

PHYLOGENY OF *RUBUS* (ROSACEAE) BASED ON NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER REGION SEQUENCES¹

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We used nuclear ribosomal DNA internal transcribed spacer region (ITS 1 – 5.8S – ITS 2; ITS) sequences to generate the first phylogeny of *Rubus* based on a large, molecular data set. We sampled 57 taxa including 20 species of subgenus *Rubus* (blackberries), one to seven species from each of the remaining 11 subgenera, and the monotypic and closely related *Dalibarda*. In *Rubus*, ITS sequences are most informative among subgenera, and variability is low between closely related species. Parsimony analysis indicates that *Rubus* plus *Dalibarda* form a strongly supported clade, and *D. repens* may nest within *Rubus*. Of the subgenera with more than one species sampled, only subgenus *Orobatus* appears monophyletic. Three large clades are strongly supported: one contains all sampled species of nine of the 12 subgenera; another includes extreme Southern Hemisphere species of subgenera *Comaropsis*, *Dalibarda*, and *Lampobatus*; and a third clade consists of subgenus *Rubus* plus *R. alpinus* of subgenus *Lampobatus*. *Rubus ursinus* appears to be a hybrid between a close relative of *R. macraei* (subgenus *Idaebatus*, raspberries) and an unidentified subgenus *Rubus* species. ITS sequences are generally consistent with biogeography and ploidy, but traditionally important morphological characters, such as stem armature and leaf type, appear to have limited phylogenetic value in *Rubus*.

Key words: biogeography; classification; internal transcribed spacer (ITS); phylogeny; Rosaceae; *Rubus*.

Rubus L. includes ~750 species (Robertson, 1974; Lu, 1983; Gu et al., 1993; Thompson, 1995) and is found on all continents except Antarctica (Focke, 1910, 1911, 1914; Gustafsson, 1942, 1943; Spies and Du Plessis, 1985; Hummer, 1996). The genus is economically and ecologically important as fruit crops, ornamentals, invasive weeds, and in early forest succession (Thompson, 1995; Hummer, 1996; Howarth, Gardner, and Morden, 1997). The most recent global taxonomic treatment of *Rubus* (Focke, 1910, 1911, 1914) included ~429 species in 12 subgenera (Table 1), the three largest being *Idaebatus* (raspberries, 117 species), *Malachobatus* (115 primarily Asian species), and *Rubus* (= *Eubatus* Focke; blackberries, ~132 species). Only three of the remaining nine subgenera have more than six species. Numerous species and infrageneric taxa have been recognized since

Focke's monograph, primarily in subgenus *Rubus* (Bailey, 1941–1945; Gustafsson, 1943; Weber, 1995).

Rubus exhibits tremendous morphological diversity including large, woody, upright species; delicate, semiherbaceous, prostrate species; and climbing species with highly reduced leaf blades (Brainerd and Peitersen, 1920; Peitersen, 1921; Gustafsson, 1943; Waugh et al., 1990). *Rubus* is one of the most taxonomically challenging genera of flowering plants (Aalders and Hall, 1966; Robertson, 1974; Lu, 1983; Richards et al., 1996), and species circumscription is complicated by hybridization, polyploidy, agamospermy, and lack of a universal species concept (Gustafsson, 1943; Weber, 1996). A recent species concept for European *Rubus* agamosperms, for example, allows as species only those biotypes whose distribution exceeds an area 50 km in diameter (Weber, 1996). In eastern North America, the number of taxa in treatments of subg. *Rubus* section *Rubus* ranges from 240 species (Bailey, 1941–1945), to 198 species (Davis, 1990), to 12 species complexes (Gleason and Cronquist, 1991).

Systematic difficulties also exist at higher infrageneric levels. Two of Focke's (1911, 1914) subgenera contain widely disjunct taxa. Subgenus *Dalibarda* has one western North American species, one western North American–eastern Asian species, one species each in the Himalayas and Tasmania, and an eastern North American endemic currently placed in the genus *Dalibarda* (Gleason and Cronquist, 1991; see below). Subgenus *Lampobatus* was originally divided into sect. *Lampobatus*, from Mexico, the West Indies, Central–South America, and the Himalayas, and sect. *Micranthobatus*, from Australia and New Zealand (Focke, 1894). Focke (1911, 1914) united sections *Lampobatus* and *Micranthobatus* into subg. *Lampobatus*, to which he added species from New Guinea and Madagascar. Kalkman (1987) suggested that spe-

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TABLE 1. List of *Rubus* subgenera and number of species based on Focke (1910, 1911, 1914), a two-letter code used in Figs. 2 and 3, and the monophyly cost (number of evolutionary steps longer than minimum-length trees required for monophyly). NA means not applicable because a single species was sampled.

Subgenus	Number of species	Code	Monophyly cost
<i>Anoplobatus</i> (Focke) Focke	6	An	15
<i>Chamaebatus</i> (Focke) Focke	5	Cb	6
<i>Chamaemorus</i> (Hill) Focke	1	Cm	NA
<i>Comaropsis</i> (Rich.) Focke	2	Co	NA
<i>Cylactis</i> (Raf.) Focke	14 (4 series)	Cy	28
<i>Dalibarda</i> (L.) Focke	5	Da	25
<i>Dalibardastrum</i> Focke	4	Ds	3
<i>Idaeobatus</i> (Focke) Focke	117 (9 sections)	Id	31
<i>Lampobatus</i> Focke	10	La	23
<i>Malachobatus</i> (Focke) Focke	115 (7 sections)	Ma	2
<i>Orobatus</i> Focke	19	Or	0
<i>Rubus</i> L. (= <i>Eubatus</i> Focke)	132 (6 sections)	Ru	4

cies of sect. *Lampobatus* be placed in subg. *Rubus* and the remaining species in the newly established subg. *Micranthobatus* (Fritsch) Kalkman. He was, however, "less certain that this is a natural (monophyletic) group than for other Malesian subgenera" (Kalkman, 1987, p. 323).

Another taxonomic problem is circumscription of *Rubus* itself. *Dalibarda repens* was described by Linnaeus (1753), who later placed it in *Rubus* as *R. dalibarda* L. Focke (1910) included this species in his *Rubus* subg. *Dalibarda* with four other species. In contrast, North American botanists have followed Linnaeus' (1753) original classification of this species and place *R. dalibarda* in the monotypic genus *Dalibarda* because of its reduced carpel number, dry fruits, and apetalous flowers (Rydberg, 1913; Bailey, 1941–1945; Fernald, 1950; Gleason and Cronquist, 1991).

Ploidy and hybridization are prevalent in *Rubus*. Only subgenera *Idaeobatus*, *Dalibarda*, and *Anoplobatus* are predominantly diploid, whereas *Dalibardastrum*, *Malachobatus*, and *Orobatus* are exclusively polyploid (Thompson, 1995, 1997). Hybridization in *Rubus* occurs mostly between closely related species (Steele and Hodgdon, 1963, 1970; Naruhashi, 1979, 1990; Kraft, Nybom, and Werlemark, 1995) and in some instances between subgenera (Gustafsson, 1942; Jennings, 1978; Weber, 1995; Alice et al., 1997). For example, Brown (1943) considered *R. ursinus* to be a cross between an ancestral Pacific blackberry and an eastern North American blackberry. Several intersubgeneric hybrids are horticulturally important (Waugh et al., 1990).

Our objectives are to infer phylogenetic relationships within *Rubus* plus *Dalibarda* using molecular data and to compare the implications of our results for *Rubus* classification with that of Focke (1910, 1911, 1914). Our sample covers much of the taxonomic/morphological diversity within the genus. We used nuclear ribosomal DNA (nrDNA) internal transcribed spacer region (ITS 1 – 5.8S – ITS 2; ITS) sequences because they have been phylogenetically useful in a wide range of taxa at generic and specific levels (Baldwin et al., 1995, and references therein; Downie and Katz-Downie, 1996; Yuan, Küpfer, and Doyle, 1996; Campbell et al., 1997; Potter, Luby, and

Harrison, 1997; Eriksson, Donoghue, and Vretblad, in press).

MATERIALS AND METHODS

Plant samples—We sampled 68 accessions from 56 *Rubus* species, including 20 members of subg. *Rubus*, 1–7 representatives of each of the remaining 11 subgenera, plus *Dalibarda repens* (Table 2). Sixteen accessions are from wild plants cultivated in arboreta, botanical gardens, or screenhouses; 18 taxa were sampled from herbarium specimens; and plants of *R. nepalensis*, *R. parvus*, and *R. tricolor* were purchased from a commercial nursery. The remaining 31 accessions were collected from the wild.

ITS sequences do not vary in each of 11 species for which we sampled two individuals (Table 2; one *R. chamaemorus* sequence is from Eriksson, Donoghue, and Vretblad, in press). Only *R. idaeus*, for which we sampled three individuals, showed intraspecific variation, with Maine and Swedish accessions differing at three nucleotide sites. North American and European *R. idaeus* differ morphologically and in *ndhF* sequences (Howarth, Gardner, and Morden, 1997), and are recognized as separate subspecific taxa (Gleason and Cronquist, 1991; Weber, 1995). Lack of within-species variation in 11 of 12 *Rubus* species indicates that loss of ITS variation from sequencing only one individual per species will be generally inconsequential.

Because of low mean ITS sequence divergence between species of *Rubus* subg. *Rubus* sect. *Rubus* (1.21%), only three of the 18 species sampled of this section were included in the final data set, which contains 40 *Rubus* species, *Dalibarda repens*, and three outgroups.

Chromosome numbers have been reported for 49 of the 56 *Rubus* species we sampled plus *Dalibarda repens*. Ploidy ranges from diploid ($x = 7$) to dodecaploid (Table 2); 38.8% are diploid, 42.9% are polyploid, and 18.3% have both diploid and polyploid counts. Of the 40 *Rubus* species plus *Dalibarda* included in the final ITS data set, 34 have chromosome counts. Ploidy ranges from diploid to octaploid; 50.0% are diploid, 38.2% are polyploid, and 11.8% have both diploid and polyploid counts.

McDade (1992) indicated that inclusion of hybrids in cladistic analysis does not affect topology unless the parents are phylogenetically distant from one another. Using morphological, chemical, and molecular data, Rieseberg and Morefield (1995) concluded that inclusion of *Helianthus* hybrid species had almost no effect on topology. All *Rubus* polyploids, except *R. ursinus*, were included in the final data set because they show levels of nucleotide polymorphism similar to those of diploid species and did not disrupt tree topology in preliminary analyses. We did exclude *R. ursinus* from most analyses, on the other hand, because ITS sequence polymorphism and morphology indicate that it is an intersubgeneric hybrid (see Discussion: Possible hybrid taxa).

Total genomic DNA was isolated using a modified CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1987) from leaf tissue collected fresh and stored at -80°C , leaves dried in silica gel desiccant, or leaves from herbarium specimens. We successfully amplified and sequenced ITS from material collected as early as 1936. Identification of specimens was verified using Focke's monograph or regional keys. Accessions from NCGR (see Table 2) were also verified by M. Thompson, Oregon State University.

