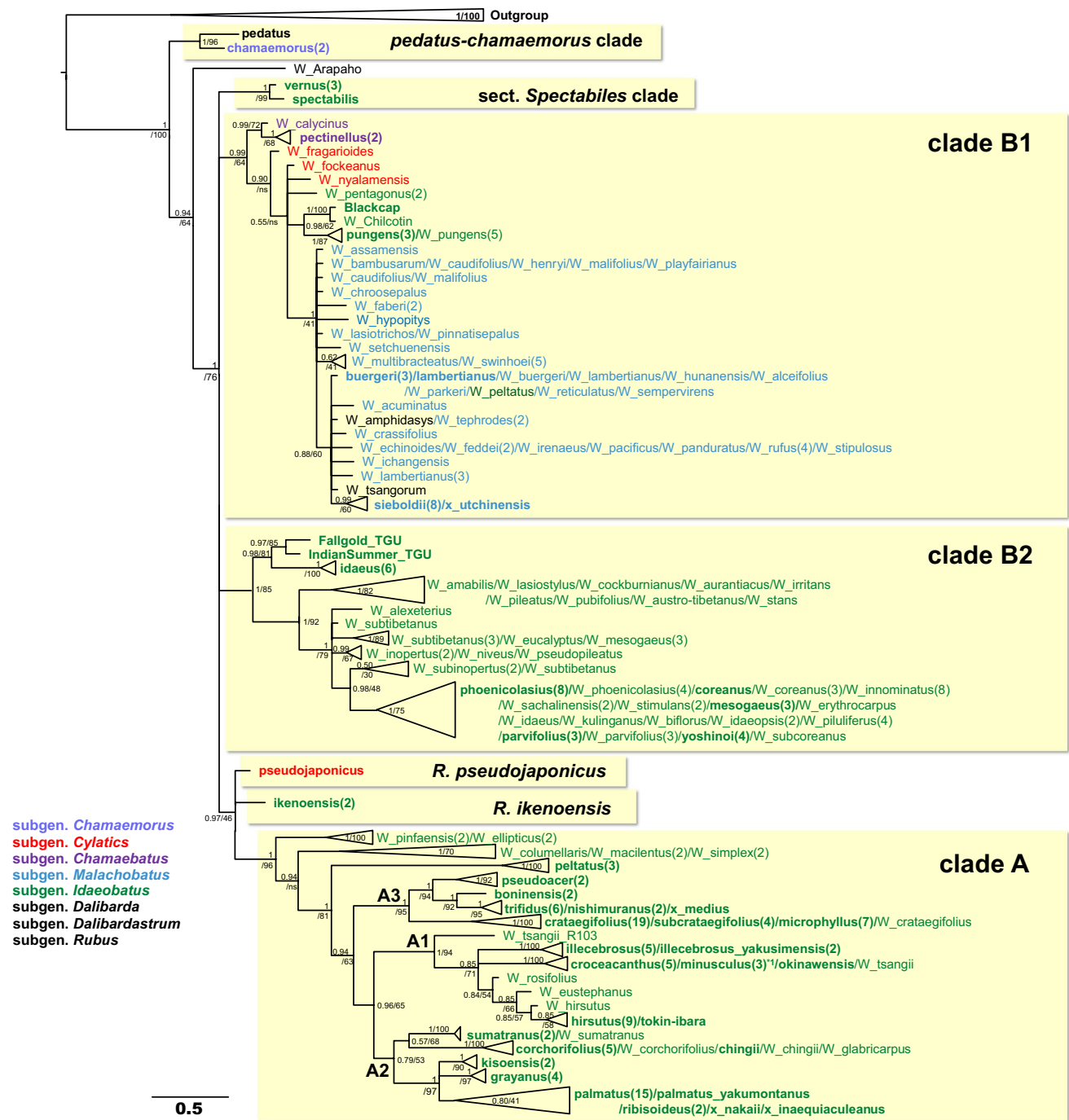
Fig. 3 Summarized phylogenetic tree estimated with BI and ML methods, based on the chloroplast DNA sequences obtained from this study (shown in bold) and from Wang et al. (2016) (with the prefix "W_"). The numbers of conspecific sequences in the same (collapsed) branches are shown in parentheses. The numbers at each node refer to the posterior probabilities/bootstrap values. An original version of the chloroplast BI and ML phylogenies is given in Online Resources 5

a lower clade with *R. × utchinensis* (*R. nesiotes × R. sieboldii*). Subclade B2 included various sections of subg. *Idaeobatus* and was further split into two lower clades, one of which was exclusively occupied by *R. idaeus* (sect. *Idaeanthi*) and cultivars. Japanese species of sect. *Parvifolii* were all included in the other.

Consensus network analysis

A consensus network combining chloroplast and ITS gene trees is shown in Fig. 4. It represents common phylogenetic structures; the *chamaemorus-pedatus* clade was the most anciently diverged, followed by the early divergence of *R. vernus*, *R. pseudojaponicus*, and *R. ikenoensis*. The rest of the Japanese *Rubus* species evolved into two major groups. The first includes Japanese species with a shoot-tip propagation system, starting with sects. *Idaeanthi and Parvifolii* of subg. *Idaeobatus*, leading to sect. *Pungentes* and subg. *Chamaebatus* and *Malachobatus*. The other is represented by species shooting from subterranean runners, leading to sects. *Corchorifolii*, *Microphylli*, *Rosifolii*, and *Peltati* of subg. *Idaeobatus*. Moreover, it represented the hybrid nature of species, such as *R.×nakaii*, *R.×medius*, and *R. nishimuranus*, which were all placed in intermediate positions between *R. trifidus* and the branches leading to the

other putative parental species, that is, *R. ribisoideus*, *R. microphyllus*, and *R. hirsutus*. *R.×utchinensis* was placed between *R. swinhoei* and *R. sieboldii*. However, the hybrid status of *R.×inaequiaculeatus* (*R. palmatus×R. ribisoideus*) was not confirmed because of close relationships between the parental species.

