Phylogeny and Biogeography of Pacific *Rubus* Subgenus *Idaeobatus* (Rosaceae) Species: Investigating the Origin of the Endemic Hawaiian Raspberry *R. macraei*[1]

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Abstract: The endemic Hawaiian raspberries *Rubus hawaiensis* and *R. macraei* (both subgenus *Idaeobatus*) had been thought to be closely related species until recent molecular studies demonstrated otherwise. These studies suggest that they are the products of separate colonizations to the Hawaiian Islands. Affinities of *R. hawaiensis* to *R. spectabilis* of western North America were clearly confirmed. However, no clear relation to *R. macraei* has been published. This study was initiated to examine species of subg. *Idaeobatus* from the surrounding Pacific region as well as species from other subgenera to better evaluate biogeographic and phylogenetic affinities of *R. macraei* by means of chromosome analysis and molecular data using the chloroplast gene *ndhF*. Results show that *R. macraei* clusters in a clade with species of blackberries, subg. *Rubus*, and of these it is most closely linked to *R. ursinus*. Chromosomally, *R. macraei* is $2n = 6x = 42$, a number that would be a new report for subg. *Idaeobatus*. However, polyploidy is common in subg. *Rubus*. Analyses indicate that *R. macraei* and *R. hawaiensis* are derived from separate colonizations from North America and that similarities between them are due to convergent evolution in the Hawaiian environment.

THE GENUS *Rubus* (Rosaceae) is a large and taxonomically complex assemblage of species consisting of several hundred sexual species to perhaps thousands of apomictic microspecies (Jennings 1988, Iwatsubo et al. 1995, Thompson 1997, Wagner et al. 1999). The most recent global treatment of *Rubus* was by Focke (1910, 1911, 1914), where the genus was divided into 12 subgenera and numerous sections and series. The two largest of Focke's subgenera included the raspberries (subgenus *Idaeobatus*) and the blackberries (subg. *Rubus* = *Eubatus* Focke). Focke originally placed 117 species into subg. *Idaeobatus*, a number that has since increased to 135 species (Thompson 1997). In subg. *Rubus*, the number has risen from Focke's original 132 species to approximately 400 sexual species and thousands of described apomictic species (Jennings 1988, Iwatsubo et al. 1995). Frequent hybridization and reproduction through apomixis has made the designation of distinct species difficult in this genus (Nybom and Schaal 1990, Nybom and Hall 1991, Kraft et al. 1996). This situation is further complicated by a large amount of phenotypic plasticity (Nybom and Schaal 1990, Nybom and Hall 1991, Kraft et al. 1996).

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Two species of *Rubus* are endemic to the Hawaiian Islands, *R. hawaiensis* and *R. macraei* (Wagner et al. 1999). Both species were placed in subg. *Idaeobatus*, section *Idaeanthi*, series *spectabiles* by Focke (1911) and share a number of notable morphological features. Both species are woody in habit, possess shredding bark, have reduced prickle concentrations, possess compound leaves with three leaflets, flowers that are pink to dusky rose-colored, and drupelets that are dark red to purple (Wagner et al. 1999). The high degree of morphological similarity supports the hypothesis that these two species are closely related to one another and as a result were thought to be derived from *R. spectabilis*, the common salmonberry from western North America (Hillebrand 1888, Fosberg 1948, Wagner et al. 1999). The differences between these two species are few compared with the similarities. *Rubus macraei* tends to grow in a prostrate decumbent manner while *R. hawaiensis* grows upward with erect branches that may become arching. Other morphological differences include the thickness of the leaves, length of the terminal leaflet, and the coloration of the abaxial surfaces of the leaves (Wagner et al. 1999; C.W.M., pers. obs.). Although these species are occasionally found growing sympatrically, no hybrids between them are known, yet natural hybrids between *R. hawaiensis* and the invasive *R. rosifolius* (both subg. *Idaeobatus*) have been documented (Randell 2000).

Given the wide morphological variation found in this genus, alternative approaches have been employed to gain insight into this difficult group, including paper chromatography fingerprinting (Haskell and Garrie 1966), isozyme techniques (Cousineau and Donnelly 1989), and characterization of anthocyanin pigments (Jennings and Carmichael 1980). Nybom et al. (1990) and Waugh et al. (1990) first employed molecular tools to study the variability in *Rubus* species using Southern hybridization analysis and DNA fingerprinting, respectively. However, neither method provided the resolution necessary to examine relationships among species and subgenera of this complex genus. Graham and McNicol (1995) used randomly amplified polymorphic DNA (RAPD) markers to examine genetic variation among some species in two *Rubus* subgenera and found that *R. macraei* did not group with other *Idaeobatus* species, but rather grouped with species of subg. *Rubus* separate from a cluster containing subg. *Idaeobatus* species. However, their analysis utilized few species of these subgenera and involved many individuals derived from artificial crosses resulting in inter- and intrasubgeneric hybrids. Further, *R. hawaiensis* was not included in this analysis to allow comparison with *R. macraei*.

Two recent studies based on sequence analysis of chloroplast and nuclear DNA have provided insight into the phylogenetic framework of this genus. Examination of the chloroplast *ndhF* region (Howarth et al. 1997) has shown the region to possess levels of variation appropriate to address questions at the species and subgeneric levels. They examined nine species from subg. *Idaeobatus* including both Hawaiian species as well as *R. spectabilis*. Their results surprisingly showed that the two Hawaiian species were not closely related to each other. As suspected, *R. hawaiensis* clustered closely with *R. spectabilis*, but *R. macraei* did not group closely with any other species in subg. *Idaeobatus*. These findings were further corroborated by Alice and Campbell (1999) in their investigation of phylogenetic relationships within *Rubus* using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA cistron. Alice and Campbell (1999) also showed that the two Hawaiian species are distantly related although *R. macraei* clustered closely to a number of other species of subg. *Idaeobatus*. Aside from a close affinity with *R. spectabilis*, *R. hawaiensis* did not cluster with any distinct group. They found a high similarity in the sequences of *R. macraei* and *R. ursinus*, a common blackberry hybrid species along the North American West Coast from northern Mexico to British Columbia. Recent investigations by Alice (2002) with ITS sequences likewise showed a close association between *R. ursinus* and *R. macraei*.

Chromosome studies have shown that the base number in *Rubus* is $x = 7$ (Graham and McNicol 1995, Iwatsubo et al. 1995, Hummer 1996, Thompson 1997). Although polyploidy is common, there is little evi-

dence of aneuploidy in the genus. Species of subg. *Idaeobatus* are largely diploid ($2n = 14$) with occasional reports of tetraploids ($2n = 28$) and rarely higher numbers (Thompson 1997). Chromosome counts performed on *R. hawaiensis* have confirmed its diploid configuration (Thompson 1995), but to date there have been no counts of *R. macraei* available. Subg. *Rubus* has a high frequency of polyploidy among the many species that have been reported, and *R. ursinus* has counts ranging from hexaploid to dodecaploid ($2n = 42$ to 84) (Thompson 1997).