Polymerase chain reaction (PCR) and DNA sequencing—PCR amplification of ITS generally followed Baldwin (1992). Double-stranded DNA was directly amplified by symmetric PCR using the ITS5 and ITS4 primers of White et al. (1990). Reaction volumes were 25 μL and contained 1.0 mg/mL bovine serum albumin (New England Biolabs, Beverly, Massachusetts), 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.9 mmol/L MgCl_2 , 200 $\mu\text{mol/L}$ each deoxynucleotide triphosphate (Stratagene, La Jolla, California), 0.3 $\mu\text{mol/L}$ oligonucleotide primer (Operon Technologies, Inc., Alameda, California), 1.0 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut), and

~25–60 ng of genomic DNA. PCR was performed in a PTC-100 thermal cycler (MJ-Research, Inc., Watertown, Massachusetts) and consisted of 40 cycles of 1 min at 97°C for template denaturation, 1 min at 48°C for primer annealing, 45 s (increased by 4 s per cycle) at 72°C for primer extension, followed by a final extension of 7 min at 72°C. PCR products were purified by gel electrophoresis in 0.8% SeaPlaque GTG agarose (FMC, Rockland, Maine) followed by band isolation. Each band containing ITS was melted at 65°C and the agarose digested with 1.0–7.5 units of β -agarase (Sigma, St. Louis, Missouri) at 39–45°C for 2.5 h. Double-stranded DNA was sequenced using the dideoxy chain termination method using an ABI PRISM[®] Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin Elmer, Norwalk, Connecticut). Samples were electrophoresed in an ABI 373A automated sequencer in a stretch gel following the manufacturer's instructions (Applied Biosystems, Inc., Foster City, California). Chromatograms were manually edited using Sequence Navigator (Applied Biosystems, Inc., Foster City, California). Primers ITS5 and ITS4 were used to sequence all samples, and in cases of potential nucleotide site polymorphism or ambiguous sequence, primers ITS3 and ITS2 (White et al., 1990) were also used. Approximately 32% of all nucleotides sequenced were derived from a single primer. All other nucleotides were verified with at least two primers. *Rubus* sequences generated in this study are available from GenBank (Table 2).

Outgroup selection—Two recent molecular phylogenetic studies of Rosaceae included genera of subfamily Rosoideae, to which *Rubus* belongs. Morgan, Soltis, and Robertson's (1994) *rbcL* phylogeny included ten Rosoideae s. s. genera and placed *Rubus* as sister to a clade including *Agrimonia*, *Rosa*, *Fragaria*, *Potentilla*, and *Alchemilla*. This clade of six genera is supported by a decay value of 1 and is sister to a well-supported clade containing *Fallugia*, *Geum*, and *Waldsteinia*. Eriksson, Donoghue, and Vretblad's (in press) ITS phylogeny included 18 Rosoideae s. s. genera and placed *Rubus* as sister to a *Fallugia*–*Geum*–*Waldsteinia* clade. This topology is supported by a decay value of 3 when several *Rubus* species are included in the analysis. Due to greater support and more extensive sampling of subfamily Rosoideae s. s., we used as outgroups single representative species of *Fallugia*, *Geum*, and *Waldsteinia* (sequences from Eriksson, Donoghue, and Vretblad, in press).

Alignment of ITS sequences—Boundaries for ITS 1 and ITS 2 in *Rubus* and *Dalibarda repens* were determined by comparison with Rosaceae sequences (Campbell et al., 1995). Sequences were aligned visually. Aligned sequences of ITS 1 and ITS 2 for *Fallugia*, *Geum*, and the five *Rubus* species exhibiting the most gaps are shown in Fig. 1. Alignment of ITS 1 sequences within *Rubus* plus *Dalibarda* required one two-base gap and six one-base gaps in most sequences. Alignment of ITS 2 sequences necessitated gaps of one to four bases at the 5' end in all species except *R. deliciosus*, and four one-base gaps in some sequences. Alignment of *Rubus* and *Dalibarda* with the outgroups required several one-base gaps and one 12-base gap (positions 118–129) in all ingroup species (Fig. 1). Multiple gaps of varying length were needed to align *Geum* and *Waldsteinia* with *Fallugia*. We determined whether insertions or deletions were responsible for gap regions based on our ITS strict consensus phylogeny.

Phylogenetic analysis—We tested for phylogenetic signal by using the RANDOM TREES option in PAUP (Swofford, 1993) and comparing the g_1 value for the distribution of tree lengths of 100 000 random trees using the critical value (at $\alpha = 0.05$) for 250 variable characters and 25 taxa. Beyond 15 taxa g_1 critical values change very little, allowing them to be used in a conservative test with more taxa (Hillis and Huelsenbeck, 1992).

Phylogenies were generated using Fitch parsimony as implemented in PAUP. Because of the number of taxa in this study, we executed HEURISTIC searches including all characters using RANDOM (1000 repli-

cates) stepwise addition of taxa followed by TBR (tree bisection-reconnection) branch swapping. To evaluate the impact of each species on number of trees found, tree length, Consistency Index (CI), Retention Index (RI), and topology, we performed taxon jackknifing (Hillis, Allard, and Miyamoto, 1993) using the HEURISTIC search option with ten replicates of RANDOM stepwise addition of taxa excluding uninformative characters. We also searched for multiple islands of equally parsimonious trees (Maddison, 1991) following methods outlined in Olmstead and Palmer (1994). Gaps were coded as missing data, a unique character state, or binary characters (presence-absence) in separate phylogenetic analyses. Character-state changes were weighted equally; to explore the effect of weighting, transversions were weighted over transitions by 2:1 and 5:1 using the step matrix option in PAUP. The transition-transversion ratio, based on our ITS strict consensus tree and calculated using MacClade (Maddison and Maddison, 1992), is 2.8:1.

Sets of equally parsimonious trees were summarized using strict consensus. Decay indices (Bremer, 1988; Donoghue et al., 1992) and bootstrap values (Felsenstein, 1985) with 500 replicates, saving up to 200 trees per replicate, were calculated as measures of support for individual clades. Decay analyses were performed with AutoDecay (Eriksson and Wikstrom, 1996) and the reverse constraint option in PAUP. To test further our results, we used topological constraint trees in PAUP to determine the monophyly cost (i.e., the number of steps beyond the most parsimonious necessary for monophyly) of *Rubus* (excluding *Dalibarda*) and each subgenus for which we sampled more than one species (except subg. *Orobatus*). Search methods were the same as those employed in taxon jackknifing. Pairwise divergence, adjusted for missing data, was calculated for all taxa in PAUP. We also mapped changes in leaf type (simple or compound), stem armature (absent, bristles, prickles, or bristles and prickles), ploidy level, and biogeographic region onto our ITS strict consensus tree using MacClade. For simplicity, we recognized only two leaf-type character states.

RESULTS

ITS length, GC content, sequence divergence, and nucleotide site variation—ITS 1 ranges from 254 to 257 base pairs (bp) in *Rubus* and *Dalibarda* and from 229 (*Geum* and *Waldsteinia*) to 270 bp (*Fallugia*) in the outgroups (Table 3). ITS 2 varies from 207 to 212 bp in all taxa sampled. Mean guanine + cytosine (GC) content is slightly higher in ITS 2 than in ITS 1 for *Rubus*, *Dalibarda*, and the outgroups. Mean pairwise divergence of sequences is higher in ITS 2 than in ITS 1 in *Rubus* and *Dalibarda* and almost identical between the ingroup and the outgroups.

Aligned sequences of ITS 1, the 5.8S gene, and ITS 2 comprise 656 characters. Missing data for the final data set are 2.91%, all in the 5.8S gene. Mean ITS nucleotide polymorphism within accessions of *Rubus* and *Dalibarda* is 0.13%. Between the ingroup and outgroups, ITS 1 shows somewhat lower levels of nucleotide site variability (43.6%) than does ITS 2 (48.4%). Of these variable sites, ~63% are potentially informative phylogenetically. A similar pattern is also found within the ingroup. Two nucleotides in the 5.8S gene distinguish *Rubus* and *Dalibarda* from the outgroups, and two are synapomorphic within *Rubus*.

In *Rubus* and *Dalibarda* there are two phylogenetically informative gaps in ITS 1: a two-base insertion (positions 47–48) and a one-base deletion (position 111) in *R. rosifolius* and *R. minusculus* (Fig. 1). One-base insertions in ITS 1 (position 218) in *R. deliciosus*, *R. trilobus*, and *R. arcticus* are homoplastic. In ITS 2, four of the five gaps are potentially informative phylogenetically. The first gap

TABLE 2. Accessions of *Rubus* species, *Dalibarda repens*, and outgroups used in this study (an asterisk denotes taxa not included in the final analysis). Subgeneric and sectional classification follow Focke (1910, 1911, 1914). Ploidy range is from Thompson (1997) and the Missouri Botanical Garden index of plant chromosome number database. Geographic origin is by country except accession 19 from the Himalayas, and the USA for which two-letter state abbreviations are used; "ex U.S.S.R." is the former Soviet Union. Herbarium vouchers include collector(s), number, and herbarium code (Holmgren, Holmgren, and Barnett, 1990). Vouchers of living collections are NCGR (United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, OR); HELSB (Helsingborg Botanical Garden, Sweden); and MOR (Morton Arboretum, Lisle, IL). Accessions 19, 20, and 31 were purchased from the Heronwood Nursery, WA, and for accessions 3b and 27, HPDL indicates the Native Hawaiian Plants DNA Library (Morden, Caraway, and Motley, 1996). Accession number is for DNA sequences in GenBank (the prefix GBAN has been added for linking the on-line version of *American Journal of Botany* to GenBank and is not part of the actual GenBank accession number). NA indicates data not available.