Estimation of divergence time

In both the ITS and chloroplast data, the UCLN was chosen by Bayes factor analysis (Online Resource 8). The estimated divergence times at representative nodes based on the UCLN model are presented in Fig. 5 and Table 2. The grown age, or the first divergence of *Rubus* including *chamaemorus-pedatus* lineage, was around 32.96–34.11 Mya (the late Eocene). The stem ages of the sect. *Spectabiles* (including *R. vernus*), *R. ikenoensis*, and *R. pseudojaponicus* were all estimated to be around 19.38–25.22 Mya (the early Miocene). The
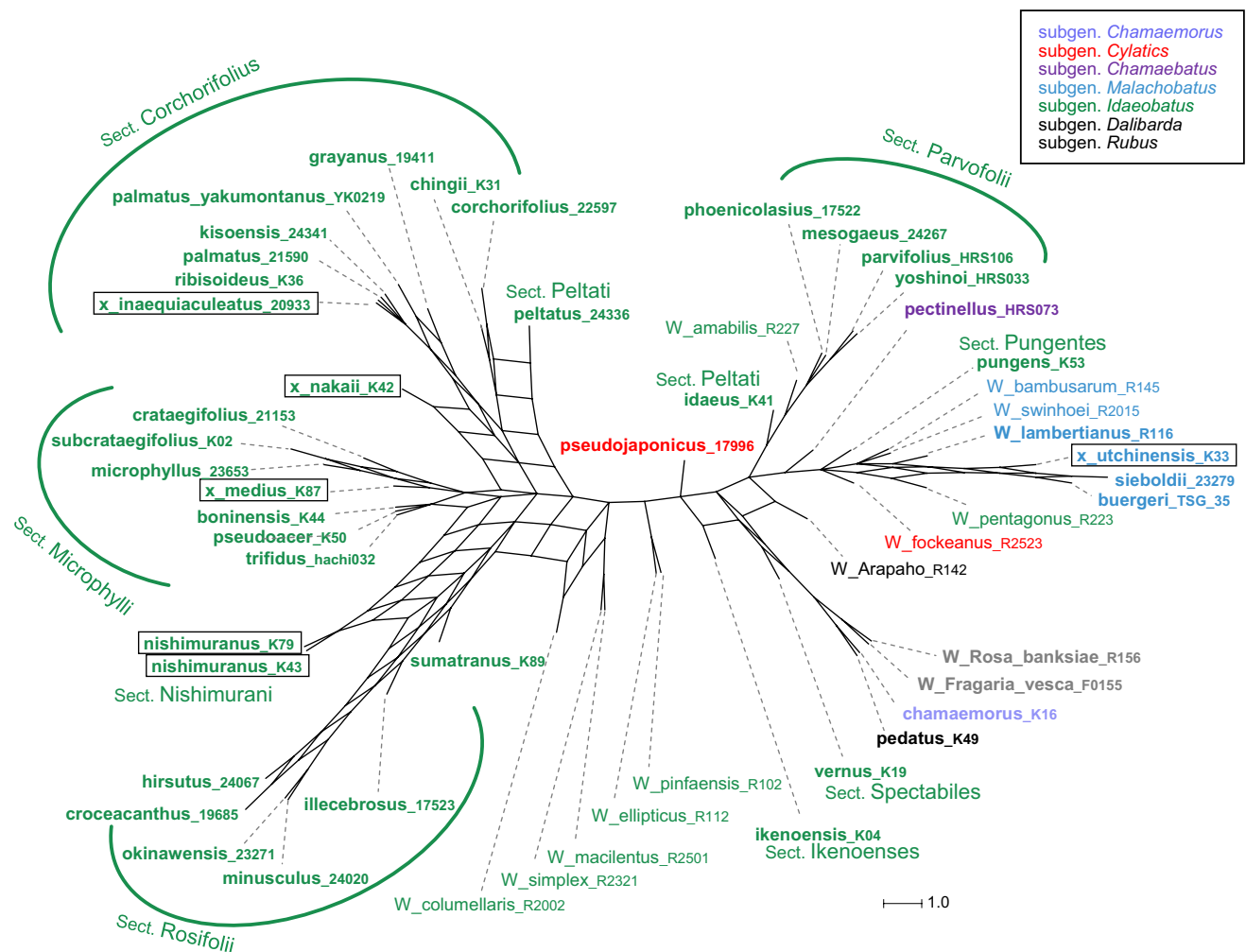
Fig. 4 Consensus network combining a chloroplast and two ITS phylogenetic trees, which are pruned to retain representative *Rubus* species along with putative hybrid species. Hybrid taxa are in boxes. Section names are indicated only for Japanese species of subg. *Idaeobatus*

stem age of the sects. *Corchorifolii*, *Rosifolii*, *Microphylli*, and *Peltati* also estimated around 17.53–21.41 Mya in the early Miocene. The crown age of the sect. *Corchorifolii* (along with *R. peltatus* or *R. sumatranus*) was around 1275–15.11 Mya, with the stem age of *R. grayanus* being approximately 6.1–7.67 Mya. Within sect. *Microphylli*, the stem and the crown ages of the thornless lineage (i.e., *R. pseudojaponicus*, *R. trifidus* and *R. boninensis*) were around 12.09–12.68 Mya and 6.85–8.29 Mya, respectively. The stem age of *R. sieboldii* was around 3.31–4.62 Mya.