The focus of the research reported here was to explore the biogeography of *Rubus* species in the northern Pacific region and to clarify relationships among these species by analyzing *ndhF* sequence variation. The *ndhF* gene has recently become of interest for systematic analyses and was used here to examine relationships among species of *Rubus*. Studies have shown this gene to be two to three times more variable than *rbcL* and twice its length (Hiratsuka et al. 1989, Sugiura 1989, Clark et al. 1995, Olmstead and Reeves 1995, Scotland et al. 1995, Terry et al. 1997). The *ndhF* gene has since been used to answer phylogenetic questions in both monocots and dicots (Olmstead and Sweere 1994, Clark et al. 1995, Kim and Jansen 1995, Olmstead and Reeves 1995, Scotland et al. 1995, Neyland and Urbatsch 1996, Smith et al. 1997, Terry et al. 1997) as well as previously in *Rubus* (Howarth et al. 1997).

Specific aims of this study were to address three questions. First, what are the closest relatives of *R. macraei* and from what geographic region did it originate? Second, what is the extent of morphological convergence that has occurred between *R. hawaiensis* and *R. macraei?* Third, how phylogenetically distinct are the *Rubus* species found in disjunct regions of Asia and North America, and what general trends of evolutionary biogeography of *Rubus* throughout the Pacific are evident? If these two regions of the Pacific are clearly separated biologically (i.e., no intercontinental dispersal), the discovery of a close relationship between the Hawaiian species and a continental species should lend substantial evidence toward the origin of these species.

MATERIALS AND METHODS

DNA Extraction and Sequence Analysis

Following the classification scheme of Focke (1910, 1911, 1914), 30 species of *Rubus*, mostly from the Pacific region and representing five subgenera, were sampled (Table 1). An emphasis was placed on the subg. *Idaeobatus* due to Focke's inclusion of both Hawaiian endemic species in this subgenus. Species were chosen for their proximity to migratory bird flight routes, chromosome number, and use in previous studies. *Rubus assamensis* (subg. *Malachobatus*) and *R. tricolor* (subg. *Dalibardastrum*) were included because these species clustered close to *R. macraei* in the ITS analysis of Alice and Campbell (1999). Morgan et al. (1994) have shown *Fallugia* to be closely related to *Rubus*, thus *F. paradoxa* was used for outgroup comparison. DNA samples were obtained through collaboration with other researchers or from extraction of fresh leaf material (Table 1). Total DNA was extracted following the protocol described by Doyle and Doyle (1987), with a few minor modifications (Morden et al. 1996). Samples were purified by cesium chloride density gradient centrifugation followed by butanol extraction to remove the ethidium bromide. Samples were dialyzed in four changes of TE (10 mM Tris, 1 mM EDTA, pH 8.0), accessioned into the Hawaiian Plant DNA Library (HPDL), and stored at −20°C (Morden et al. 1996, Randell and Morden 1999).

The 3′ portion of the *ndhF* gene (corresponding to nucleotide 1024 of tobacco *ndhF* and a region downstream from the *ndhF* terminus and internal to *orf350*) was amplified from each species using the polymerase chain reaction (PCR). The 3′ region of the gene has been shown to evolve more rapidly than the 5′ end (Olmstead and Sweere 1994, Clark et al. 1995, Kim and Jansen 1995, Neyland and Urbatsch 1996, Howarth et al. 1997). Amplifications consisted of 100 µl reactions with 1 × buffer, 2 mM MgCl, 0.128 mM dNTP, 1 µM of each amplifying primer, 2 units Taq DNA polymerase (Fisher Biotech), and ca. 50–100 ng DNA. Cocktails were exposed to the following amplification con-

TABLE 1

Accessions of *Rubus* Used in This Study (Chromosome Numbers, Natural Range of the Species, Source Location, and Accession Numbers in the Hawaiian Plant DNA Library [HPDL] [Morden et al. 1996, Randall and Morden 1999])

Species	Subgenus	Chromosome No.[a]	Natural Range	Accession No.	Source Location[b]	HPDL
R. parviflorus Nutt.	*Anoplobatus*	2*x*	W. North America	*Luffman 217*	CCPPC	1173
R. tricolor Focke	*Dalibardastrum*	4*x*	China	*L. Alice sn*[c]		1453
R. corchorifolius L. f.	*Idaeobatus*	2*x*	Eastern Asia	USDA-1770.001	NCGR	1344
R. coreanus Miq.	*Idaeobatus*	2*x*	Eastern Asia	USDA 1438.01	NCGR	1339
R. ellipticus Smith	*Idaeobatus*	2*x*	Eastern Asia	*Howarth s.n.*	Hawai'i, HI	270
R. hawaiensis A. Gray	*Idaeobatus*	2*x*	Hawaiian Islands	*Gardner s.n.*	Hawai'i, HI	208
R. hirsutus Thunb.	*Idaeobatus*	2*x*	Eastern Asia	1040.001	NCGR	1340
R. idaeus L.	*Idaeobatus*	2*x*				
subsp. *idaeus*	*Idaeobatus*	2*x*	Europe	*Luffman 213*	CCPPC	1172
subsp. *melanolasius* (Dieck) Focke	*Idaeobatus*	2*x*	North America	*Morden 1206*	Utah	293
R. ikenoensis A. Leveille & Vaniot	*Idaeobatus*	2*x*	Japan	USDA-1421.001	NCGR	1347
R. illecebrosus Focke	*Idaeobatus*	2*x*	Japan	USDA-1422.001	NCGR	1348
R. leucodermis Douglas ex Torr. & Gray	*Idaeobatus*	2*x*	W. North America	USDA-12.002	NCGR	1345
R. macraei A. Gray	*Idaeobatus*	This study	Hawaiian Islands	*Gardner s.n.*	Hawai'i, HI	207
				Anderson s.n.	Maui, HI	1135
R. niveus Thunb.	*Idaeobatus*	2–3*x*	Eastern Asia	*Morden 1212*	Maui, HI	306
R. occidentalis L.	*Idaeobatus*	2*x*	E. North America	*L. Alice R16*[c]		1450
R. palmatus Thunb.	*Idaeobatus*	2*x*	Japan	USDA-2.001	NCGR	1346
R. phoenicolasius Maxim.	*Idaeobatus*	2*x*	Eastern Asia	*L. Alice 96-2*[c]		1451
R. pungens Cambess.	*Idaeobatus*	2*x*	Eastern Asia	USDA-46.002	NCGR	1349
R. rigidus Sm.	*Idaeobatus*	Unknown	South Africa	*Morris s.n.*	South Africa	1207
R. rosifolius Smith	*Idaeobatus*	2*x*	SW Pacific	*Morden 1182*	Kaua'i, HI	258
R. sachalinensis A. Leveille	*Idaeobatus*	4*x*	Eastern Asia	USDA-167.001	NCGR	1341
R. spectabilis Pursh	*Idaeobatus*	2*x*	W. North America	*Luffman 219*	CCPPC	1174
				Gardner s.n.	UH-Mānoa	209
R. strigosus (Michx.) Maxim	*Idaeobatus*	2*x*	North America	*Luffman 186*	CCPPC	1171
R. sumatranus Miq.	*Idaeobatus*	2*x*	S. Pacific and Asia	USDA-1668.000	NCGR	1343
R. assamensis Focke	*Malachobatus*	4*x*	Eastern Asia	USDA-1701[c]	NCGR	1447
R. argutus Link	*Rubus*	2–3*x*	E. North Amercia	*Gardner s.n.*	UH-Mānoa	1133
R. cuneifolius Pursh	*Rubus*	2–4*x*	E. North America	*Morris s.n.*	South Africa	1206
R. ursinus Cham. & Schldl.	*Rubus*	6, 8–12*x*	W. North America	*L. Alice 47–90*		
Fallugia paradoxa (D. Don) Endl.	—	4*x*	W. North America	*7943*[d]	RSABG	