	Taxon	Ploidy	Origin	Voucher	Accession number
1.	<i>Dalibarda repens</i> L. (= <i>R. dalibarda</i> L.)	2x	ME-USA	Alice 97-4, MAINE	GBANAF055748
	Subg. <i>ANOPLOBATUS</i>				
2.	<i>R. deliciosus</i> Torr.	2x-3x	OK-USA	1021, NCGR/Alice 98-1, MAINE	GBANAF055733
3.	<i>R. odoratus</i> L.	2x	ME-USA	Alice R14, MAINE	GBANAF055734
4a.	<i>R. parviflorus</i> Nutt.	2x	MI-USA	Richards 666, MAINE	GBANAF055735
4b.	<i>R. parviflorus</i>		WA-USA	Luffman 217, HPDL 1173	GBANAF055736
5.	<i>R. trifidus</i> Thunb.	2x	Japan	3, NCGR/Alice 98-2, MAINE	GBANAF055737
6.	<i>R. trilobus</i> Moc. et Sesse.	NA	Mexico	Ruiz 889, MO	GBANAF055738
	Subg. <i>CHAMAEBATUS</i>				
7.	<i>R. nivalis</i> Douglas	2x	OR-USA	1374, NCGR/Alice 98-3, MAINE	GBANAF055739
8.	<i>R. pectinellus</i> Maxim.	6x	Japan	Jutla & Fujino 680, MO	GBANAF055797/ GBANAF055798
	Subg. <i>CHAMAEMORUS</i>				
9a.	<i>R. chamaemorus</i> L.	8x	ME-USA	Alice R17, MAINE	GBANAF055740
9b.	<i>R. chamaemorus</i>		Sweden	R. Eriksson s.n., GH/S	GBANU90803
	Subg. <i>COMAROPSIS</i>				
10.	<i>R. geoides</i> Sm.	4x	Argentina	Dudley et al. 1538a, MO	GBANAF055799/ GBANAF055800
	Subg. <i>CYLACTIS</i>				
11.	<i>R. arcticus</i> L.	2x-3x	Sweden	T. Eriksson 701, S	GBANAF055741
12a.	<i>R. humulifolius</i> C. A. Mey.	2x-4x	ex U.S.S.R.	Barkalov & Bezdeleva s.n., MO	GBANAF055742
12b.	<i>R. humulifolius</i>		Finland	1173, NCGR/Alice 98-4, MAINE	GBANAF055743
13.	<i>R. minusculus</i> H. Lév. et Van.	2x	Japan	161, NCGR/Alice 98-5, MAINE	GBANAF055744
14.	<i>R. pubescens</i> Raf.	2x	ME-USA	Alice R15, MAINE	GBANAF055745
15a.	<i>R. saxatilis</i> L.	4x	Sweden	T. Eriksson 719, S	GBANAF055746
15b.	<i>R. saxatilis</i>		Yugoslavia	918, NCGR	GBANAF055747
	Subg. <i>DALIBARDA</i>				
16.	<i>R. gunnianus</i> Hook.	NA	Tasmania	Wells 96-1, MAINE	GBANAF055749
17.	<i>R. lasiococcus</i> A. Gray	2x	OR-USA	Merello et al. 827, MO	GBANAF055750
18.	<i>R. pedatus</i> Sm.	2x	Canada	Alice 96-1, MAINE	GBANAF055751
	Subg. <i>DALIBARDASTRUM</i>				
19.	<i>R. nepalensis</i> (Hook.f) Kuntze	4x	Himalayas	Alice 97-1, MAINE	GBANAF055752
20.	<i>R. tricolor</i> Focke	4x	China	Alice 97-2, MAINE	GBANAF055753
	Subg. <i>IDAEOBATUS</i>				
	Sect. <i>Corchorifolii</i>				
21.	<i>R. crataegifolius</i> Bunge	2x	Korea	16, NCGR/Alice 98-6, MAINE	GBANAF055754
	Sect. <i>Idaeanthi</i>				
22a.	<i>R. idaeus</i> L.	2x	ME-USA	Alice R8, MAINE	GBANAF055755
22b.	<i>R. idaeus</i>		Sweden	T. Eriksson 735, S	GBANAF055756
22c.	<i>R. idaeus</i>		Sweden	T. Eriksson 773, S	GBANAF055757
23.	<i>R. occidentalis</i> L.	2x	ME-USA	Alice R16, MAINE	GBANAF055758
24.	<i>R. phoenicolasius</i> Maxim.	2x	DC-USA	Alice 96-2, MAINE	GBANAF055759
	Sect. <i>Rosifolii</i>				
25a.	<i>R. rosifolius</i> Sm.	2x	Seychelles	Eurard 11660, MO	GBANAF055760
25b.	<i>R. rosifolius</i>		HI-USA	Dibble & Dibble 6800, MAINE	GBANAF055761
	Sect. <i>Spectabiles</i>				
26.	<i>R. hawaiiensis</i> A. Gray	2x	HI-USA	399, NCGR/Alice 98-7, MAINE	GBANAF055762
27.	<i>R. macraei</i> A. Gray	NA	HI-USA	Gardner s.n., HPDL 207	GBANAF055763

TABLE 2. Continued.

	Taxon	Ploidy	Origin	Voucher	Accession number
	Subg. <i>LAMPOBATUS</i>				
28.	<i>R. alpinus</i> Macfad.	NA	Jamaica	F. W. H. & L. G. 14319, GH	GBANAF055764
29.	<i>R. australis</i> G. Forst.	NA	New Zealand	Gardner 1539, MO	GBANAF055801/ GBANAF055802
30.	<i>R. moorei</i> F. Muell.	NA	Australia	Streimann 8207, GH	GBANAF055765
31.	<i>R. parvus</i> Buchanan	4x	New Zealand	Alice 97-3, MAINE	GBANAF055766
	Subg. <i>MALACHOBATUS</i>				
	Sect. <i>Acuminati</i>				
32.	<i>R. lambertianus</i> Ser.	4x	China	Boufford & Bartholomew 23955, MO	GBANAF055796
	Sect. <i>Elongati</i>				
33a.	<i>R. assamensis</i> Focke	4x	China	Zhong-tao et al. 8700118, MO	GBANAF055803/ GBANAF055804
33b.	<i>R. assamensis</i>		China	1701, NCGR	GBANAF055805/ GBANAF055806
34.	<i>R. tephrodes</i> Hance	4x	China	Yao 9231, MO	GBANAF055767
	Sect. <i>Lineati</i>				
35.	<i>R. lineatus</i> Reinw.	NA	Bhutan	Grierson & Long 1950, GH	GBANAF055768
	Subg. <i>OROBATUS</i>				
36.	<i>R. nubigenus</i> Kunth	6x	Ecuador	1257, NCGR	GBANAF055769
37.	<i>R. roseus</i> Poir.	6x	Ecuador	Luteyn & Quezada 14402, MO	GBANAF055770
	Subg. <i>RUBUS</i>				
	Sect. <i>Floribundi</i>				
38.	<i>R. robustus</i> C. Presl.	2x	Bolivia	Steinbach 247, GH	GBANAF055771
	Sect. <i>Rubus</i> (Sect. <i>Moriferi</i>)				
39a.	* <i>R. allegheniensis</i> Porter	2x-3x	ME-USA	Alice R1, MAINE	GBANAF055772
39b.	<i>R. allegheniensis</i>		MO-USA	Alice 60, MAINE	GBANAF055773
40.	* <i>R. argutus</i> Link	2x-3x	FL-USA	Alice & Judd 15, MAINE	GBANAF055774
41.	* <i>R. bifrons</i> Vest	4x	TN-USA	Alice 98-9, MAINE	GBANAF055775
42.	<i>R. caesius</i> L.	4x	Sweden	Karlen 243, S	GBANAF055776
43.	* <i>R. canadensis</i> L.	2x-3x	ME-USA	Alice & Campbell 98-10, MAINE	GBANAF055777
44.	<i>R. cuneifolius</i> Pursh	2x-4x	AL-USA	Alice 5, MAINE	GBANAF055778
45.	* <i>R. divaricatus</i> P. J. Müll.	3x-4x	Germany	Martensen 2618.32	GBANAF055779
46.	* <i>R. hispidus</i> L.	2x-5x	ME-USA	Alice R9, MAINE	GBANAF055780
47a.	* <i>R. nessensis</i> Hall	4x	Germany	Martensen 2718.34	GBANAF055781
47b.	<i>R. nessensis</i>		Sweden	s.n., HELSB	GBANAF055782
48.	* <i>R. pedemontanus</i> Pinkw.	5x	Germany	Martensen s.n.	GBANAF055783
49.	* <i>R. pensilvanicus</i> Poir.	4x	ME-USA	Alice R5, MAINE	GBANAF055784
50.	* <i>R. sanctus</i> Schreb.	2x	Greece	T. Eriksson 714, S	GBANAF055785
51.	* <i>R. sapidus</i> Schltld.	NA	Mexico	Moore Jr. & Wood Jr. 4166, GH	GBANAF055786
52a.	* <i>R. setosus</i> Bigelow	2x-3x	ME-USA	Alice 113, MAINE	GBANAF055787
52b.	<i>R. setosus</i>		NH-USA	Alice 112, MAINE	GBANAF055788
53.	* <i>R. sulcatus</i> Vest	4x	Germany	Martensen 1325.12	GBANAF055789
54a.	* <i>R. trivialis</i> Michx.	2x	IL-USA	Alice 55, MAINE	GBANAF055790
54b.	<i>R. trivialis</i>		SC-USA	Alice 33, MAINE	GBANAF055791
55.	<i>R. ulmifolius</i> Schott	2x	France	190-84, MOR	GBANAF055792
56.	* <i>R. vigorosus</i> Müll. et Wirt.	4x	Germany	Martensen 2518.32	GBANAF055793
	Sect. <i>Ursini</i>				
57a.	* <i>R. ursinus</i> Cham. et Schltld.	6x-12x	OR-USA	197, NCGR/Alice 98-8, MAINE	GBANAF055794
57b.	<i>R. ursinus</i>		WA-USA	47-90, MOR	GBANAF055795
	OUTGROUPS				
58.	<i>Fallugia paradoxa</i> (D. Don) Endl.	4x	NM-USA	Hill 14684, GH	GBANU90805
59.	<i>Geum urbanum</i> L.	6x	Sweden	T. Eriksson 655, GH	GBANU90802
60.	<i>Waldsteinia fragarioides</i> (Michx.) Tratt.	NA	SC-USA	Hill & Soblo 21384, GH	GBANU90822/ GBANU90823

occurs after a series of cytosine residues near the 5' end (Fig. 1, positions 15–18) and was not used in our analysis because unambiguous positional homology could not be determined and several accessions exhibit length variability. The second gap (position 26) cannot be classified as an insertion or deletion, or as plesiomorphic or apo-

morphic, because it varies in the outgroups. The presence of a cytosine residue is, however, diagnostic of clades B and F (see below for composition of clades), subg. *Orobatus*, and *R. pectinellus* + *R. nepalensis*. The third gap is a deletion (position 90) that is potentially homoplastic and synapomorphic for clades B and C, and subg. *Oro-*