Phylogenetic signal and ancestral state reconstruction

Both Blomberg's *K* and Pagel's λ suggested significant phylogenetic signals (at $p = 0.01$) of leaf compoundness, stem thorniness, and vegetative reproduction in both phylogenies. Phylogentic signal was also significant for fruit color in the ITS phylogeny, but in the chloroplast phylogeny, this was only the case for Pagel's λ (Table 3). Ancestral state reconstructions (Fig. 6, Online Resource 9) suggested that non-reddish (white, yellow/orange, and black) fruit colors are descendant traits in *Rubus*. The ancestral state of leaf shape was incongruent between genes, where common ancestors of the core *Rubus* were prone to have ternate and digitate-prone leaves in the ITS and chloroplast phylogenies, respectively. However, they commonly demonstrated a trend toward specialized descendants in clade A with either simple or pinnately compound leaves. The unarmed lineages in the core *Rubus* were suggested to have developed multiple times from a common armed ancestor of the core *Rubus*. The results also indicated an ancestral polymorphism of vegetative reproductive traits and character fixation in the descendant lineages of subg. *Idaeobatus*.

Discussion

Ancestral positions of *Rubus pedatus* and *R. chamaemorus*

The ancestral positions of *R. pedatus* and *R. chamaemorus* have already been shown in many previous studies (Alice and Campbell 1999; Carter et al. 2019; Michael 2006), and their sister relationships have been found frequently (e.g., Michael 2006; Carter et al. 2019). *Rubus chamaemorus* has many different characteristics from *R. pedatus*, such as leaf shape (simple vs. digitate), sex expression (unisexual vs. bisexual), and ploidy level (octaploid vs. diploid), although both are herbaceous perennial plants with red, pulpy fruits and a northern geographic distribution. *Rubus chamaemorus* may have originated from a common ancestor with *R. pedatus* by polyploidization, which drove the morphological

changes. Michael (2006) suggested the possibility of allopolyploid origin of *R. chamaemorus* based on its two divergent alleles in the nuclear *GBSSI* gene, implying that further genomic studies are required to understand its speciation. The estimated divergence of this lineage in the early Oligocene (33–34 Mya) could be supported by the occurrence of fossils in Eurasia during the Oligocene (Bozukov et al. 2008; Pavlyutkin et al. 2011), although it was much more ancient than originally reported (Carter et al. 2019).

Early divergence of *Rubus vernus*, *R. pseudojaponicus*, and *R. ikenoensis*

The major clade comprising the rest of *Rubus*, hereafter referred to as the "core *Rubus*" clade, was identical to 'clade A' in Alice and Campbell (1999) and 'groups 3–8' in Carter et al. (2019). Three northern species in Japan within the core *Rubus* clade, *R. vernus*, *R. pseudojaponicus*, and *R. ikenoensis*, were assigned to early diverged lineages, which deviated during the end of the Oligocene and the early Miocene (Fig. 5 and Table 2).

Early divergence and monophyly of the sect. *Spectabiles* clade confirmed the primitive status of this section, which has distinctive thornless (or mostly thornless) bodies, trifoliate (sometimes trilobed) leaves, and pinkish-purple petals. The sister polytomy in this clade illustrates their migration history. Independent derived mutations (data not shown) in *R. vernus* and *R. hawaiensis* suggest they immigrated independently from their ancestral area (*R. spectabilis*). *Rubus vernus* most probably migrated via the Bering land bridge (Wen et al. 2016) during the late Miocene.

Rubus pseudojaponicus, R. arcticus, and *R. pubescens* of subg. *Cylactis* share the traits of being perennial herbs with unarmed stems and three- or five-lobed leaves, and that they are distributed across the Arctic or subarctic regions of the Northern Hemisphere; *R. pseudojaponicus* is limited to the Japanese and Southern Kurile Islands, *R. arcticus* sensu lato has a circumpolar distribution, and *R. pubescens* is restricted to North America. The sister relationship between *R. pseudojaponicus* and *R. arcticus*, which is allied with *R. pubescens*, may suggest a biogeographic origin of this group in the North American continent and migration into Asia.