[a] From Thompson (1997).

[b] NCGR, USDA-ARS National Clonal Germplasm Repository, Corvallis, Oregon; CCPPC, Canadian Clonal Plant Propagation Centre, Ontario, Canada; RSABG, Rancho Santa Ana Botanic Garden, California.

[c] DNA samples provided by Lawrence Alice, Western Kentucky University.

[d] DNA sample provided by Rodger Evans, University of Toronto. Flowering voucher at Royal Ontario Museum Herbarium (TRT).

TABLE 2

Primers Designed for This Study, Their Location Within the Gene, Sequence, Length, and GC Content

Primer	Relative Tobacco Coordinates	Sequence	Length	GC Content
F 1417	1417–1440	TTCTATTCAATGTCTCTATGGGGT	24	9/24
F 1696	1696–1717	GGAATTCCTTTCTTCAATCAGG	22	9/22
F 1982	1982–2003	TATATGATTGGTCATATAATCG	22	6/22

Note: Primers were designed to increase amplification efficiency and to more effectively cover the area being sequenced. Primer coordinates reflect the corresponding positions in the tobacco *ndhF* sequence (Olmstead et al. 1993).

ditions: one cycle of 3 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C; 28 cycles of 1.5 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C with 5 sec added per cycle; and one cycle of 1.5 min at 94°C, 1.5 min at 50°C, and 6 min at 72°C. Amplification primers were the same as those used previously for *Rubus* (Howarth et al. 1997). PCR products (4 μl) were visualized on 1.5% agarose gels to assure proper amplification. The remaining PCR product was purified in Ultrafree-MC filters (Millipore Corporation, Bedford, Massachusetts) using the manufacturer's specifications. Concentrations of purified products were determined using UV spectroscopy (Sambrook et al. 1989).

The dideoxy sequencing method of Sanger et al. (1977) utilized *Rubus*-specific sequencing primers designed for increased sequencing efficiency and to more effectively cover the area being sequenced (Table 2). Final solutions containing 50–100 ng of purified double-stranded PCR product and 3.2 picomoles of primer were sequenced at the University of Hawai'i Biotechnology/Molecular Biology Instrumentation and Training Facility, using an ABI 373A automated sequencer with Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Biosystems Inc., Foster City, California). The computer-generated sequences were compared visually with the resultant chromographs to verify results.

Sequences were visually aligned and comparisons among sequences made using the Wisconsin Genetics Computer Groups Sequence Analysis Package. Percentage similarities were calculated using the "Gap" function included in that package. Insertion/deletion events (indels) and indeterminable bases were coded as missing data. Neighbor joining and parsimony analyses were performed using PAUP 4.0b8 (Swofford 1996). Parsimony searches were conducted with all characters weighted equally. Starting trees were found by random stepwise addition with 10 replicates using the quick swapping option on all trees. MULTREES and the TBR branch swapping algorithm was in effect for the search. Bootstrap analyses (Felsenstein 1985) were done with 1000 replicates for both neighbor joining and parsimony searches and decay analysis (Bremer 1988) for parsimony searches. Biogeographic distributions among species were evaluated using MacClade 3.0 (Maddison and Maddison 1992).

Cytological Analysis

The chromosome number of *R. macraei* was determined from mitotic configurations in root tips that were excised and pretreated in a saturated aqueous solution of paradichlorobenzene (PDB) for ca. 5 hr at 4°C. The root tips were then fixed in absolute alcohol:glacial acetic acid (3:1) at room temperature for 2 hr and stored at −20°C. For microscopic analysis, the root tips were hydrolyzed in concentrated HCl:95% EtOH (1:1) for 5 min, rinsed in tap water, and squashed on a microscope slide in a drop of acetocarmine mixed with Hoyer's medium (Beeks 1955). Cells with well-spread, countable chromosomes at metaphase morphology were found in six preparations.

FIGURE 1. Alignment of the terminal 18 bp and a portion of the downstream sequence of *Rubus ndhF*. Underscored bases are the stop codon for each species. Dashes (–) are missing bases (gaps) in the aligned sequence. Single letter abbreviations for amino acids are given on the first line corresponding to the *R. coreanus* DNA sequence. The arrow in the *R. palmatus* sequence corresponds to the possible position of a 3-bp gap.

RESULTS

Sequence Analysis

Approximately 1200 base pairs (bp) were sequenced, beginning with base 1023 (corresponding to the tobacco sequence [Olmstead et al. 1993]) and extending beyond the stop codon. The resulting aligned sequence was 1179 bp in length. The first 242 bases were excluded from further analysis, because complete sequences in this region were only available for half of the species. There were only six base changes shared by two or more species that were found within this region, and only three of these were informative in clades that were well defined by the remainder of the sequence. The region beyond the stop codon was omitted from most analyses because of frequent indels resulting in dubious alignment of this region. The third residue of the stop codon, or this relative position in the alignment, was the last base included in the phylogenetic analysis. The remaining 776 bases were used in the phylogenetic analyses, revealing 141 variable sites, 41% of which were phylogenetically informative.