	ITS 1	10	20	30	40	50	60	70	80	
FALLUGIA	TCGAAACCTG	CCCAGCAGAA	CGACCCGAGA	ACTTGTTC	ACGCTC--GG	GGACGAAGAG	TCTTGTGACT	CCTCGTCCCC		
GEUM			.C..AA....	.A.....G..G..	G.C....-C	G...T...T		
deliciosus	...T.....	C	.A.....	..T--..	.G....G..	...CAC.G..-		
rosifolius		A.....A.....	.A..TAG..	.G....G..	...A..G..-		
parvus		A.....	..T--..	.G....G..	...CAC.G..	...A....-		
caesius		A.....	..T--..	.G....G..	...TC.G..-		
macraei		A.....	..T--..	.G....G..	...ACAG..T		
		90	100	110	120	130	140	150	160	
FALLUGIA	TTTCTCGGG	AG-CAAAGCA	TGTGTGTTG	ATGCATTGT	GCCCAAGGGT	CAAGTGCTCC	CACGCAGCCG	ACCCTCCCGA		
GEUM	GC.....	G.GT.C---	CGC.....GAA--	...T..T..		
deliciosusG...-G	.CT.....	T....C--G.-...G	...CGGAA..T..G		
rosifolius	...C....	G.G..G-TTG	.CT.....	...C--G.-...G	...TAGAA..T..G		
parvusG...-TAG	.CT.....	C....C--G.-...G	...TAGAA..G		
caesiusG...-T.G	.CT...-C.	T....C--G.-...G	...TTGAA..T..G		
macraeiA.	.GA..-T.G	.CC.....	T....C--G.-...G	...TGGAA..T..G		
		170	180	190	200	210	220	230	240	
FALLUGIA	GCGTACAAAC	GAACACCGGC	GTGAATTGGG	CCAAGGAACT	TGAATGAAAG	AGCRTT-CCC	TCGT-CGTCC	CGGAAACGGT		
GEUMT.....G...-	...-A....	T....A..		
deliciosusG..T..	C.A.-....		
rosifoliusT	..T.....G...-	C...-....C		
parvusA	..T.....G...-T	CT...-....		
caesiusT.....G...-	C..C...C.		
macraeiT.....G...-A	C.C.-....		
		250	260	270						
FALLUGIA	GTGCGTGC	GTGGTTTCGT	CATCTTCAAT	ATGTC						
GEUM	...CT...						
deliciosusG						
rosifolius	...C...G	T....A..	T.....						
parvus	...AG	T....A..						
caesius	...G	TG...A..						
macraei	...G	T....A..						
		ITS 2	10	20	30	40	50	60	70	80
FALLUGIA	GTCGTTGYCC	CCCC---AA	CCTCCCTCGG	GAGTTGGCG	GGACGGATGA	TGGCTCCCG	TGTGCTTGGT	CACGCGGTTG		
GEUM	...C...C	...T---G	AT.C...T		
deliciosus	...C...C	...CCC..	.CT.-...	AT...AT.T....	...CT..	...AT....		
rosifolius	...C...C	...---G	.C.T-...A....	...T....	...T....	...T....		
parvus	...C...C	.T...-GTA...	...C...CT.	...T....	...T....		
caesius	...C...C	...---G	A.C-...CT..	...A....	...A....		
macraei	A.....C	...C--..	.C.-...	...T....CT..	...T....	...T....		
		90	100	110	120	130	140	150	160	
FALLUGIA	GCATAAAAT	CAAGTCCTCG	GCGAYTAACG	CCACGACAAT	CGGTGGTTGT	CAAA-CCTCT	GTTGCCTGTC	GTGTGCGTGT		
GEUM	...T...C	...T...T	...C...C-....C		
deliciosus	...C...C	...C...C	...C...C-....C		
rosifolius	...A...A	...T...T	...C...T	T.....	...-....	...A...	...T...		
parvus	...-...-	...-...-	...C...C	TC...A	...-....	...TT.C.C	...C...C		
caesius	...-...-	...-...-	...C...C-....	...-....	...A...	...C...C		
macraei	...A...A	...T...T	<u>TTTG</u>G...G	...-....	...C...C	...C...C		
		170	180	190	200	210				
FALLUGIA	GTCGTTTGGG	GTCTCGCTGA	CCCATTGCGC	ATCACTTT-G	TT-GTNCCTT	CAACG				
GEUM	A...C.C...	.C..T.T...T...	...G...				
deliciosus	...A.C.A.	.G...AA..	A.T..G.T..	...GA.CA-	.CGA.G...				
rosifolius	...AAC.A.	.A.C.AA..	A...ACT.T	...GA.C-	.CGA.G...				
parvus	...AAC.A.	.G...CACA.	A...GTT..	G...GA.C-	.CGA.G...				
caesius	...AGC.A.	.G...CACA.	A...GTT..	G...GA.C-	.CGA.G...				
macraei	...AAC.A.	.G...AG...	A...GCT..	TGA..C-	.CGA.G...				

Fig. 1. Aligned sequences of ITS 1 and ITS 2 for *Fallugia*, *Geum*, and the five *Rubus* species exhibiting the most gaps. *Rubus* species are in lowercase letters and outgroups are in uppercase. Dots represent the same nucleotide present in *Fallugia* and dashes represent gaps. The four underlined residues in ITS 2 at sites 103–106 are found only in *R. macraei* and in *R. ursinus* (not shown) as part of a nucleotide site polymorphism (site 106 appears fixed for the *R. macraei* nucleotide).

TABLE 3. ITS sequence characteristics of outgroups and *Rubus* plus *Dalibarda*. For *Rubus* and *Dalibarda* (ingroup) length includes range, and mean and SD are given for GC content and sequence divergence. I.D. = insufficient data.

ITS characteristics	ITS 1	5.8S	ITS 2
Length (in base pairs)			
<i>Fallugia</i>	270	164	209
<i>Geum</i>	229	164	207
<i>Waldsteinia</i>	229	164	208
<i>Rubus</i> plus <i>Dalibarda</i>	254–257	164	207–212
GC content (%)			
Outgroups	55.9 ± 0.23	I.D.	57.9 ± 0.51
Ingroup	55.4 ± 1.07	54.8 ± 0.19	57.4 ± 1.38
Sequence divergence (%)			
<i>Fallugia</i> and <i>Geum</i>	11.2	I.D.	7.4
<i>Fallugia</i> and <i>Waldsteinia</i>	10.9	I.D.	5.1
<i>Geum</i> and <i>Waldsteinia</i>	4.7	I.D.	3.2
Ingroup and outgroups	14.1 ± 2.06	1.9 ± 0.37	14.5 ± 1.74
Within ingroup	5.2 ± 1.80	0.16 ± 0.308	7.2 ± 3.16
Variable nucleotide sites			
Ingroup and outgroups	120	7	104
Within ingroup	102	4	96
Potentially phylogenetically informative sites			
Ingroup and outgroups	76	3	65
Within ingroup	55	2	54

batus. The fourth gap (position 135) is a one-base insertion and synapomorphic for clade B.

Phylogeny of *Rubus*—Phylogenetic signal in the final ITS data set is significant ($P < 0.01$) based on the value of the g_1 statistic (-0.983). Heuristic searches including only ITS characters and gaps coded as missing data generated 208 equally parsimonious trees requiring 445 evolutionary steps (strict consensus in Fig. 2). Excluding uninformative sites, the CI is 0.579, and the RI is 0.745. Regression analysis of log-transformed CIs against number of taxa predicts an expected CI for 44 taxa of 0.344 (Sanderson and Donoghue, 1989). Thus, levels of homoplasy in our ITS data set are lower than expected.

All trees based on ITS data place *Rubus* and *Dalibarda repens* in the same clade (Fig. 2). This clade is strongly supported by a bootstrap value of 100%, a decay value of 19, and the presence of two ovules per carpel (the outgroups have one ovule).

Three clades with more than three species within the ingroup (A, B, and C; Fig. 2) are supported by bootstrap values >93% and decay values greater than 3. Clade A contains all sampled species of nine of the 12 subgenera and excludes *R. chamaemorus* of the monotypic subg. *Chamaemorus*, and all but one sampled member each of subg. *Anoplobatus* (*R. trifidus*) and subg. *Dalibarda* (*R. gunnianus*). Clade B includes *R. geoides* of subg. *Comaropsis* from southern South America, *R. gunnianus* of subg. *Dalibarda* from Tasmania, and three species of subg. *Lampobatus* from Australia and New Zealand. Clade C is one branch of a weakly supported trichotomy with *R. nivalis* of subg. *Chamaebatus* plus two subg. *Orobatus* species. Clade B consists of four subg. *Rubus* species, representing two of the six sections (but not *R. ursinus* of sect. *Ursini*, not shown), plus *R. alpinus* of subg. *Lampobatus*.

For purpose of discussion, three other clades with more than three species are named (D, E, and F; Fig. 2). Clade D contains representatives of three subgenera: *R. minusculus* of subg. *Cylactis*; *R. rosifolius* and *R. crataegifolius* of subg. *Idaeobatus*; and *R. trifidus* of subg. *Anoplobatus*. Clade E includes four species of subg. *Idaeobatus*, *R. idaeus*, *R. macraei*, *R. occidentalis*, and *R. phoenicolasius*, plus *R. saxatilis* of subg. *Cylactis*. Clade F has three of the four species of subg. *Malachobatus* sampled plus *R. tricolor* of subg. *Dalibardastrum*.

For each subgenus in which we sampled more than one species (except the monophyletic subg. *Orobatus*), we used topological constraint trees in PAUP to force monophyly. Of the nine subgenera we tested, four produced minimum-length trees two to six steps longer than Fig. 2, and the remaining five subgenera each required at least 15 additional steps (Table 1). Therefore, it is unlikely that Focke's (1910, 1911, 1914) subgenera *Anoplobatus*, *Cylactis*, *Dalibarda*, *Idaeobatus*, and *Lampobatus* are monophyletic. If highly divergent species are not constrained to belong to their respective subgenus, monophyly cost is reduced.

Taxon jackknifing identified removal of *R. nepalensis* as the exclusion that most markedly reduces the number of trees recovered and increases resolution in clade A. Removal of this species yields four equally parsimonious trees of length 441. All nodes are resolved in the strict consensus tree (Fig. 3), except for one trichotomy each in clades C and F. However, the newly resolved nodes are poorly supported with bootstrap values <50% and decay values of 1 (not shown in Fig. 3). Clade F is weakly united with *R. pectinellus* of subg. *Chamaebatus*. Clade F + *R. pectinellus* is sister to a clade containing two groups. The first group has *R. lineatus* of subg. *Malachobatus* + clade E. The second group contains three large clades. One clade includes (*R. arcticus* + *R. pu-*