In contrast, *R. ikenoensis* formed a unique lineage in which no related species were identified. This species resembles *R. pseudojaponicus* but is distinguished by its biennial stems, bristly stem hairs, petioles, peduncles, and nodding flowers without petals (Naruhashi 2001). The taxonomic treatment of *R. ikenoensis* remains controversial. The species was first classified as a synonym of *R. defensus* in sect. *Saxatiles* (subg. *Cylactis*) and then transferred to sect. *Pungentes*. Currently, it is in the monotypic section *Ikenoenses* (subg. *Idaeobatus*). Our results support the argument on the uniqueness of this species by Naruhashi (2001), although
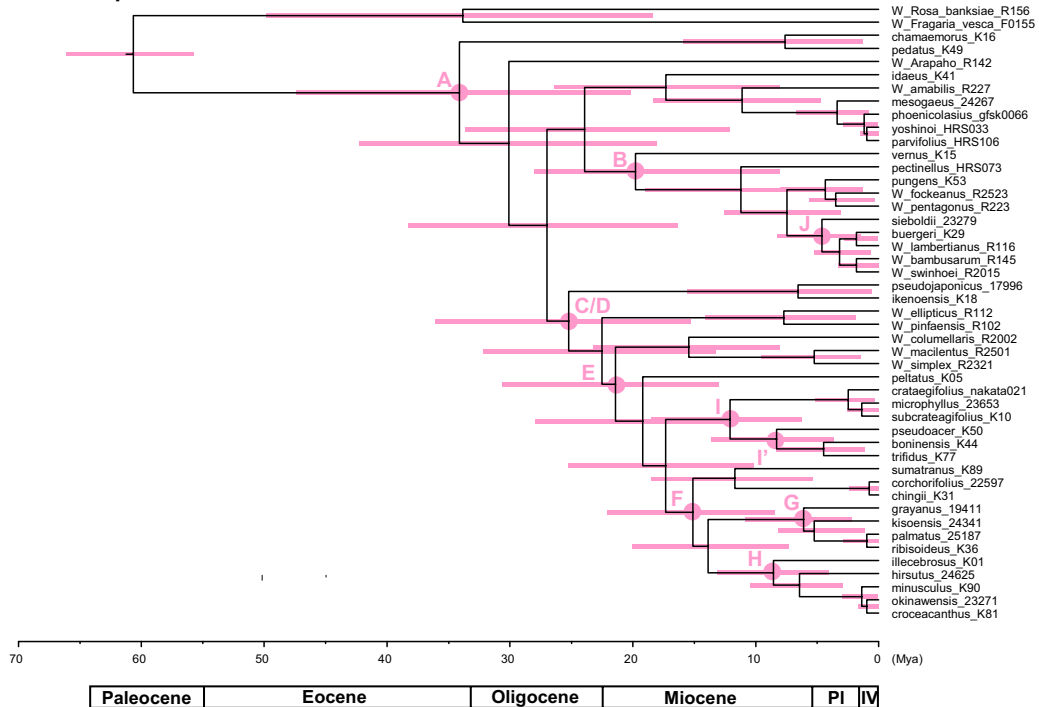
a ITS
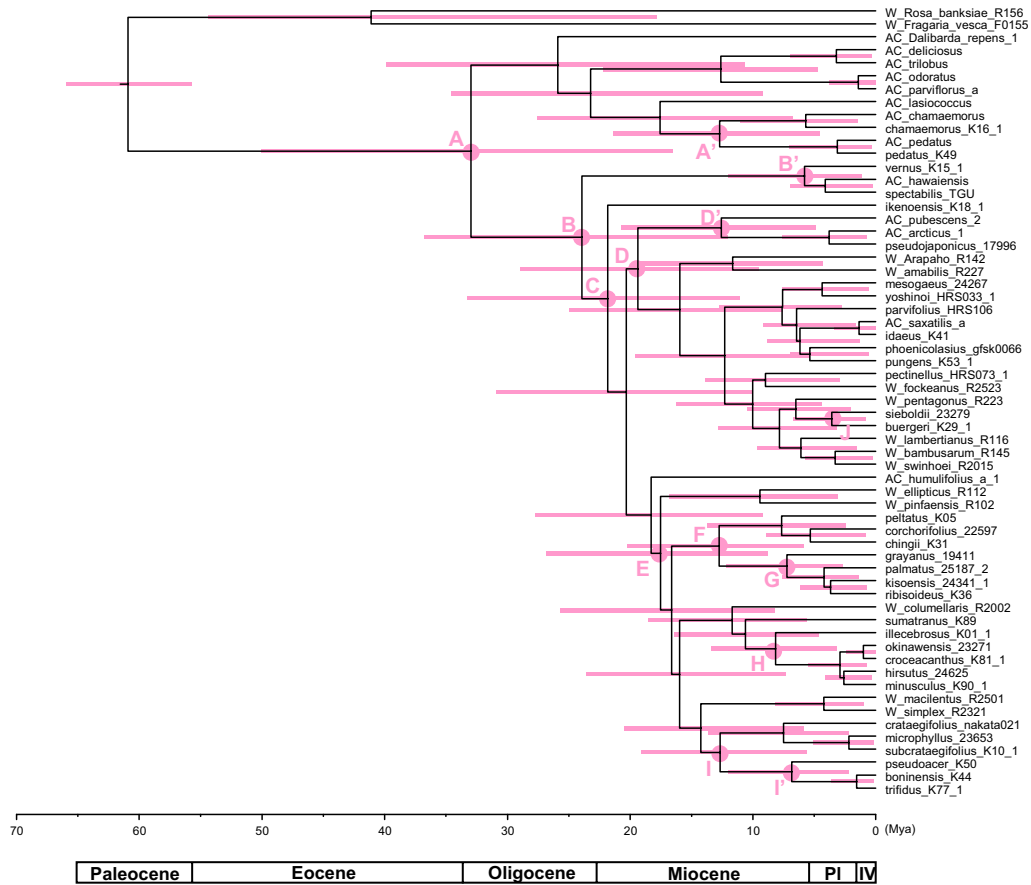
b Chloroplast

◄**Fig. 5** Bayesian divergence time estimates of Japanese *Rubus* species in millions of years ago (Mya) based on **a** ITS and **b** chloroplast sequences. Pinkish bars at each node indicate 95% highest posterior density (HPD) intervals. The numerical average estimates of divergence time (with 95% HPD) for the representative node (the lettered nodes) are given in Table 2. Abbreviation of the periods: Pl—Pliocene, IV—Quaternary. Mean divergence time and 95% HPD for lettered nodes (A–J) were given in Table 2

the taxonomic placement within subg. *Idaeobatus* should be revised.