Although largely not used in the phylogenetic analysis, the sequence in the region of the stop codon is of evolutionary interest (Figure 1). All species strongly diverged after the stop codon and seven species had mutations at or near the stop codon. Three species, *R. palmatus*, *R. assamensis*, and *R. tricolor*, had modifications to the synonymous stop codon (TAA to TAG or TGA) that did not change the length or composition of the protein. *Rubus palmatus* also had four other point mutations immediately preceding the stop codon that are unique to this species. An equally parsimonious alignment is hypothesized with a 3-bp deletion (indicated by the arrow in Figure 1) resulting in a small gap that would shift the stop codon back one codon position relative to the alignment given here. All species of subg. *Rubus* plus *R. macraei*, *R. rigidus*, and *R. rosifolius* share a deletion of a single base pair immediately following the stop codon. Deletions of resi-

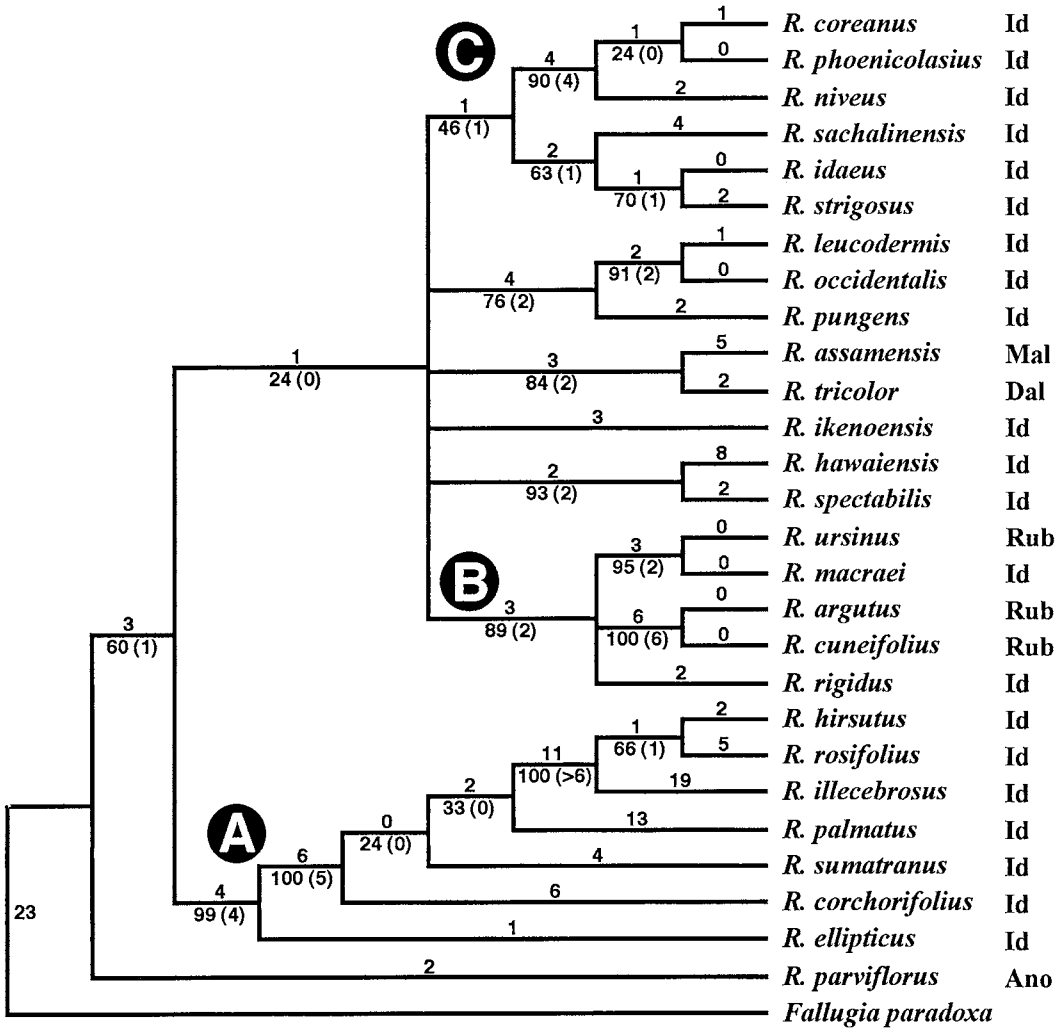

FIGURE 2. Phylogenetic relations among *Rubus* species based on *ndhF* sequence divergence using Wagner parsimony. One of 788 equally most parsimonious trees of 169 steps. Consistency index was 0.894 and homoplasy index was 0.106. Numbers above branches are branch lengths; numbers below branches are bootstrap values with 1000 replications and decay values (in parentheses). Letters A, B, and C refer to clades discussed in the text. Subgenera to which species belong are labeled as follows: Ano, *Anoplobatus;* Dal, *Dalibardastrum;* Id, *Idaeobatus;* Mal, *Malachobatus;* Rub, *Rubus.*

dues at or preceding the stop codon in *R. niveus*, *R. hawaiensis*, *R. spectabilis*, and one accession of *R. macraei* from Maui caused a frame shift resulting in an alteration of the position of the stop codon in these species. The deletions in *R. hawaiensis* and *R. spectabilis*, and that of *R. macraei*, although resulting in a nearly identical sequence and position of the stop codon, are believed to have occurred separately and have been discussed in detail by Howarth et al. (1997).

Parsimony analysis revealed 788 equally most parsimonious trees of 169 steps with a consistency index of 0.800 (excluding uninformative characters) and a retention index of 0.882 (Figure 2). The most basal species in

the analysis was *R. parviflorus* (subg. *Anoplobatus*) although its separation from the remainder of *Rubus* was weakly supported. There were three clusters of species present, and the relations depicted among them suggest that subg. *Idaeobatus* is polyphyletic. Clade "A" consists of a strongly supported group of seven Asian species in subg. *Idaeobatus*. The weakly supported sister group to clade "A" consists of two smaller clusters: clade "B" is strongly supported and includes all subg. *Rubus* species plus *R. macraei* and *R. rigidus*, and clade "C" consists of subg. *Idaeobatus* species including the cultivated raspberry *R. idaeus*. Other notable species combinations that are a part of the clade "B" and "C" polytomy include a clade with *R. hawaiensis* and *R. spectabilis* as well as a clade with *R. assamensis* (subg. *Malachobatus*) and *R. tricolor* (subg. *Dalibardastrum*). Although further resolution of these clades was not possible with this search, species in these clusters are closely related and their branches are strongly supported.

A strict consensus of all trees in the parsimony search caused those clades with a decay index of zero to collapse into a polytomy. All three labeled clades and the other species combinations are united at this polytomy but remain intact. The neighbor joining tree (Figure 3) was similar to that produced by parsimony. Although completely resolved, short branches in the tree are not supported, as indicated by the low or absent bootstrap values. These two trees would be nearly identical if branches with less than 50% bootstrap support were collapsed.