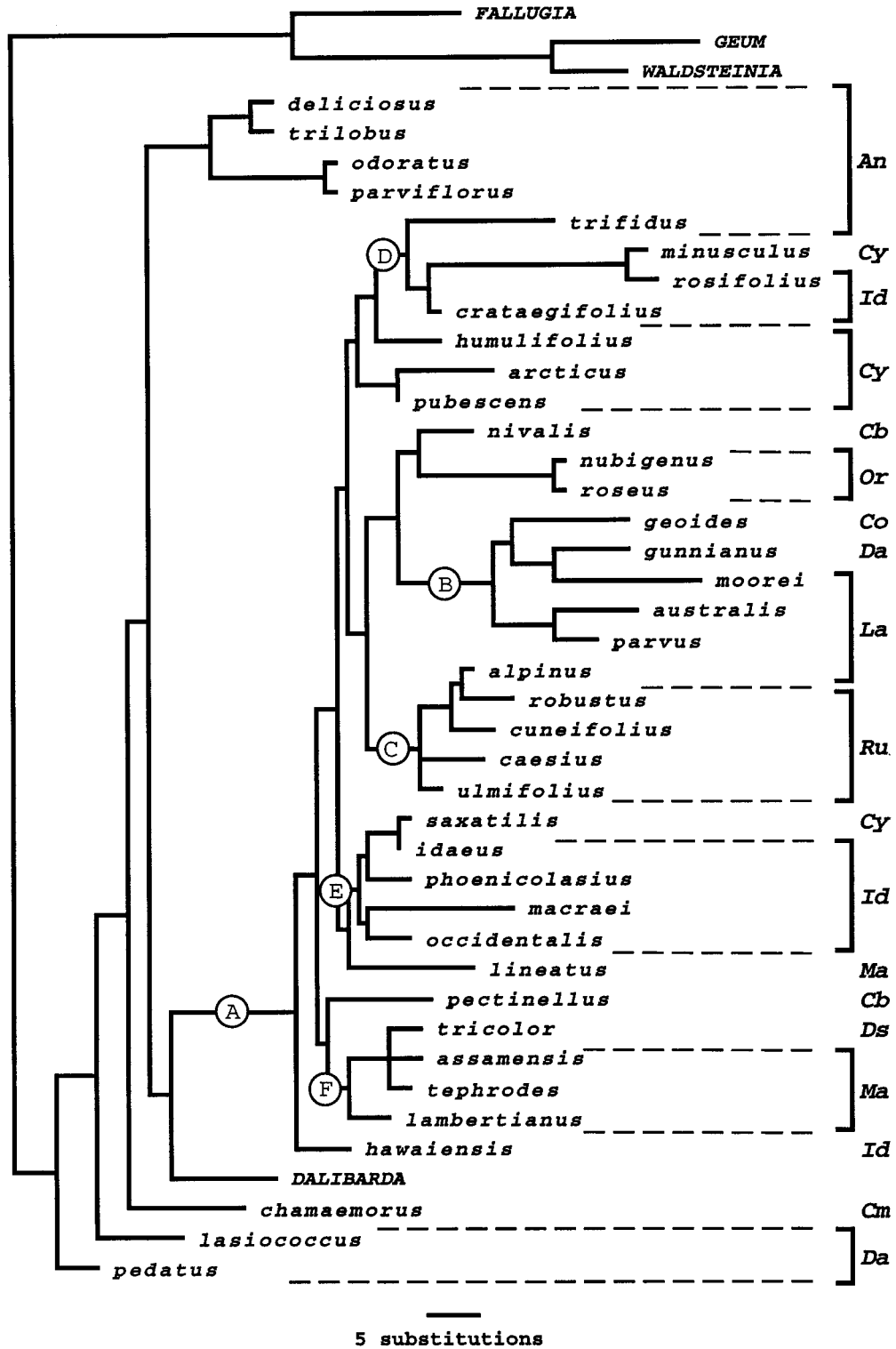


Fig. 3. Strict consensus phylogram of four equally parsimonious trees of length 441 containing 39 *Rubus* taxa (excluding *R. nepalensis*), *Dalibarda repens*, and three outgroups based on ITS sequences with gaps coded as missing data. The branch leading to *Rubus* plus *Dalibarda* has 31 nucleotide substitutions. *Rubus* species are in lowercase. Major clades are labeled A–F on branches. See Fig. 2 caption for *Rubus* subgenera codes.

consensus (not shown) topology is identical to Fig. 2 except that (*R. pectinellus* + *R. nepalensis*) form a trichotomy with *R. lineatus* and clade F, and clade C is sister to (clade B + (*R. nivalis* + subg. *Orobatus*)). Bootstrap and decay support for this topology are marginally stronger than in Fig. 2.

If transversions are weighted over transitions by 2:1, the strict consensus tree is basically identical to Fig. 2. With a 5:1 weighting scheme there is some increase in resolution; clades A through F are maintained and clade C is allied with (clade F + *R. lineatus*). In an unweighted analysis with gaps coded as binary characters, clade C is associated with (clade B + (*R. nivalis* + subg. *Orobatus*)). Other differences using a 5:1 weighting scheme include: (1) *R. odoratus* + *R. parviflorus* are separated from *R. trilobus* + *R. deliciosus*; (2) *R. chamaemorus* is united with *R. pedatus*; and (3) *R. crataegifolius* + (*R. rosifolius* + *R. minusculus*) are separate from *R. trifidus* and *R. humulifolius*. These differences are weakly supported.

DISCUSSION

ITS divergence and phylogenetic information in *Rubus*—In *Rubus* and *Dalibarda*, length and GC content of ITS 1 and ITS 2 (Table 3) are similar to those of other reported angiosperm sequences (Baldwin et al., 1995). Although several small gaps (1–4 bp) are present in each spacer, nucleotide substitution appears to be the main source of variability. Pairwise divergence of *Rubus* and *Dalibarda* sequences ranges from 0.4 to 9.4% in ITS 1 and from 0.0 to 16.0% in ITS 2. These ITS 1 divergence values are similar, or slightly lower, than those reported by Baldwin et al. (1995) in *Astragalus*, *Fouquieria*, *Gilia* sect. *Giliandra*, and *Viburnum*. ITS 2 divergence is slightly higher in *Rubus* and *Dalibarda* than in these four groups. In contrast, ITS 1 and ITS 2 divergence values in all these genera are markedly lower than those in *Gentiana* (5.0 to 48.9% in ITS 1 and 1.1 to 45.3% in ITS 2; Yuan, Küpfer, and Doyle, 1996). The percentage of aligned characters that are potentially phylogenetically informative is 20.0% in ITS 1 and 25.1% in ITS 2 in *Rubus* and *Dalibarda*, but $\leq 18\%$ in *Astragalus*, *Fouquieria*, *Gilia* sect. *Giliandra*, and *Viburnum*. However, the numbers of potentially phylogenetically informative characters in *Rubus* plus *Dalibarda* ITS 1 and ITS 2 are almost identical to each other (Table 3).

Previous molecular systematic studies of *Rubus*—Three previous molecular studies, two of which were explicitly phylogenetic, focused on three economically important subgenera: *Anoplobatus*, flowering raspberries; *Idaeobatus*, raspberries; and *Rubus*, blackberries, and are generally congruent with our ITS results except in the placement of *R. parviflorus* (see below). These studies used chloroplast DNA (cpDNA) restriction fragment length polymorphism (Waugh et al., 1990), random amplified polymorphic DNA (RAPD) markers (Graham and McNicol, 1995), and *ndhF* sequences (Howarth, Gardner, and Morden, 1997) and each included ≤ 24 taxa and no more than 14 species. Our study is the first molecular phylogenetic study of *Rubus* subgenera based on a large taxonomic sample.

Congruence between ITS-based phylogeny and traditional *Rubus* classification—All ingroup species excluded from clade A (Figs. 2, 3) have been classified outside *Rubus* by some workers (see Bailey, 1941–1945). ITS data do not preclude exclusion of these species from *Rubus* given the lack of resolution and weak support for basal nodes. However, high bootstrap and decay values on the branch connecting *Rubus* and *Dalibarda* with the outgroups suggest a close relationship. Although one might recognize basal species in Fig. 2 as distinct from *Rubus* based on ITS data, we prefer to treat them (not including *Dalibarda repens*) as congeneric on morphological grounds. All ingroup species sampled have two ovules (the outgroups have one), and with the exception of *Dalibarda*, have fleshy aggregates of drupelets.

More evidence is necessary to determine the phylogenetic position of *Dalibarda repens*. This species is the fifth branch in the tree from the ingroup/outgroup node and thus appears to be nested within *Rubus*, although bootstrap support is $< 50\%$ and decay values are only 1 or 2 for the first four nodes within the *Rubus* plus *Dalibarda* clade. Hence, *Dalibarda* could be the sister genus to *Rubus* or part of an unresolved basal complex. If *Dalibarda* is constrained as sister to *Rubus*, minimum-length trees are only three steps longer than those in Fig. 2. *Dalibarda repens* has been excluded from *Rubus* due to dry fruits, apetalous flowers, and reduced carpel number (5–10), but some *Rubus* species, such as *R. pedatus*, have only 3–6 carpels (Bailey, 1941–1945).

ITS data do not support monophyly of *Rubus* or of any subgenus for which we sampled more than one species except subg. *Orobatus* (Figs. 2, 3). In the sections that follow, we discuss this incongruence between our ITS phylogeny and Focke's (1910, 1911, 1914) classification for each subgenus.

Subg. *Anoplobatus*—ITS data clearly show that subg. *Anoplobatus* is not monophyletic; when it is constrained to be so, 15 steps are added to minimum-length trees in Fig. 2. Instead, the five species we sampled are divided into a New World group of four species that is strongly excluded from clade A and Japanese *R. trifidus*, which is allied to certain subg. *Idaeobatus* species.

ITS data indicate that New World members of subg. *Anoplobatus* are monophyletic and divided into two clades (Figs. 2, 3). One clade includes *R. odoratus* (eastern North America) and *R. parviflorus* (western North America), and the other contains *R. deliciosus* (southwestern North America) and *R. trilobus* (Mexico and Guatemala). Rydberg (1913) treated both species pairs as separate genera: *Rubacer* (*Rubus odoratus* and *R. parviflorus*) and *Oreobatus* (*Rubus trilobus* and *R. deliciosus*). Rydberg segregated these two genera from *Rubus* on the basis of different styles, stigmas, receptacles, stem architecture, bark, and leaf morphology. Waugh et al. (1990), who sampled three species of subg. *Anoplobatus*, found *R. odoratus* and *R. deliciosus* to be sister taxa, but *R. parviflorus* was basal in a clade with three Asian subg. *Idaeobatus* species. Howarth, Gardner, and Morden (1997) also found that *R. parviflorus* nested within subg. *Idaeobatus*, although they sampled only one species of subg. *Anoplobatus*. These results conflict with ITS data, which strongly unite *R. odoratus* and *R. parviflorus*, apart

from Asian subg. *Idaeobatus* species. The position of *R. parviflorus* in trees of Howarth, Gardner, and Morden (1997) is spurious because of rooting. When *Fallugia* is used as outgroup in the *ndhF* analysis, *R. parviflorus* is sister to the remaining *Rubus* species sampled (representing subgenera *Dalibardastrum*, *Idaeobatus*, *Malachobatus* and *Rubus*), in agreement with our ITS topology (C. Morden, personal communication, University of Hawai'i). Morphologically, *R. parviflorus*, together with other subg. *Anoplobatus* species, differs from most subg. *Idaeobatus* species in its unarmed stems and simple leaves with adnate stipules. Cluster analysis of RAPD markers (Graham and McNicol, 1995) placed *R. deliciosus* at the base of their *Rubus* phenogram, in agreement with ITS data.

Rubus trifidus strongly nests within clade A near certain Asian species of subg. *Idaeobatus* (Figs. 2, 3). Satomi and Naruhashi (1971) considered *R. trifidus* seeds to be identical to the *R. idaeus* type and noted that *R. odoratus* seeds are distinct from all Japanese species sampled. Naruhashi (1980) transferred *R. trifidus* to subg. *Idaeobatus* near *R. crataegifolius*, an alliance supported by ITS data.

Subg. Chamaebatus—ITS data indicate that the two species we sampled of this subgenus, *R. nivalis* (northwestern North America) and *R. pectinellus* (eastern Asia), occur within clade A (Figs. 2, 3) but may not be closely related. When monophyly of subg. *Chamaebatus* is forced, six steps are added to minimum-length trees in Fig. 2. Subgenus *Chamaebatus* contains five simple-leaved, prickly stemmed, prostrate species (Focke, 1910, 1914); the three members we did not sample occur in Mexico, the Himalayas, and eastern Asia. *Rubus nivalis* appears related to clade B and the subg. *Orobatus* clade. *Rubus pectinellus* is weakly united with *R. nepalensis* (Fig. 2) or sister to clade F when *R. nepalensis* is excluded (Fig. 3). *Rubus nivalis* and *R. pectinellus* are similar morphologically but differ in ploidy level: *R. nivalis* is diploid; *R. pectinellus* is hexaploid (Thompson, 1997).