Phylogenetic relationships between major *Rubus* lineages

The ITS and chloroplast phylogenies displayed similar phylogenetic structures. Major incongruence was the unresolved clade B in the ITS phylogenies, which split into two distinct clades (clades B1 and B2) in the chloroplast phylogenies (Figs. 2 and 3), and in the recent studies based on nuclear genomic information (Carter et al. 2019; Wang et al. 2016). This is likely due to incomplete lineage sorting in the ITS region, because similar clades with B1 and B2 in the chloroplast phylogenies were also recovered in recent studies based on nuclear genomic information (Carter et al. 2019; Wang et al. 2016), and in "partial ITS phylogeny" constructed based on our original sequences (not shown). Nevertheless, as visualized in the consensus network (Fig. 4), we can conclude that Japanese *Rubus* evolved into two major groups (subdivided into three major groups), which overlap with those (groups 4, 5, and 7) displayed by Carter et al. (2019).

The monophyletic lineage (clade A) containing the four sections, *Microphylli*, *Rosifolii*, *Corchorifolii*, and *Peltati* (subg. *Idaeobatus*), has been detected in many previous studies (Alice and Campbell 1999; Morden et al. 2003; Okada et al. 2020; Wang et al. 2016; Yang et al. 2012), however, this has not received sufficient attention. This mostly East Asian lineage exclusively has subterranean runners as a form of vegetative reproduction (Nesme 1985; Suzuki 1990, 1987). In contrast, species of clade B (B1 and B2), except for *R. idaeus,* propagated by rooting at the apices of their stems (Naruhashi 2001). As stated later, these two contrasting strategies in vegetative reproduction in *Rubus* may have existed as an ancestral polymorphism and specialized in these descendant lineages (Fig. 6). Presently, the divergence of this lineage can be traced back to the early Miocene (18–21 Mya), which might be an era of *Rubus* diversification (Fig. 5, Table 2). This is consistent with the European fossil records, which suggested that *Rubus* had diverse and common elements by the late Miocene (DeVore and Pigg 2007; Huang et al. 2015).

Tip-rooting lineages of *Idaeobatus* and subg. *Malachobatus/Chamaebatus*

Although the chloroplast phylogenies recover two clades (B1 and B2), which mostly divide the polyploidy subg. *Malachobatus* and *Chamaebatus* from the others, both clades are still taxonomically complex, covering several subgenera and polyploidy levels (Fig. 3). In particular, the placement of *R. pungens* (sect. *Pungentes*) near the subg. *Malachobatus/Chamaebatus* was not congruent with the nuclear phylogenies in previous studies (Carter et al. 2019; Morden et al. 2003; Wang et al. 2016), nor supported by any morphological traits, and was therefore considered to be the result of ancient introgression following hybridization or incomplete lineage sorting.

The sister relationships between the two Asian *Chamaebatus* members were clearly resolved in both phylogenies. *Rubus pectinellus* is a hexaploidy, perennial creeping plant with semi-woody prickly stems and simple leaves and is only distinguished from *R. calycinus* by its deeply digitately divided stipules. However, New World species of subg. *Chamaebatus* belonged to a different clade, suggesting the need for taxonomic revisions.

The only robust inference for species-level divergence was the derived monophyletic lineage of *R. sieboldii* within subg. *Malachobatus*. This warm-temperate species evolved recently from a species complex of *Malachobatus*, probably in the Pleistocene (Fig. 5, Table 2). A cytogenetic study by Iwatsubo and Naruhashi (1993) considered this species to be an allotetraploid of hybrid origin. Although the hybrid nature of this species has not been proven, the recent divergence may have been triggered by such an event.

Some insights into the species circumscription of Japanese *Malachobatus* were also obtained. For example, although rare, locally endemic *R. nesiotes* was not available, the phylogenetic position of *R.×utchinensis* (*R. nesiotes*×*R. sieboldii*) implies a close affinity between *R. nesiotes* and *R. swinhoei*. Hybridization, perhaps with *R. buergeri*, was suspected for the Japanese accession of *R. lambertianus*; therefore, we employed a Chinese accession for some analyses. The hybrid may have arisen by chance in a botanical garden or may have occurred in its small populations in Japan. Of the diverse taxa of subg. *Idaeobatus* within this group, it is noteworthy that the chloroplast phylogenies revealed a distinctive divergence of *R. idaeus* and its allies, which exceptionally have subterranean runners in this clade (Nesme 1985). Moreover, the taxonomically controversial lineage comprising *R. idaeus* and *R. saxatilis* (subg. *Cylactis*) in the ITS phylogenies (Alice and Campbell 1999) might reflect

Table 2 Mean divergence time estimates (Mya) and the 95% highest posterior density (HPD) intervals of the representative nodes (lettered nodes in Fig. 5) for Japanese *Rubus* species and lineages based on ITS and chloroplast sequences

Nodes	Phylogenetic events	Mean [95% HPD] estimates of divergent time (Mya)	
		ITS	Chloroplast
A	Divergence of "core *Rubus*"	32.96 [16.550.04]	34.11 [20.1847.42]
A'	Vicariance of *R. chamaemorus*	5.71 [1.4510.98]	–
B	Divergence of sect. *Spectabiles* (*R. vernus*)	23.94 [12.0436.75]	19.79 [8.0226.43]
B'	Vicariance of sect. *Spectabiles*	5.81 [1.111.98]	–
C	Divergence of *R. ikenoensis*	21.84 [11.0333.24]	25.22 [15.2436.04]
D	Divergence of *R. pseudojaponicus* and its allies	19.38 [9.5628.97]	
D'	Vicariance of *R. pedatus* and its allies	3.12 [0.357.64]	–
E	Divergence of underground-branching sections of subgen. *Idaeobatus*	17.53 [8.8426.89]	21.41 [12.9730.59]
F	Divergence of primitive *Corchorifolii* species (+ *R. peltatus* or *R. sumatranus*)	12.75 [5.8920.28]	15.11 [8.4122.08]
G	Divergence of *R. grayanus*	7.67 [2.712.15]	6.1 [2.1610.86]
I	Divergece of thornless *Microphylii* lineage	12.68 [5.5919.12]	12.09 [6.2118.46]
I'	Divergence between *R. trifidus* group and *R. pseudoacer*	6.85 [2.1811.99]	8.29 [3.6713.62]
H	Divergence of *R. illecebrosus*	8.16 [3.213.43]	8.54 [4.0313.12]
J	Divergence of *R. sieboldii*	3.31 [0.816.69]	4.62 [1.488.24]