The relationship between *Rubus macraei* and *R. ursinus* is close, as indicated by the high bootstrap value (95%) and nearly identical sequences (Table 3). It is also clear that *R. rigidus*, usually considered a member of subg. *Idaeobatus*, has stronger affinities to other subg. *Rubus* species, a relationship also found in ITS sequences (L. Alice, pers. comm.). It is of interest to note that there is more sequence divergence between *R. hawaiensis* and *R. spectabilis* than between *R. macraei* and *R. ursinus*.

A closer examination was made of "clade C" to clarify relationships among species and varieties of *R. idaeus*. Alignment of the region downstream from the stop codon is much more certain for these few taxa, and this region (all positions downstream from the stop codon in Figure 1) was used in the further analyses here. Neighbor joining (Figure 4) and parsimony analyses gave identical results that show Asian *R. sachalinensis* to be basal within this clade, followed by the European raspberry *R. idaeus*, and the two North American taxa (*R. strigosus* and *R. idaeus* subsp. *melanolasius*) forming a terminal clade. *Rubus idaeus* is paraphyletic in this analysis, and the relationships among these closely related species and subspecies should be further explored.

Chromosome Number

Root tip cells of *R. macraei* revealed 42 chromosomes in six separate preparations. The base chromosome number of $x = 7$ for *Rubus* has been previously reported and apparently characterizes all known populations of the genus (Graham and McNicol 1995, Iwatsubo et al. 1995, Hummer 1996, Thompson 1997). Thus, *R. macraei* is considered to be a hexaploid with $2n = 6x = 42$. It would be desirable, however, to confirm the pairing behavior of chromosomes at meiosis.

Morphological Comparison

The morphological differences and similarities of the two endemic Hawaiian *Rubus* species and their closest continental relatives are reviewed in Table 4. Most of the characteristics listed for the Hawaiian species are either identical to those of their continental counterpart or are within the variability of the North American species. However, there are a few important morphological similarities between the two Hawaiian species that are not found within the natural variation of the continental species.

Morphological features that tend to vary between the two geographic species pairs are hair density, flower color, fruit shape, and fruit size. The concentration of trichomes seen on the stems and on the adaxial and abaxial leaf surfaces of both Hawaiian *Rubus*

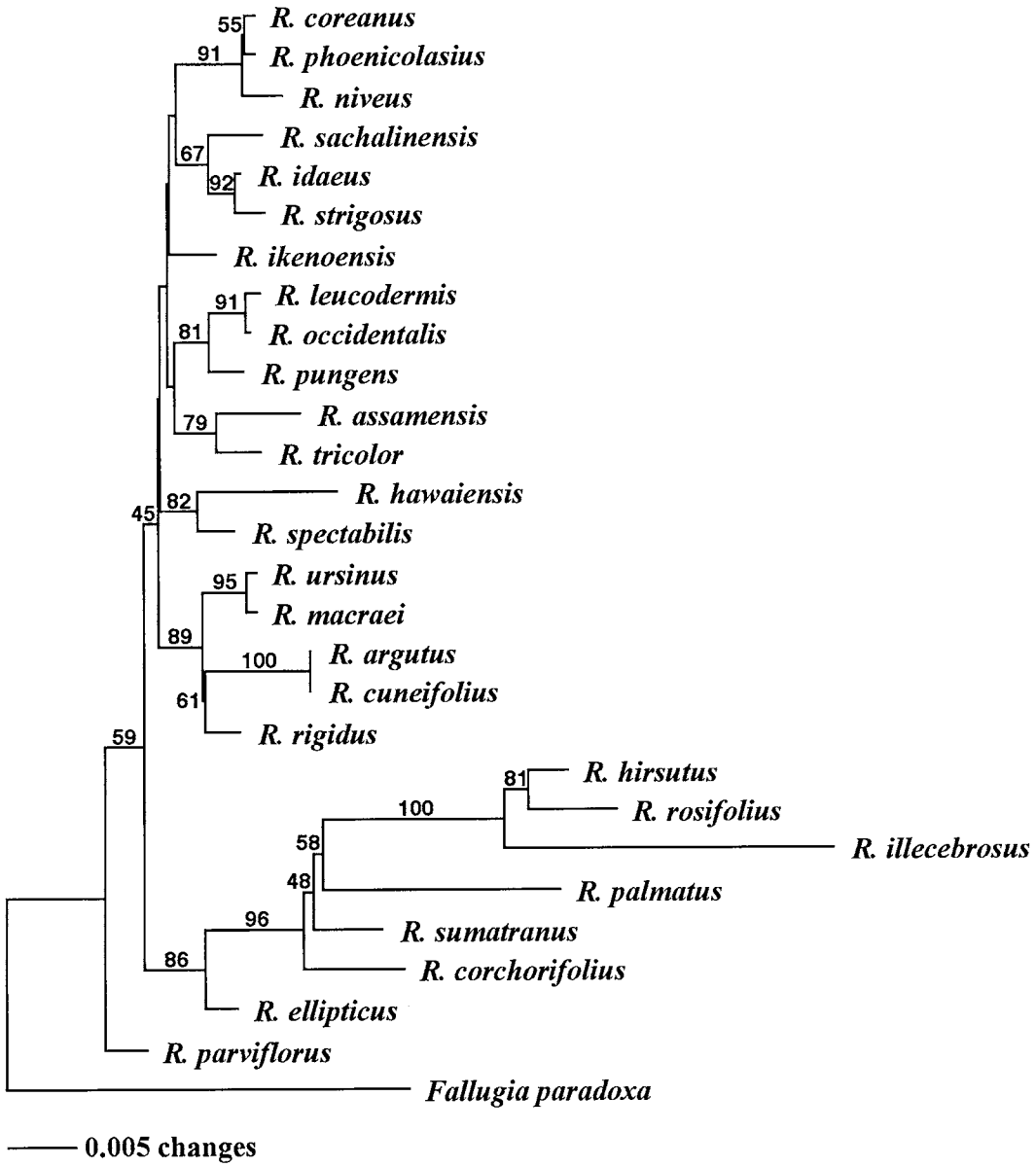

FIGURE 3. Phylogenetic relationships among *Rubus* species based on *ndhF* sequence divergence using neighbor joining. Numbers are bootstrap values associated with that branch based on 1000 replications.

species is greater than that seen on either of the closest continental ancestors. The size of the fruit is another distinctive feature that sets the Hawaiian species apart from their continental ancestors. Both Hawaiian species have very large fruit, ranging from 2.5 to 5 cm in length. This large size is not observed in the continental species, with both species having an upper size limit of 2 cm, an entire 0.5 cm less than the smallest Hawaiian fruits.