Subg. Chamaemorus—Relationships of this monotypic subgenus are not fully resolved by ITS beyond excluding it from clade A (Figs. 2, 3). All species outside clade A have simple leaves and unarmed stems with the exception of *R. pedatus*, which has five-foliate leaves, and *R. lasiococcus*, which has simple or ternate leaves. Furthermore, nonclade A species are diploid (the triploid count of *R. deliciosus* is considered aberrant by Thompson, 1997) except for octaploid *R. chamaemorus*, the circumpolar cloudberry or baked-apple-berry, which also differs from other species excluded from clade A in its dioecy. The four other dioecious species we sampled, *R. parvus*, *R. australis*, and *R. moorei* of subg. *Lampobatus* and *R. ursinus* of subg. *Rubus* (not shown), all nest within clade A.

Subg. Comaropsis—*Rubus geoides*, the single species of subg. *Comaropsis* that we sampled, is strongly nested in clade B (Figs. 2, 3). This species was once placed in genus *Dalibarda* but later united with another southern South American species in subg. *Comaropsis* (Focke,

1910). Except for its prickly petioles, *Rubus geoides* morphologically resembles the unarmed *R. gunnianus* of subg. *Dalibarda*, which is related based on ITS data.

Subg. Cylactis—ITS data show that this subgenus of 14 species is clearly polyphyletic (Figs. 2, 3); forcing its monophyly yields minimum-length trees 28 steps longer than those in Fig. 2. We sampled one species from each of three of Focke's (1910, 1914) series and two species from the fourth. ITS data, including a synapomorphy in the 5.8S gene, indicate that *R. arcticus* (series *Arctici*) and *R. pubescens* (series *Saxatiles*) are closely related. The *R. arcticus* + *R. pubescens* clade is part of a multichotomy (Fig. 2) or sister to *R. humulifolius* (subg. *Cylactis*, series *Humulifolii*) + clade D (Fig. 3).

Rubus saxatilis (subg. *Cylactis*, series *Saxatiles*) is sister to *R. idaeus* in clade E, and differs from Swedish *R. idaeus* at only two sites, one of which is polymorphic and includes the *R. idaeus* residue. The alternative residue is an autapomorphy. *Rubus saxatilis* may be a tetraploid derivative of *R. idaeus*, but it lacks the primary character for distinguishing raspberries: dehiscence of the fruits without the receptacle.

The fifth sampled member of subg. *Cylactis*, *R. minusculus*, is tightly linked (100% bootstrap and a decay value of 18) with *R. rosifolius* of subg. *Idaeobatus*. These Asian diploids also share two gaps, pinnately compound leaves, and weak prickles. Satomi and Naruhashi (1971) and Naruhashi (1980) recognized the close relationship of these two species, which are considered synonymous in the Missouri Botanical Garden's TROPICOS database (based on the Flora of China checklist). Constraining monophyly of subg. *Cylactis* excluding *R. minusculus* adds only six steps to minimum-length trees. Thus, our ITS results suggest that *R. minusculus* should be removed from subg. *Cylactis*.

Polyphyly of subg. *Cylactis* (Figs. 2, 3) is consistent with its morphological heterogeneity. Leaf type ranges from simple in *R. humulifolius*, to ternate in *R. arcticus*, *R. pubescens*, and *R. saxatilis*, and to pinnately compound in *R. minusculus*. Stem armature varies from prickly in *Rubus humulifolius*, *R. saxatilis* and *R. minusculus*, to unarmed in *R. arcticus* and *R. pubescens*.

Subg. Dalibarda—This subgenus of five species (Focke, 1910, 1914), four of which we sampled, is not monophyletic based on our ITS data (Figs. 2, 3). Three of the species, western North American *R. lasiococcus*, western North American-eastern Asian *R. pedatus*, and the eastern North American endemic *R. dalibarda* (= *Dalibarda repens*), are excluded from clade A, but do not form a monophyletic group. *Rubus pedatus* is sister to all *Rubus* species sampled plus genus *Dalibarda*, but bootstrap support is <50% and the decay value is only 2. Placement of *R. lasiococcus* (Bailey, 1941–1945) and *R. pedatus* (Bailey, 1941–1945; Naruhashi, 1980; Lu, 1983) in subg. *Cylactis* instead of subg. *Dalibarda* as proposed by Focke (1910), strongly conflicts with ITS data. *Rubus pedatus* has been placed in the genus *Dalibarda* and also in the genus *Comaropsis* (see Bailey, 1941–1945). A relationship with genus *Dalibarda* is supported here, but not with *R. geoides* of subg. *Comaropsis*. *Dalibarda repens* tenuously nests within *Rubus* and

might instead be the sister of *Rubus* (as previously discussed).

Tasmanian *R. gunnianus*, the fourth species of subg. *Dalibarda* that we sampled, occurs in clade B with species of subgenera *Comaropsis* and *Lampobatus* from the extreme Southern Hemisphere. Separation of *R. gunnianus* from other subg. *Dalibarda* species is justified based on bootstrap and decay values (Fig. 2) and addition of 25 steps to minimum-length trees when monophyly of subg. *Dalibarda* is forced.

Subg. Dalibardastrum—Both sampled species of this subgenus nest in different lineages of clade A (Fig. 2). Chinese *R. tricolor* is part of a trichotomy with *R. assamensis* and *R. tephrodes* of subg. *Malachobatus*. This weakly supported group combines divergent morphologies: *R. tricolor* has bristly, prostrate stems and *R. assamensis* and *R. tephrodes* have prickles and/or bristles and upright stems.

The other subg. *Dalibardastrum* species we sampled, Himalayan *R. nepalensis*, is weakly allied to *R. pectinellus* (Fig. 2) and may be of hybrid origin (see Possible hybrid taxa below). Graham and McNicol (1995) showed that *R. nepalensis* clustered with *R. coreanus* Miq., an eastern Asian species of subg. *Idaeobatus*, but *R. nepalensis* has trifoliate leaves and weak, bristly, prostrate stems, while *R. coreanus* has 5–7 leaflets and stout, prickly, upright stems (Ohwi, 1965). Forcing the monophyly of subg. *Dalibardastrum* adds only three steps to the shortest trees. The strict consensus topology with subg. *Dalibardastrum* (not shown) constrained to be monophyletic is identical to Fig. 3 except that *R. nepalensis* and *R. tricolor* are sister species, and uncertain support for relationships of *R. nepalensis* and *R. tricolor* leave unresolved the status of subg. *Dalibardastrum*.

Subg. Idaeobatus—Our ITS phylogeny indicates that subg. *Idaeobatus* is polyphyletic with three apparent lineages (Figs. 2, 3), a finding consistent with its occurrence on six continents. Thus, the raspberry fruits separating from the receptacle may have evolved at least three times. Waugh et al. (1990) and Howarth, Gardner, and Morden (1997) also found subg. *Idaeobatus* to be polyphyletic, forming at least two distinct groups.

Subgenus *Idaeobatus* species *R. crataegifolius* and *R. rosifolius* are members of clade D (Figs. 2, 3), which has uniform seed morphology (Satomi and Naruhashi, 1971; *R. rosifolius* seeds were not studied), but is otherwise diverse morphologically. *Rubus rosifolius* and *R. minusculus* of subg. *Cylactis* have pinnately compound leaves and weak prickles. The remaining two clade D species, on the other hand, have simple leaves, and only *R. crataegifolius* has prickles. When monophyly is imposed on subg. *Idaeobatus*, 31 steps are added to minimum-length ITS trees in Fig. 2. However, exclusion of *R. rosifolius* and *R. crataegifolius* from the constraint tree, reduces the monophyly cost to 14 and six, respectively. Thus, separation of *R. rosifolius* and *R. crataegifolius* from other subg. *Idaeobatus* species is supported.

Rubus hawaiiensis (subg. *Idaeobatus*) is sister to the remaining taxa in clade A (Figs. 2, 3), but bootstrap and decay support are low. *Rubus hawaiiensis* was classified by Focke (1911, 1914) in sect. *Spectabiles* with three

other Hawaiian species and their hypothesized North American continental relative, *R. spectabilis* Pursh (not sampled). Only two native species, *R. hawaiiensis* and *R. macraei*, are currently recognized in Hawai'i (Wagner, Herbst, and Sohmer, 1990). Neither ITS nor *ndhF* (Howarth, Gardner, and Morden, 1997) support a close relationship of these two species, suggesting that two colonization events (both from western North America; see below) are responsible for Hawaiian *Rubus*. Yet, minimum-length ITS trees only three steps longer than Fig. 2 are obtained if *R. hawaiiensis* and *R. macraei* are constrained to be sister taxa.

Subg. Lampobatus—ITS data indicate that the four species we sampled of this subgenus, *R. australis* and *R. parvus* (both of New Zealand), *R. moorei* (Australia), and *R. alpinus* (West Indies and Central–South America), are divided into two groups (Figs. 2, 3). The first three species were originally placed in sect. *Micranthobatus* (Focke, 1894) and unite with Tasmanian *R. gunnianus* of subg. *Dalibarda* and *R. geoides* of subg. *Comaropsis* to form the well-supported clade B. Members of this clade are diverse morphologically, including species with simple leaves and unarmed stems (*R. parvus*), species with simple or ternate leaves and prickly petioles (*R. geoides*), and species with compound leaves and prickly stems (*R. moorei*). *Rubus gunnianus* and *R. moorei* are apparently more closely related to each other than either is to the New Zealand species *R. parvus* and *R. australis*.

Rubus alpinus, initially put in sect. *Lampobatus* with several Mexican/West Indian species plus one Himalayan species (Focke, 1894), is more closely related to species of subg. *Rubus*, in agreement with Rydberg (1913) and Kalkman (1987). ITS data therefore suggest that *R. alpinus* should be removed from subg. *Lampobatus* and that *R. geoides* and *R. gunnianus* should be included.

Subg. Malachobatus—This primarily Asian subgenus, in which we sampled four species, is not monophyletic based on ITS data (Figs. 2, 3). Three of the four species, *Rubus assamensis*, *R. tephrodes*, and *R. lambertianus*, are united with *R. tricolor* of subg. *Dalibardastrum* in the weakly supported clade F. Species of clade F are Asian, tetraploid, simple-leaved, and armed with prickles and/or bristles.

Rubus lineatus, the fourth sampled species of subg. *Malachobatus*, is part of a multichotomy in clade A (Fig. 2). When *R. nepalensis* is excluded from analysis, *R. lineatus* is sister to clade E (Fig. 3). However, if transversion-weighting is employed or gaps are included, *R. lineatus* is allied with clade F (not shown). *Rubus lineatus* differs from most subg. *Malachobatus* species in its palmately compound leaves and occasionally unarmed stems. Constraining subg. *Malachobatus* to be monophyletic requires only two additional steps. Thus, additional data are necessary to evaluate the monophyly of this subgenus.