Table 3 The results of phylogenetic signal tests (Blomberg's *K* and Pagel's *λ*) for five morphological traits against ITS and chloroplast phylogenies

Traits	ITS				Chloroplast			
	K	*p*	*λ*	*p*	*K*	*p*	*λ*	*p*
Petal color	0.132	0.135	0.547	0.028	0.055	0.97	0.000	1
Fruit color	**0.300**	**0.004**	**0.625**	**< 0.001**	0.107	0.112	**0.436**	**< 0.001**
Leaf shape	**0.259**	**0.001**	**0.804**	**< 0.001**	**0.316**	**0.001**	**0.945**	**< 0.001**
Stem prickles	**0.501**	**0.001**	**0.606**	**< 0.001**	**0.405**	**0.001**	**0.405**	**< 0.001**
Vegetative reproduction	**0.764**	**0.001**	**1.000**	**< 0.001**	**0.870**	**0.001**	**1.000**	**< 0.001**

Values suggesting significant phylogenetic signals were shown in bold

speciation events through hybridization and polyploidization. In contrast, the poor phylogenetic resolution of sect. *Parvifolii* in all phylogenies might be attributed to interspecific hybridization, slow evolutionary rates, polyphyletic species, or taxonomic complications.

Divergence of subg. *Idaeobatus* shooting from subterranean runners

Sects. *Peltati*, *Microphylli*, *Rosifolii*, *Corchorifolii*, and *Nishimurani* of subg. *Idaeobatus* commonly have subterranean runners and white flowers in a cluster or corymbs and exclusively have specialized in either simple or pinnately compound leaves (Naruhashi 2001; Wang et al. 2016). They are a major component (20 species) of the Japanese *Rubus*, representing more than half of the species. However, their phylogenetic positions have not been fully considered in previous studies.

The long branch lengths among the inner subclades suggested distinct divergence among the lineages. They mostly corresponded with the sectional classification, except for the incongruent positions of four early-diverging species, including *R. peltatus* (*Peltati*), *R. sumatranus* (*Rosifolii*), *R. corchorifolius*, and *R. chingii* (*Corchorifolii*). It could simply be viewed as the placement of *R. peltatus* in the ITS phylogeny, and *R. sumatranus* in the chloroplast phylogeny, close to these two *Corchorifolii* species. The relatedness among *R. peltatus*, *R. corchorifolius*, *and R. chingii* in the ITS phylogenies may reflect some evolutionary processes through shared characteristics, such as solitary nodding flowers, pubescent ovaries, and simple leaves. The position of *R. sumatranus* nested within the *Corchorifolii* clade in the chloroplast phylogenies is a bit more counterintuitive, but it may be supported by the presence of yellow to orange fruit color. All these species are distributed in western Japan and south-eastern Asia (to subtropical Asia for *R. sumatranus*), which are known to be home to the relics of temperate plant flora (Tang et al. 2018), which may have promoted interspecific hybridization or organelle capture at an early stage of diversification.
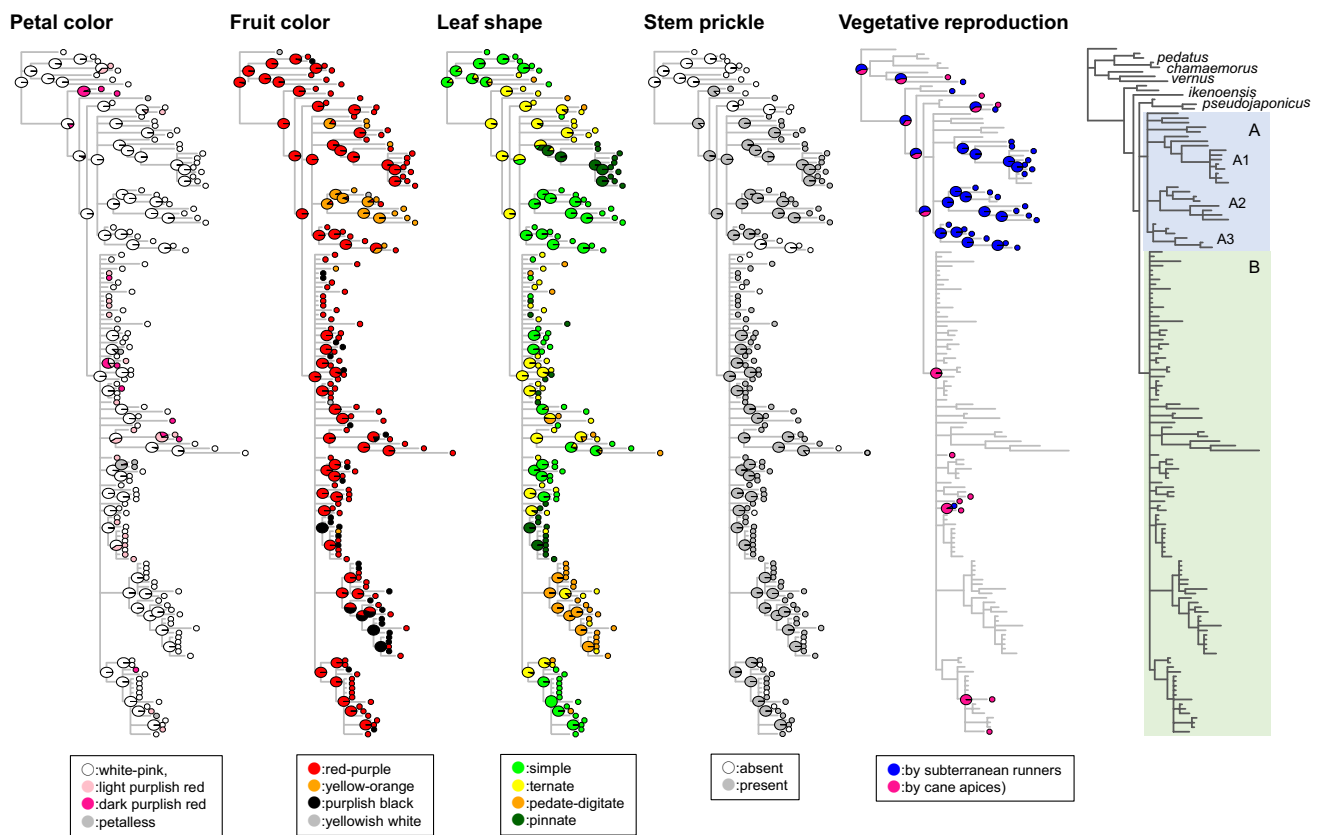
Fig. 6 The results of ancestral trait reconstruction for five morphological traits mapped onto the pruned ITS phylogenies. The BI trees were pruned to retain one accession from the species for which the trait data were obtained. Pie charts on each node show the probability of each character state

