

PHYLOGENETICS OF *BUCKLEYA* (SANTALACEAE)
BASED ON ITS SEQUENCES OF NUCLEAR
RIBOSOMAL DNA

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ABSTRACT. *Buckleya* (Santalaceae) is a hemi-parasitic, shrubby genus with two species in China, one in Japan, and one in the southeastern United States. Phylogenetic relationships among these species are controversial and have not been tested using molecular data. In this study we used sequences of the internal transcribed spacer region of nuclear ribosomal DNA to test previous phylogenetic hypotheses. Two sister species pairs are well supported: *B. distichophylla* plus *B. graebneriana*, and *B. lanceolata* plus *B. henryi*. Sequence differences and morphological characters support the recognition of *B. lanceolata* and *B. henryi*. Sequence divergence between *B. distichophylla* and *B. graebneriana* is twice as high as that between *B. lanceolata* and *B. henryi*. These results are most consistent with the treatment proposed by Carvell and Eshbaugh. Biogeographically, one of the Chinese species (*B. graebneriana*) is most closely related to the eastern North American species (*B. distichophylla*), while the other Chinese species (*B. henryi*) is allied with the Japanese species (*B. lanceolata*). Maximum likelihood analyses do not reject clock-like evolution of nrDNA ITS spacers in *Buckleya*, and divergence times may date to the Late Miocene and Pliocene.

Key Words: biogeography, *Buckleya*, Santalaceae, ITS, phylogeny, molecular clock

species locally common in east central China. In Japan, *B. lanceolata* (Siebold & Zucc.) Miq. is endemic to Honshu, Shikoku (Carvell and Eshbaugh 1982), and perhaps also to Kyushu (Hara 1959; Horikawa 1976). Both *B. henryi* Diels and *B. graebneriana* Diels are endemic to China. The former is more widely distributed (Gansu, Shaanxi, Anhui, and Zhejiang) than the latter (Gansu, Shaanxi, and Henan).

Species of *Buckleya* form haustoria on a variety of hosts, including *Taxus*, *Pinus*, *Chamaecyparis*, *Abies*, *Cryptomeria*, and some hardwoods, both in nature and in culture (Kusano 1902; Musselman and Mann 1978; Piehl 1965). *Buckleya* is of minor economic value; the fruits are rich in starch and have long been pickled and sold as a condiment in both China and Japan. The young leaves and the bark are reported to be poisonous and irritating to the skin (Howard 1984). *Buckleya* can be distinguished easily from other genera of the Santalaceae by its distichous leaf arrangement and elliptic or rounded fruits with apical foliaceous bracts. There is little doubt that *Buckleya* is monophyletic on the basis of these distinctive morphological traits, and this is also supported by a broader phylogenetic analysis based on nrDNA internal transcribed spacer (ITS) sequences (Li and Nickrent, unpubl. data).

Buckleya was discovered by Thomas Nuttall in 1816 (Nuttall 1818), and placed in the genus *Borya* Willd. (Oleaceae). *Borya* was later replaced by the conserved name *Forestiera* Poir. (Oleaceae). Torrey (1843), using material collected by S. B. Buckley, correctly noted its affinity to the Santalaceae. By the beginning of the twentieth century all four species had been described (Diels 1900; Miquel 1870).

Pilger (1935) placed the four species in two sections on the basis of bract persistence before fruit maturity: sect. *Eubuckleya*, characterized by deciduous bracts and including only the North American *Buckleya distichophylla*; and sect. *Quadriala*, defined by enlarged and persistent bracts and containing all three Asiatic species. Carvell and Eshbaugh (1982, p. 33), however, disagreed with Pilger's taxonomy and proposed instead that *B. henryi* and *B. lanceolata* are closely related and that "*Buckleya distichophylla* and *B. graebneriana* differ significantly from this group and do not appear to show a close affinity to each other."

In this study we tested both Pilger's and Carvell and Eshbaugh's hypotheses on interspecific relationships using sequences

Table 1. Specimens used in this analysis (S = staminate, P = pistillate). Vouchers are deposited in A.