Subg. Orobatus—The two Ecuadorian species we sampled, *R. nubigenus* and *R. roseus*, of this primarily South American subgenus form a strongly supported clade (Figs. 2, 3), including one synapomorphy in the 5.8S

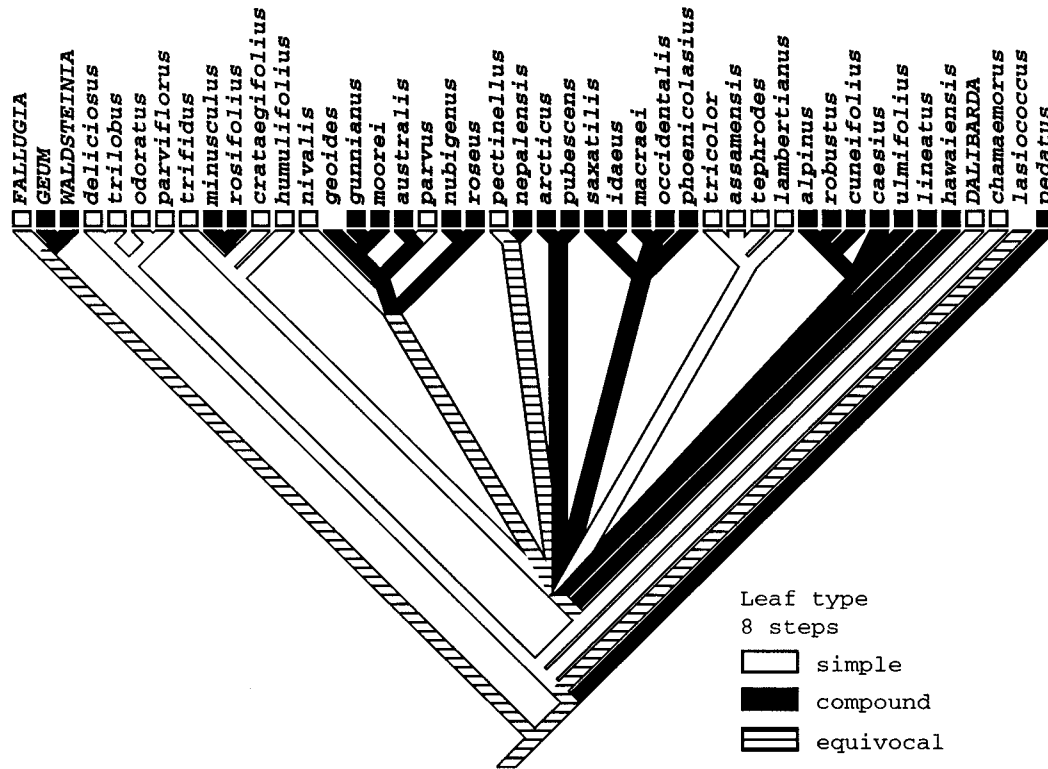


Fig. 4. Topology of Fig. 2 tree with leaf type mapped. For simplicity no distinction was made between digitate, ternate, and pinnate leaves. Missing squares at branch tips indicate variability in this character.

gene, and differ by only two transitions and a one-base deletion in *R. nubigenus*.

Subg. Rubus—Based on ITS data, sampled species of this subgenus form a well-supported clade (clade C; Figs. 2, 3) that includes *R. alpinus* of subg. *Lampobatus* but excludes *R. ursinus* (not shown). Section *Rubus* is not monophyletic because North American *R. cuneifolius* of sect. *Rubus* is more closely related to South American *R. robustus* of sect. *Floribundi* than to European *R. ulmifolius* of sect. *Rubus*. Previous molecular data show that subg. *Rubus* sect. *Rubus* species are closely related. Three sampled sect. *Rubus* species in Waugh et al. (1990) formed a monophyletic group and four species in Graham and McNicol (1995) also clustered together using RAPD markers. Approximately 1050 bp of *ndhF* sequence show no variability between *R. argutus* and *R. cuneifolius* of subg. *Rubus* sect. *Rubus* (Howarth, Gardner, and Morden, 1997).

Six nucleotide sites distinguish North American and European species of sect. *Rubus*, and five species (*R. allegheniensis*, *R. divaricatus*, *R. nessensis*, *R. sapidus*, and *R. sulcatus*) are polymorphic for at least one of these sites. Separate analysis of *Rubus* subg. *Rubus* species (excluding *R. ursinus*) with *R. chamaemorus*, *R. odoratus*, and *Dalibarda repens* as outgroups produces a highly unresolved strict consensus tree (not shown), but does recover a clade of New World species plus European *R. nessensis*. If, however, the five polymorphic taxa noted above are removed, BRANCH AND BOUND searches yield 20 most parsimonious trees of length 94 (CI =

0.820, RI = 0.893). The strict consensus tree (not shown) indicates that European subg. *Rubus* species form a weakly supported clade that is sister to a New World clade (79% bootstrap value) with South American *R. robustus* of sect. *Floribundi* sister to North American sect. *Rubus* species. These polymorphic species could be hybrids (three of the five are tetraploid) of recent origin. Alternatively, they could be ancient and ancestral species wherein concerted evolution has failed to homogenize ITS repeats (Campbell et al., 1997), and lineage sorting or biased gene conversion has resulted in distinct New World and European lineages.

Congruence between ITS data and nonmolecular features—Morphology

—We mapped leaf type and stem armature, which are commonly used in *Rubus* classification (Focke, 1910, 1911, 1914; Bailey, 1941–1945), onto the Fig. 2 topology. We recognized only two leaf-type character states, simple and compound, and for simplicity did not distinguish among digitate, ternate, and pinnate leaves. The most parsimonious mapping of leaf type onto the ITS strict consensus tree (Fig. 4) requires eight evolutionary steps and is congruent with some clades, but not others. New World subg. *Anoplobatus* species (*R. odoratus*, *R. parviflorus*, *R. deliciosus*, and *R. trilobus*) and clade F species have simple leaves, and clade C and E species have compound leaves. However, clades B and D include simple-leaved and compound-leaved species. The presence of simple and ternate leaves in *R. lasiococcus* and other species also indicates that leaf

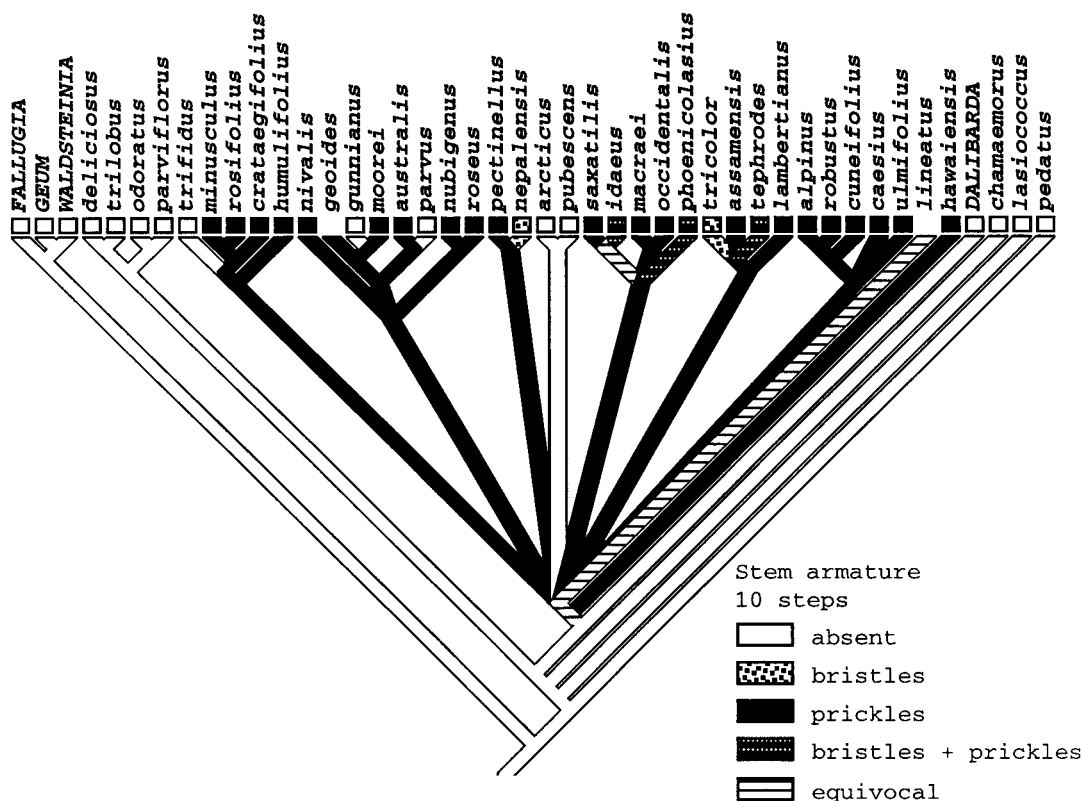


Fig. 5. Topology of Fig. 2 tree with stem armature mapped. Missing squares at branch tips indicate variability in this character.

type is phylogenetically plastic and therefore of limited value in *Rubus* at the subgeneric level.

Stem armature shows somewhat greater congruence with ITS data than leaf type; mapping of this character requires ten steps but includes four character states rather than two for leaf type (Fig. 5). Most clade A species have armed stems, while all species excluded from clade A do not. Of clades B–F, only clade E is not homoplastic for this character. Clade F species, for example, have bristles and/or prickles. Clade C (Figs. 2, 3) appears to include only prickly stemmed species, but bristly stemmed species excluded from the final data set also occur in clade C.

It has been suggested that evolution in Rosaceae (Kalkman, 1988) and *Rubus* (Lu, 1983) proceeded from woody to herbaceous and from compound to simple leaves. Lu (1983) considered subg. *Idaeobatus* to be the most primitive group and subg. *Chamaemorus* the most advanced. ITS data conflict with these hypotheses: primarily semi-herbaceous, simple-leaved species occupy basal positions in our trees. Of the species excluded from clade A, only *R. pedatus* and occasionally *R. lasiococcus* have compound leaves, and only the New World subg. *Anoplobatus* clade (*R. odoratus*, *R. parviflorus*, *R. deliciosus*, and *R. trilobus*) has woody stems.

Ploidy level—Base chromosome number is phylogenetically informative among Rosaceae subfamilies (Morgan, Soltis, and Robertson, 1994), and ploidy is largely congruent with our *Rubus* ITS phylogeny (Fig. 6). Five or possibly six of the seven species excluded from clade A (Figs. 2, 3) with known chromosome counts are dip-

loid, clade D species are diploid, clade F species are tetraploid, and members of the subg. *Orobatus* clade are hexaploid. In contrast, ploidy in clades C and E ranges from diploid to tetraploid.