Sect. *Microphylli* is a newly proposed taxon (Naruhashi 2001) for species previously classified in sect. *Corchorifolii*, based on the inflorescence of erect corymbs, and the monophyletic clade demonstrated in this study justifies this taxonomic treatment. Within this clade, the distinct clade of *R. pseudoacer*, *R. boninensis*, and *R. trifidus* notably suggests the ecological divergence of the thornless lineage that may have taken place during the mid-Miocene (Fig. 5, Table 2). Of these, warm-temperate species (*R. trifidus* and *R. boninensis*) have specialized properties; they are both unarmed evergreen plants that have glabrous receptacles, and they only appear different in the glabrous calyx and orange fruit of *R. trifidus*. *R. trifidus* is subendemic, found only in Japan and Korea, and is abundant in the volcanic zones of the Izu Peninsula and Izu Islands, whereas *R. boninensis* is only reported from the Iwo Jima Islands, which are the southern Izu Islands. *R. boninensis* was considered to be a species derived from *R. trifidus*, as indicated by their progenitor-derivative relationship in the ITS phylogenies. In contrast, *R. pseudoacer* is an endemic species confined to the cool-temperate subalpine zones of central and western Japan, and it has morphological characteristics similar to those of the rest of the *Microphylli* species, such as deciduous leaves

and pubescent receptacles. The divergence between these ecologically differentiated lineages potentially occurred 7–8 Mya in the late Miocene (Fig. 5, Table 2), which corresponded with the formation of the volcanic zone in Japan (Maruyama et al. 1997). The rest of the *Microphylli* species showed no apparent phylogenetic structure, although the ITS partial phylogeny showed an evolutionary trend from *R. crataegifolius* to *R. microphyllus*.

Within the well-supported clade of derived *Rosifolii* species, phylogenetic distinctiveness of *R. illecebrosus* and its varieties was in accordance with their unique traits, including annual and glabrous stems, in contrast to other perennial shrubs.

The monotypic section *Peltati* (*R. peltatus*) with unique morphological features, including large peltate leaves and stalked torus (receptacles), was supported in our chloroplast phylogenies; however, these findings are inconsistent with those of Wang et al. (2016), who placed *R. peltatus* within the *Malachobatus* clade. We attribute this to possible misidentification with other species of subg. *Malachobatus* with peltate leaves.

The monophyletic clade comprising *R. grayanus*, *R. palmatus*, *R. kisoensis*, *R. ribisoideus*, and their allies suggested

a recent radiation of *Corchorifolii* species. Within this derived lineage, the most southerly distributed species, *R. grayanus,* was the earliest diverged, presumably during the late Miocene (6–8 Mya). However, Mimura et al. (2014) proposed a much more recent divergence (ca. 1 Mya) of *R. grayanus* based on multiple nuclear loci. Although this incongruence may have arisen due to methodological differences, post-divergence hybridization and introgression may have resulted in heterogeneous genealogies during speciation, as suggested by our data (*R. palmatus* var. *yakumontanus*) and Mimura et al. (2014). Another distinct clade was formed for *R. kisoensis* in the chloroplast phylogenies. This is a locally endemic species in Central Honshu and has been treated as a variety of *R. palmatus*. The monotypic clade in the chloroplast sequences might justify its independent taxonomic status, although further studies are needed to reveal the species status.

Trait evolution in Japanese *Rubus*

Although ancestral trait reconstructions based on limited taxon sampling might be biased, it could at least provide some insights, especially on trait evolution in subg. *Idaeobatus* (Fig. 5, Online Resource 9). As suggested by Alice and Campbell (1999), thorns evolved in the core *Rubus* and were lost multiple times. Similarly, fruit colors other than red–purple were all derived traits that occurred independently.