TABLE 3

Sequence Similarities (in Percentages) between the Two Hawaiian Species of *Rubus* and Their Closest Continental Ancestors

Species	2	3	4
1. *R. macraei*	97.9	99.9	98.5
2. *R. hawaiensis*	—	96.3	99.0
3. *R. ursinus*	—	—	97.2
4. *R. spectabilis*	—	—	—

Note: The approximate average percentage similarity between all of the species was 96.8.

The previous features were unique to the Hawaiian species, but there are other morphological features that may demonstrate convergence between them. Although there is little difference seen between *R. hawaiensis* and *R. spectabilis* in the comparison of flower color, there is a large difference seen between *R. macraei* and *R. ursinus*. *Rubus macraei* displays dark pink to rose-colored flowers, as does *R. hawaiensis*, thus differing from *R. ursinus*, which has white to pale pink flowers. This same pattern is seen in fruit shape, where *R. hawaiensis*, *R. spectabilis*, and *R. macraei* all produce ovoid fruit (Jepson 1925, Wagner et al. 1999), contrasting with *R. ursinus* that has oblong to ovate (Jepson 1925) or conical (Munz 1974) fruit.

DISCUSSION

Chromosome Number and Evolutionary Origin of R. macraei

Knowledge of chromosome numbers of the world's *Rubus* species is of interest in many fields of study including breeding, cytotaxonomy, evolution, and phylogenetics (Thompson 1997). Although the ploidy levels in some species groups are quite complex, the basic chromosome number of $x = 7$ has been found consistently throughout the world's *Rubus* populations as well as most subfamily Rosoideae sensu stricto to which *Rubus* belongs (Graham and McNicol 1995, Iwatsubo et al. 1995, Hummer 1996, Thompson 1997). The majority of subg. *Idaeobatus* species are diploid (79%), with some that are both diploid and triploid (13%) or allotetraploid (6%), and two species reported to have high ploidy levels (13x and 18x [Table 5]). *Rubus hawaiensis* is known to be diploid (Thompson 1995). However, if the taxonomic placement of *R. macraei* in subg. *Idaeobatus* is considered to be its true phylogenetic affinity, it is the only hexaploid known in the subgenus. In contrast, subg. *Rubus*, with which *R. macraei* clusters in this study, has a diverse array of chromosome numbers reported, and 75% of the species are tetraploid or of higher ploidy (Thompson 1997) (Table 5).

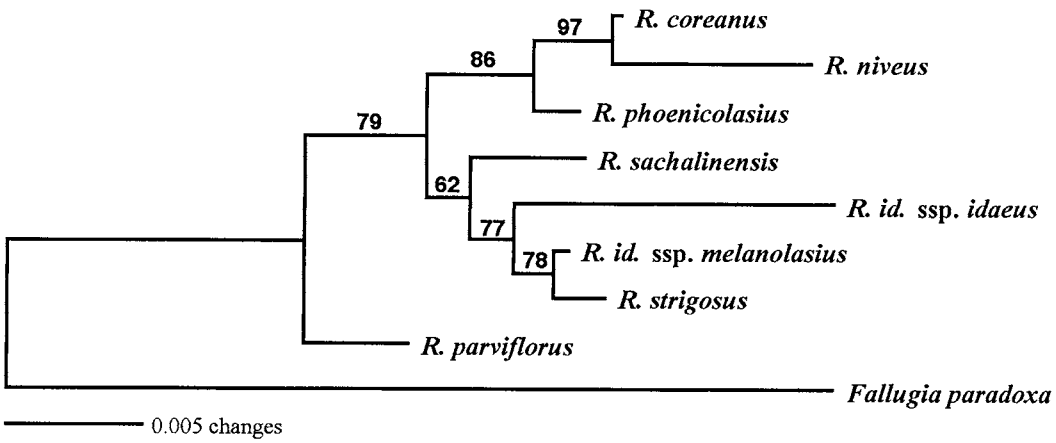

FIGURE 4. Phylogenetic relations among species in "clade C" with the expanded data set using neighbor joining. Numbers are bootstrap values associated with that branch based on 1000 replications. Data set also included the 70 sequence positions downstream from the predominant stop codon in Figure 1.

TABLE 4

Morphological Comparisons between the Two Endemic Hawaiian Species of *Rubus* and Their Closest Known Ancestors (Note the Difference in Hair Density, Petal Color, Fruit Shape, and Fruit Size [Morphological Determinations from Hedrick 1925, Jepson 1925, Abrams 1944, Munz and Keck 1959, Munz 1974, Hickman 1993, and Wagner et al. 1999])

Trait	*R. hawaiensis*	*R. macraei*	*R. spectabilis*	*R. ursinus*
Habit	Shrub	Shrub	Shrub	Clambering
Bark	Shredding	Shredding	Shredding	N/A
Stems	Erect	Prostrate	Erect	Variable
Primocanes/floricanes	Similar	Similar	Similar	Different
Prickles	Slender	Slender	Small or unarmed	Straight, bristlelike
Hairs	Moderately dense	Dense	Sparse	Glabrous to pubescent
Hair type	Pilose	Tomentose	Glabrous to pilose	Tomentose
Leaf type	Trifoliate	Trifoliate	Trifoliate	Simple, trifoliate, or pinnate
Leaflet number	3	3	3	(1) 3–5 (7)
Leaf thickness	Thin	Somewhat thick	Thin	Not noted
Leaf shape	Ovate	Ovate	Ovate	Ovate
Terminal lf. length (cm)	8–15 (21)	5.1–8.5 (11)	3–10 (17)	(2) 5–12
Terminal lf. width (cm)	5–10 (15)	3–6 (9)	4–10	Almost as broad
Leaf hairs (adaxial)	Pilose to tomentose	Sparsely pilose	Glabrous to pubescent	Glabrous to pubescent
Leaf hairs (abaxial)	Tomentose	Tomentose	Glabrous to pilose	Glabrous to canescent
Leaflet margins	Irregularly serrate	Irregularly serrate	Irregularly serrate	Irregularly serrate
Leaflet lobing	Weak	Weak	Shallow	Shallow
Petiole length (cm)	(1.3) 2.5–3.5	0.4–3	1–9	2–5
Petiole hairs	Tomentose	Tomentose	Glabrous	Tomentose
Petiole prickles	Occasional	Occasional	Usually unarmed	Prickly
Stipule presence	Present	Present	Present	Present
Stipule shape	Linear to lanceolate	Linear to lanceolate	Linear	Linear lanceolate
Stipule length (cm)	1–1.8	0.8–1.5	Not noted	Not noted
Inflorescence	Corymbose	Corymbose	Usually single	Corymbose
Flowers per inflor.	1–4 (8)	1–8	1–2 (4)	2–15
Flowers	Perfect	Perfect	Perfect	Dioecious
Pedicel length (mm)	13–30	8–55	Not noted	"Long"
Pedicel hairs	Pilose to tomentose	Tomentose	Glabrous	Gray tomentose
Sepal length (mm)	8–17	10–16	10	Not noted
Sepal hairs (adaxial)	Tomentose	Tomentose	Adpressed hairs	Tomentose
Sepal hairs (abaxial)	Tomentose	Tomentose	Adpressed hairs	Tomentose
Sepal apex	Acuminate to caudate	Acuminate to caudate	Long	Short acuminate
Petal color	Dark pink to rose	Dark pink to rose	Reddish purple	White to pale pink
Petal shape	Obovate	Obovate	Obovate to elliptic	Obovate to elliptic
Petal length (mm)	13–18	13–16	10–15 (20)	5–25
Fruit color	Yellow or red to dark purple	Dark red to dark purple	Yellow or red	Dark purple to black
Fruit shape	Ovoid	Ovoid	Ovoid	Oblong to spheric
Fruit length (cm)	2.5–5	2.5–4	1.5–2	Up to 2