Biogeography—We used our ITS-based phylogeny to examine hypotheses of the origin of *Rubus*. Lu (1983) hypothesized that southwestern China is the center of origin of *Rubus* because of the high diversity of species found there (194 species, especially in subgenera *Idaeobatus* and *Malachobatus*), number of subgenera, and morphological variation. Kalkman (1988) postulated a Gondwanan origin for Rosaceae, including *Rubus*. Figure 7 shows that most species at the base of our strict consensus tree are neither Chinese nor Gondwanan. *Rubus pedatus* extends from western North America to far eastern Asia, *R. chamaemorus* is circumpolar, and the other species excluded from clade A are North American–Mexican/Guatemalan. Thus, an origin of *Rubus* in southwestern China or Gondwanaland seems unlikely. A western North American or far eastern Asian origin (e.g., Japan or eastern Russia) is more probable in light of ITS data given the distribution of basal species in our ITS strict consensus tree.

Possible hybrid taxa—Because of its blackberry-like fruit, *R. ursinus* has been placed in subg. *Rubus* (Focke, 1911, 1914; Rydberg, 1913; Bailey, 1941–1945). Brown (1943) and Jennings (1995) proposed a hybrid origin of *R. ursinus* but did not consider a raspberry as one of its parents. In our analysis, *R. ursinus* is sister to the Ha-

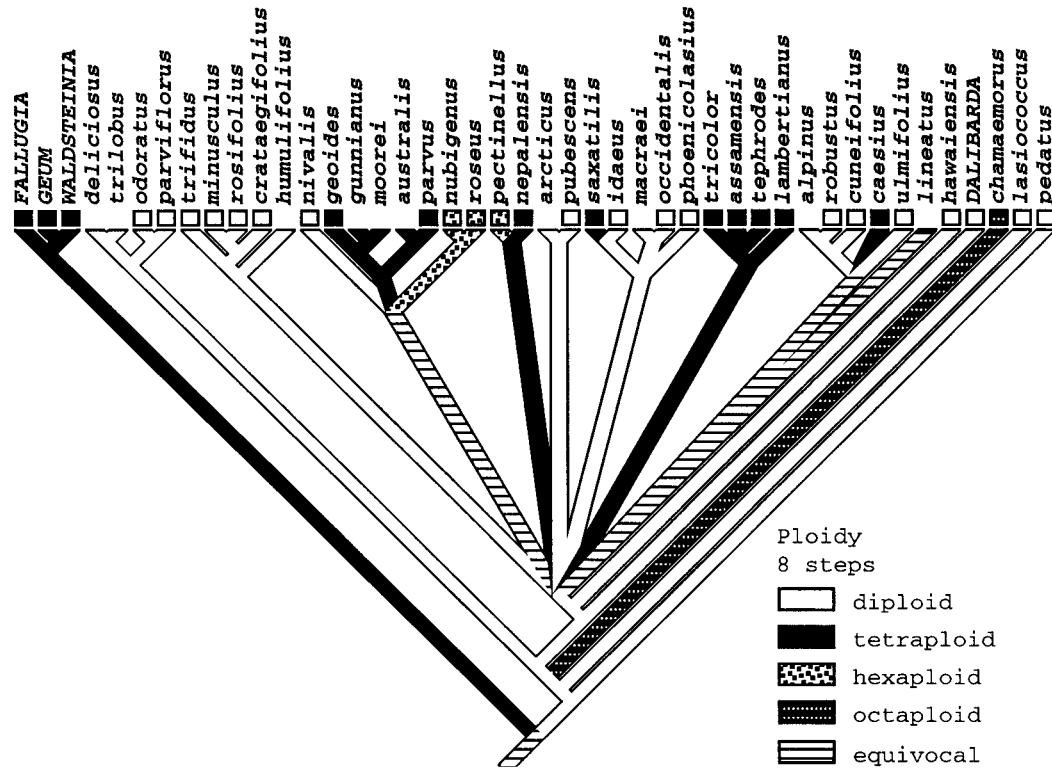


Fig. 6. Topology of Fig. 2 tree with ploidy mapped. Ploidy of *Waldsteinia* is from *W. ternata*, not *W. fragarioides*, which we sampled for ITS. Ploidy of *Geum* represents the lowest chromosome count reported in the genus (tetraploid); *G. urbanum*, included in our study, is hexaploid. Chromosome counts for the three outgroups are from the Missouri Botanical Garden's index of plant chromosome number database. Missing squares at branch tips indicate variability in this character or data not available.

waiian endemic *R. macraei* of subg. *Idaeobatus* (tree similar to Fig. 2 not shown), the two taxa differing by only three nucleotides. At six sites in ITS 2 (39, 103-105, 131, 177; Fig. 1), *R. ursinus* is polymorphic and includes, as part of the polymorphism, the *R. macraei* nucleotide. Because residues at positions 103-106 in ITS 2 are diagnostic for *R. macraei* and the putative hybrid *R. ursinus* as part of a polymorphism (site 106 appears fixed for the *R. macraei* nucleotide), it is hypothesized that *R. macraei* (or a close relative that we have not sampled) was involved in the origin of *R. ursinus*. Based on this relationship between western North American *R. ursinus* and Hawaiian *R. macraei*, it is probable that *R. macraei* originated in western North America.

Taxon jackknifing identified one species, *R. nepalensis*, whose removal markedly reduces the number of equally parsimonious trees recovered and increases resolution in clade A (see Results). This impact on tree topology could be explained by a hybrid origin of *R. nepalensis* (McDade, 1992; Campbell et al., 1997) or by homoplasy. We examined 21 potentially phylogenetically informative characters that might create the instability introduced by this taxon. For six homoplastic characters (the remaining 15 are largely autapomorphic for terminal clades), patterns of relationship of *R. nepalensis* are particularly complex. This species is least similar to clades B and D (Fig. 2) at the six homoplastic sites and shares five nucleotides with clade F, four with clade E, three with clade C, and two with *R. arcticus* + *R. pubescens*. Based on ITS sequence divergence, *R. nepalensis*

is closest to *R. idaeus*, *R. saxatilis*, and *R. tricolor*. The first two species are sister taxa in clade E, and *R. tricolor* is in clade F.

Homoplasy in these six characters alone may be responsible for the reduction in tree number and increased resolution in clade A when *R. nepalensis* is excluded. We removed each of the six homoplastic characters individually and ran HEURISTIC searches in PAUP to determine their impact on tree number. Two characters had no effect, one increased tree number to 3068, two reduced tree number to 48 and 36, and character 68 in ITS 2 (Fig. 1) reduced tree number to 12 and tree length by nine.

In conclusion, our ITS results show that Focke's (1910, 1911, 1914) classification of *Rubus* contains mostly non-monophyletic subgenera although several groups are strongly supported. Subgenus *Rubus* (including *R. alpinus* of subg. *Lampobatus* and excluding *R. ursinus*) forms clade C (Fig. 2), and extreme Southern Hemisphere species form clade B. Furthermore, it appears that *R. minusculus* is closely related to *R. rosifolius* and should be removed from subg. *Cylactis*, Tasmanian *R. gunnianus* should be allied with Australian and New Zealand subg. *Lampobatus* species, and *R. trifidus* is not a member of subg. *Anoplobatus*. Although our results do provide strong phylogenetic signal for some infrageneric clades in *Rubus*, we sampled only 56 of the ~750 *Rubus* species. Biogeographic and ploidy level variations are generally more consistent with ITS-based trees than leaf type and stem armature, which are highly homoplastic and of limited phylogenetic value among *Rubus* subgenera.

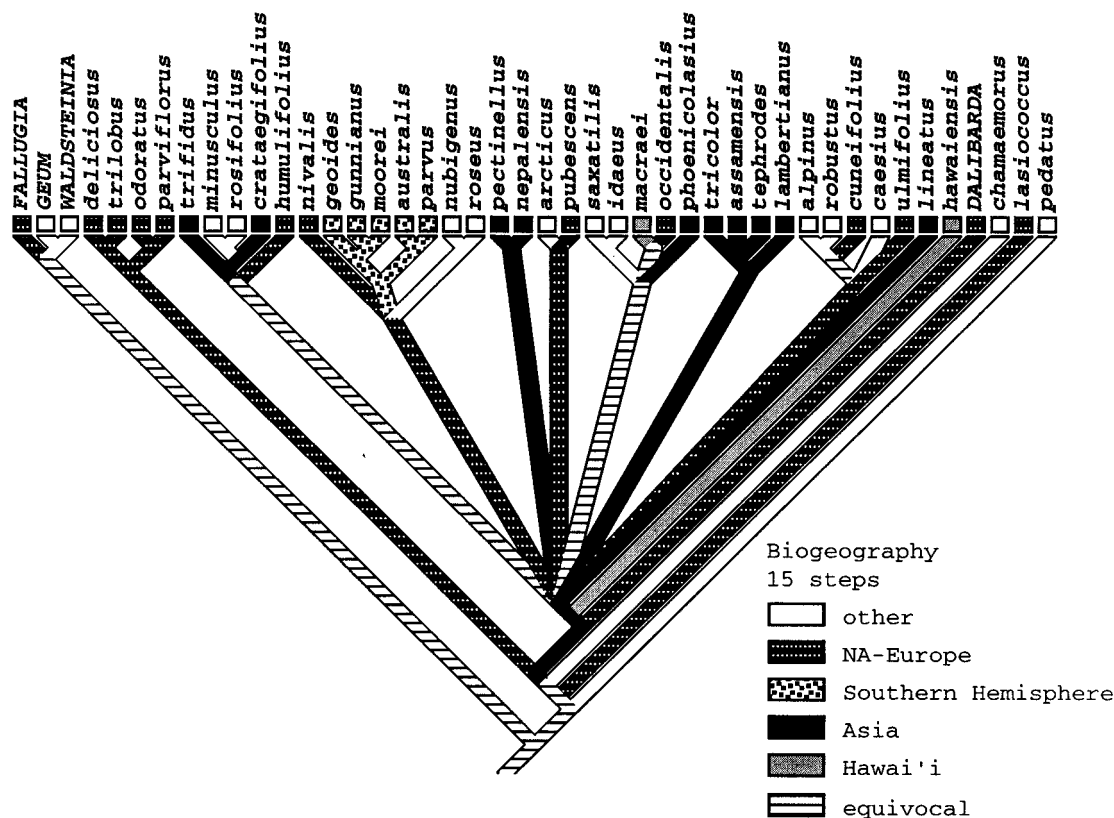


Fig. 7. Topology of Fig. 2 tree with biogeographic region mapped. "Other" represents noncoded regions or two or more coded regions. "NA-Europe" is North America, Mexico plus Guatemala, and/or Europe. "Southern Hemisphere" includes Australia, New Zealand, Tasmania, and southern South America.

To resolve phylogenetic relationships within *Rubus*, additional sampling of species, particularly those of large and disjunct subgenera, is needed. Because gene trees may not represent species trees (Doyle, 1992; Kellogg, Appels, and Mason-Gamer, 1996), more data are critical for confident determination of organismal relationships in *Rubus*. Levels of homoplasy in key morphological characters, such as leaf type and stem armature, encourage further use of molecular data. Given that weak support of several nodes in ITS-based trees is due largely to a limited number of characters, either a faster evolving or longer nuclear DNA region should be sought. Hybridization within and between *Rubus* subgenera and prevalence of polyploidy mandate use of a plastid gene. These data might provide a more robust phylogeny and allow one to address the possible origins of polyploid *Rubus* taxa.

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