The expected ancestral trait of ternate leaves or equivocal state of the core *Rubus* was concordant with that of Alice and Campbell (1999), as exemplified by the occurrence of various types of leaves in the earliest-diverging lineages of *Rubus*. Moreover, the independent treatment of compound leaf types in this study may propose a novel perspective, that is, the evolution from labile leaf traits with simple to ternate/digitate leaves to specialized simple or pinnately compound leaves.

The specialization of traits was also inferred for vegetative reproduction. Ancestral polymorphism was represented by the co-occurrence of both traits within the *chamaemorus-pedatus* clade, the *pseudojaponicus-arcticus* clade, and the *idaeus-saxatilis* clade, whereas descendant lineages of subg. *Idaeobatus* (clade A, B1, and B2) propagated almost exclusively by cane apices or by subterranean runners. Unfortunately, it was difficult to reveal the process of character fixation, as vegetative reproductive traits are only described in limited species and not available for the relevant foreign species from any literature.

Biogeography of Japanese *Rubus*

Distribution of *Rubus* in Japan is well understood and is considered to be primarily controlled by climatic conditions (Naruhashi and Satomi 1972), with some apparent phylogenetic constraints, such as those seen in subg. *Malachobatus*, which is confined to warm-temperate to subtropical areas. However, the results of our study provide important insights into the biogeography of Japanese *Rubus*.

For example, six species, *R. chamaemorus*, *R. pedatus, R. pseudojaponicus*, *R. vernus*, *R. ikenoensis*, *and R. idaeus,* are northerly distributed species confined to subalpine zones in Japan. Except for *R. idaeus*, they all belonged to the earliest diverged lineages of *Rubus*. With the exception of *R. ikenoensis*, these species or their allies occur widely in the subarctic regions and high mountains of the Northern Hemisphere. The estimated divergence times of Japanese and American lineages (3–6 Mya, Fig. 5 and Table 2) agreed well with the ages of intercontinental vicariance reported in angiosperms, suggesting that the divergence of these northern species may have been instigated by the formation of the Bering Strait (Wen et al. 2016). The phylogenetic independence of *R. ikenoensis* is quite interesting from a biogeographic viewpoint. Northern Honshu, which *R. ikenoensis* is confined to, also harbors a few endemic monotypic genera, such as *Pteridophyllum* and *Ranzania* (Hotta 1974). *Rubus ikenoensis* may be regarded as an old endemic species.

Another biogeographic feature is the high species diversity of a lineage of subg. *Idaeobatus* (sects. *Microphylli*, *Rosifolii*, *Corchorifolii*, and *Peltati*). All of them belong to an Asian taxon (Focke 1910), and their monophyly represents an Asian radiation of the lineage propagating by subterranean runner during the early Miocene.

Interestingly, two major lineages in sect. *Microphylli* showed contrasting biogeographic patterns: the thornless lineage comprised of subendemic (*R. trifidus*) or endemic species (*R. pseudoacer* and *R. boninensis*), whereas the other lineage contained common, widely distributed species (*R. microphyllus*, *R. subcrataegifolius*, and *R. crataegifolius*). Moreover, the species of the former lineage are ecologically divergent, that is, *R. trifidus* and *R. boninensis* are warm-temperate species in insular or coastal habitats, whereas *R. pseudoacer* is confined to mountainous cool-temperate or subalpine regions in western Japan. These distributions suggest that the thornless species had been established and survived in specialized habitats within the Japanese Archipelago since the middle Miocene.

Similarly, sect. *Corchorifolii* also included two major lineages with contrasting biogeographic patterns. The early diverged species (*R. corchorifolius* and *R. chingii*) showed relictual distributions in western Japan and China, whereas all the other common species were proven to be derived and newly evolved. This finding implies that there were two major stages of the evolution of *Corchorifolii* species, with the second phase of divergence occurring in the late Miocene or later.

The results of the phylogenetic analysis also represent diverse evolutionary backgrounds of endemic species. As stated above, *R. ikenoensis, R. pseudoacer*, and *R. illece-brosus* can be regarded as old endemics. Based on their sister relationships with allied species, *R. vernus* and *R. pseudojaponicus* were considered to be of vicariant origin. Of all the local endemics, an oceanic island species *R. boninensis* was derived from *R. trifidus*. In contrast, *R. sieboldii* was suggested to be a new endemic species of unknown origin that diverged during the late Miocene to Pliocene.

Conclusions

This molecular phylogenetic study based on ITS and chloroplast sequences provided significant insights into the phylogenetic origins of Japanese *Rubus*. This revealed the ancient origins of most of the northern species, along with their intercontinental vicariance and phylogenetic uniqueness (of *R. ikenoensis*). It also displays the two major evolutionary groups (subdivided into three) of the rest of the Japanese *Rubus*, and the diversification process within them, particularly within a lineage of subg. *Idaeobatus*, which specializes in subterranean runners as a form of vegetative reproduction.

Information on Electronic Supplementary Material

Online Resource 1. List of samples.
Online Resource 2. Primers used in this study.
Online Resource 3. List of data from published studies.
Online Resource 4. Best-fit models of substitution.
Online Resource 5. ITS phylogenies based on BI and ML methods.
Online Resource 6. Chloroplast phylogenies based on BI and ML methods.
Online Resource 7. Partial chloroplast phylogenies based on BI and ML methods.
Online Resource 8. Marginal likelihood estimates of each molecular clock model.
Online Resource 9. The results of ancestral trait reconstruction.

Declarations

Conflict of interest The authors declare that they do have no conflict of interest.

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