TABLE 5

Ploidy Levels of the 11 Currently Accepted Subgenera of the Genus *Rubus*

Subgenus	Species in Subgenus	Species Counted	Ploidy Levels
Anaplobatus	6	4	2*x*
Chamaebatus	6	3	2*x* (1), 6*x* (2)
Chamaemorus	1	1	6*x*
Comaropsis	2	1	4*x*
Cylactis	16	10	2*x* (6), 2*x* & 3*x* (1), 2*x* & 4*x* (2), 4*x* (1)
Dalibarda	5	2	2*x*
Dalibardastrum	12	3	4*x* (2), 6*x* (1)
Idaeobatus	135	70	2*x* (55), 2*x* & 3*x* (9), 4*x* (4), 13*x* (1), 18*x* (1)
Malachobatus	127	40	4*x* (27), 6*x* (5), 8*x* (6), 14*x* (2)
Micranthobatus	12	5	4*x*
Orobatus	18	6	6*x* (6)
Rubus	100s to 1000s	312	2*x* (20%), 3*x* (5%), 4*x* (60%), 5*x* (3%), 6*x* (5%), 7*x* (1%), 8*x* (2%), 9*x* (2%), 10*x* (1%), 11*x* (1%), 12*x* (2%)

Note: Number of species, or percentage thereof, for given ploidy levels are in parentheses. Data extracted from Thompson (1997).

The hexaploid nature of *R. macraei* reported here is relevant to the assessment of the relationship of *R. macraei* and *R. ursinus*. *Rubus ursinus* is thought to be of hybrid origin, with chromosome numbers ranging from hexaploid to dodecaploid. Darrow (1955) considered it to be the result of a cross between species of subgenera *Rubus* and *Idaeobatus*, although others (Brown 1943, Jennings 1995) did not consider subg. *Idaeobatus* species to be involved in its formation. Sequence analysis indicates that there is incongruence in the placement of these species. Phylogenetic analysis using the nuclear ribosomal RNA ITS placed *R. ursinus* and *R. macraei* with other species of subg. *Idaeobatus* (*R. leucodermis* and *R. occidentalis* [Alice and Campbell 1999, Alice 2002]). However, results of this study demonstrate that *R. ursinus* and *R. macraei* are closely related to other members of subg. *Rubus* and distantly related to *R. leucodermis* and *R. occidentalis*. These findings support the hypothesis that *R. ursinus* is of hybrid origin between subgenera *Idaeobatus* and *Rubus*.

The close genetic affinity of *R. macraei* to *R. ursinus* (similarly high ploidy level and high sequence identity of both ITS and *ndhF*) suggests that these species may have descended from a common ancestor following this hypothesized hybridization and subsequent polyploidization. It is likely that the original hybrid polyploid dispersed to and colonized the Hawaiian Islands and eventually evolved into what is now recognized as *R. macraei*. Whether this original colonist was in fact *R. ursinus* or an extinct predecessor of *R. ursinus* is unknown. An alternative scenario is that *R. macraei* evolved as a new species in North America, dispersed to Hawai'i, and then became extinct in North America. However, this is a less parsimonious explanation and does not seem as likely. Alternatives that suggest that the hybrid precursor to *R. macraei* and *R. ursinus* occurred in the Hawaiian Islands and then recolonized North America are unfounded.

Biogeography of the Included Rubus *Species*

The well-defined clade comprising seven Asian *Idaeobatus* species (Figure 5) suggests that, in general, there are few dispersal events connecting the populations of North America and Asia. It has long been thought that birds carried seeds of colonizing species in their digestive tract to the Hawaiian Islands after fruit consumption (Carlquist 1974), and this is the likely vector for the genus worldwide. No bird flight paths link the North American

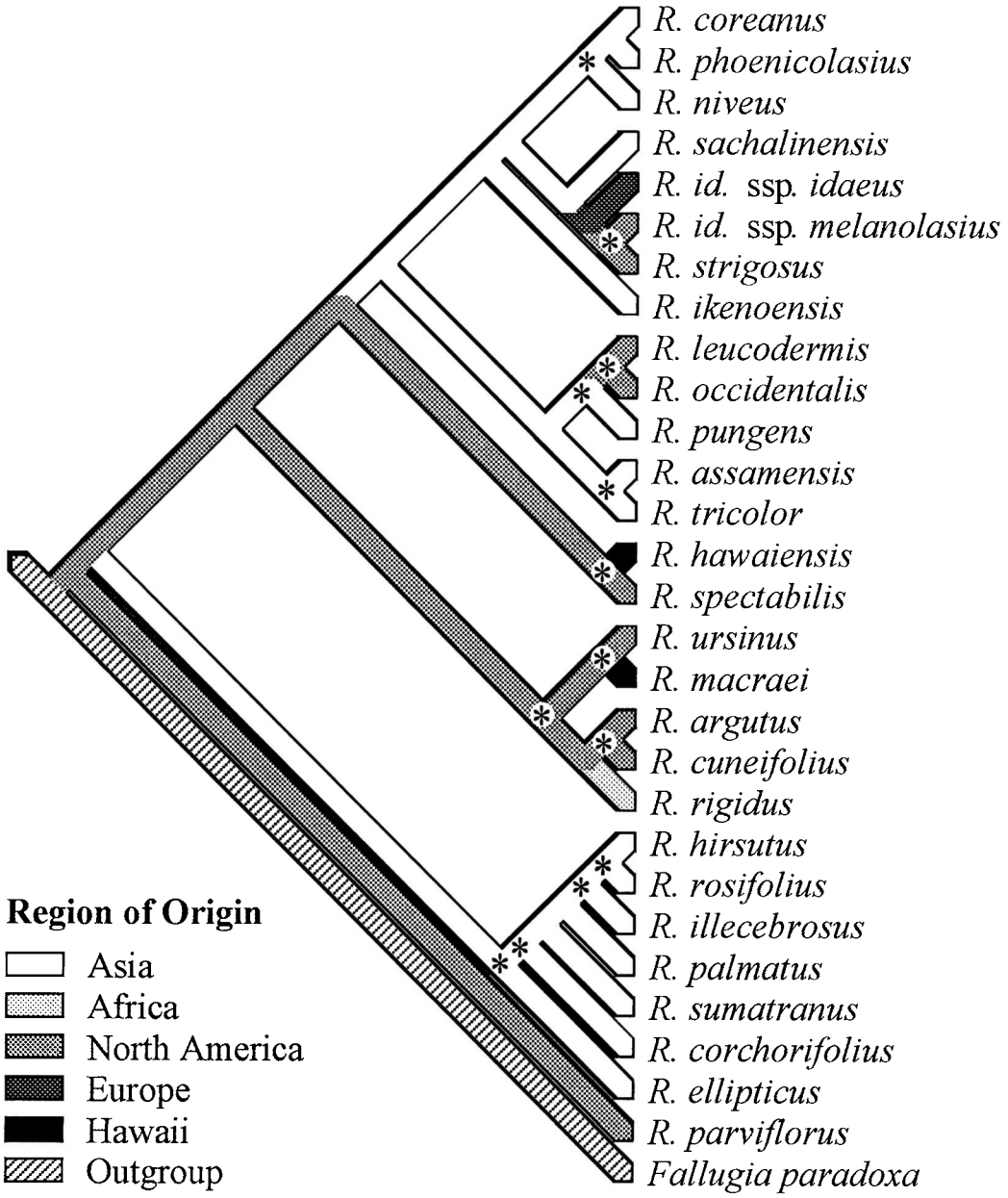

FIGURE 5. Biogeographic relations among species. Tree configuration is that of the neighbor joining analyses (Figures 3 and 4). Asterisks indicate those nodes for which bootstrap values were ≥75%.

and Asian continents, although flight paths between both continents and oceanic islands, including the Hawaiian Islands, are known (Berger 1972, Kloeckner et al. 1982, Williams and Williams 1988, Johnson et al. 1989, Gwinner 1990). The results of this study strongly point toward North America as the likely point of origin for both Hawaiian

endemics. It is important to note that the ranges of the continental *Rubus* species do not necessarily overlap with a migrant bird route, though there are frequent sightings each year of North American birds not usually found in the Islands (Conant et al. 1991).

The origin of the widespread genus *Rubus* has been speculated to be western North America or far eastern Asia (Alice and Campbell 1999), although the center of diversity of the genus is in Southwest China (Lu 1983, Gu et al. 1993). An Asian origin is also consistent with results in this study for *R. idaeus*, an economically important raspberry, and its close relatives *R. sachalinensis* and *R. strigosus*. *Rubus strigosus* and *R. sachalinensis* were both previously classified as subspecies of *R. idaeus* by Focke (1910, 1911, 1914) and have since been elevated to species rank. The Asian species (*R. sachalinensis*) was basal to these species in the clade, and the North American taxa (*R. idaeus* subsp. *melanolasius* and *R. strigosus*) were most derived. This distribution is consistent with the hypothesis that this lineage originated in Asia with a *R. sachalinensis*-like ancestor that migrated across the continent to Europe before colonizing North America. This broadly dispersed species that is found throughout the Northern Hemisphere is most closely related to an Asian group of *Idaeobatus* species (Figure 5), suggesting an Asian origin for this economically important species.

Extent of Convergence between the Hawaiian Species

The comparison of a large set of commonly noted morphological features of the two Hawaiian *Rubus* species and their continental ancestors reveals a small number of features in the Hawaiian species that are not seen within the natural variation of the mainland species. These features include hair density, flower color, fruit shape, and fruit size, all of which may be directly related to the survivability or reproductive success of the colonist in a new environment. Because these distinctive morphological characters are similar in both Hawaiian species and the species are subjected to similar environmental constraints in their sympatric distribution, the features linking them are likely due to morphological convergence.

It is interesting that *R. hawaiensis* displays more sequence divergence from *R. spectabilis* than *R. macraei* does from *R. ursinus*. However, the morphological differences between *R. macraei* and *R. ursinus* seem to be greater than those of the other pair. The *ndhF* chloroplast gene is likely not affected by the environmental pressures that were responsible for the changes in the features of the flowers, fruits, and pubescence. Since colonization in the Hawaiian Islands, morphological evolution has undoubtedly taken place at a much higher rate than has molecular evolution in the chloroplast. A conservative estimate of chloroplast rates of change is 1.0×10^{-9} synonymous substitutions per site per year (Wolfe et al. 1987) or 1 base change per 1000 sites over the past one million years. As such, few changes should be anticipated between the Hawaiian species and their North American counterparts. Greater sequence divergence between *R. hawaiensis* and *R. spectabilis* suggests that *R. hawaiensis* is a much earlier colonist to the islands than is *R. macraei*. This is borne out also by the broader distribution of *R. hawaiensis* (Kaua'i, Maui, and Hawai'i) than *R. macraei* (Maui and Hawai'i) in the island chain. A further caveat of this is that the *R. hawaiensis* colonist likely arrived at an older island such as Kaua'i (5.1 million years old) before Maui (1.3 million years old) or Hawai'i (0.43 million years old) were formed and later dispersed to the younger islands (island ages from Carson and Clague 1995), whereas the *R. macraei* progenitor colonized Hawai'i or Maui.

Conclusions

In this study we were able to answer four important questions on the origin and evolution of the Hawaiian endemic raspberries, *R. macraei* in particular. First, both Hawaiian endemic species, *R. macraei* and *R. hawaiensis*, are derived from separate colonists originating from western North America, and these colonists are of apparent different phyloge-

netic affinities. Second, there has been a tremendous degree of morphological convergence that has taken place between these two species such that each has characters markedly different from those of their continental ancestors. Until recently (Wagner et al. 1999), this has been interpreted as evidence for a very close relationship between these species. Third, genetic differences between each species and their closest continental ancestor suggest that *R. hawaiensis* colonized the archipelago long before *R. macraei*. Fourth, because of the polyploid nature of *R. macraei*, it is likely derived from a polyploid species not unlike *R. ursinus*. We speculate that it is allopolyploid in origin (hybridization with subsequent doubling of the chromosomes that has probably occurred twice). The origin of *R. ursinus* has also been questioned, and it may be of hybrid origin between subgenera *Idaeobatus* and *Rubus* (Alice 2002), but this hypothesis as well as additional evidence regarding the parentage of *R. macraei* needs further study.

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