Sample	Species	Collector	Locality	Note	GenBank#
1	<i>Buckleya henryi</i>	Boufford et al. 26105	Henan, China	S	AF291906
2	<i>B. henryi</i>	Boufford et al. 26290	Henan, China	P	AF291900
3	<i>B. henryi</i>	Boufford et al. 26201	Henan, China	P	AF291907
4	<i>B. lanceolata</i>	Yonekura et al. 95537	Fukushima, Japan	S	AF291904
5	<i>B. lanceolata</i>	Boufford et al. 25361B	Miyage, Japan	P	AF291903
6	<i>B. lanceolata</i>	R. Nishimura 909	Honshu, Japan	P	AF291902
7	<i>B. graebneriana</i>	Boufford et al. 26521	Henan, China	P	AF291899
8	<i>B. graebneriana</i>	Boufford et al. 26520	Henan, China	P	AF291905
9	<i>B. distichophylla</i>	Boufford et al. 5/4/98	Tennessee, U.S.A.	P	AF291901
10	<i>Thesium impletum</i>	Kim Steiner, s.n.	Natal, South Africa		AF291908

of the ITS region of nuclear ribosomal DNA. This region has been widely used for resolving relationships at lower taxonomic levels (Baldwin et al. 1995; Li and Donoghue 1999; Li et al. 1997; Liston et al. 1999). Fossils of *Buckleya* are unknown, but we have used sequence information to provide a range of estimated times of divergence.

MATERIALS AND METHODS

Plant material. Nine individuals were sampled from recently collected herbarium specimens of the four species of *Buckleya*: *B. distichophylla* (one pistillate individual), *B. graebneriana* (two pistillate individuals), *B. henryi* (one staminate and two pistillate individuals), and *B. lanceolata* (one staminate and two pistillate individuals). *Thesium impletum* Hill (Santalaceae) was included in the analysis for rooting purposes; DNA of this species was kindly provided by Dan Nickrent of Southern Illinois State University (Table 1).

Molecular techniques. About 20 mg of dried leaf material were removed from each of the specimens. The DNA was ex-

tracted using the Qiagen Plant DNAeasy Kit (Qiagen Inc., Santa Clarita, CA), following the manufacturer's instructions. The polymerase chain reaction (PCR) was performed using the primer pairs ITS4 and ITS5 of White et al. (1990) in a Perkin Elmer thermocycler. Each 25 μ l reaction included 50–100 ng genomic DNA, 2.5 μ l of 10 \times PCR buffer (Gibcol BRL, Grand Island, NY), 0.8 μ l of 50 μ M MgCl₂, 4 μ l of dNTP (2.5 μ M), 1 μ l of 10 μ M primers, 1–1.5 units of Taq polymerase (Gibcol BRL), and an appropriate amount of distilled water. The PCR thermocycler program consisted of 35 cycles of denaturation at 94°C (30 sec.), annealing at 55°C (60 sec.), and extension at 72°C (115 sec.), followed by a 7 min. extension at 72°C. The PCR products, identified by comparison with the low mass DNA size marker (Gibcol BRL), were purified in a 1% agarose gel, and were then extracted using a Qiagen gel extraction kit (Qiagen Inc., Santa Clarita, CA). Purified PCR product was used directly as a template for sequencing reactions using a cycle sequencing kit (Applied Biosystems, Foster City, CA) and primers ITS4 and ITS5 of White et al. (1990) and ITS3B (Baum et al. 1998). The sequences were obtained using an ABI 377 Automated Sequencer, analyzed using Sequencher 3.0 (Gene Codes Corp., Inc., Ann Arbor, MI), and then aligned manually.

Phylogenetic analyses. Parsimony analyses were conducted using the exhaustive search algorithm in PAUP* (version 4.0b3, Swofford 2000). All characters were equally weighted and character state changes were unordered. Gaps were treated as missing. The sister group of *Buckleya* is unclear, since intergeneric relationships in the Santalaceae have not been analysed in detail. However, in a phylogenetic analysis of Santalales, which included 12 genera of Santalaceae *s.l.*, *Thesium* appeared to be most closely related to *Buckleya* (Nickrent et al. 1998). *Thesium impeditum* was therefore included for rooting. Decay analysis (Bremer 1988; Donoghue et al. 1992) and five hundred replicate bootstrap analyses (Felsenstein 1985) were conducted to assess relative support for clades.

Estimate of divergence time. To determine whether the ITS region in *Buckleya* evolved in a clocklike fashion or not, the maximum likelihood ratio (MLR) test was conducted using PAUP* 4.0b3 following Baum et al. (1998). One individual from

Table 2. ITS sequence divergence in *Buckleya* (Kimura 2-parameter distance $\times 100$, calculated using PAUP*).

Species	1	2	3	4	5	6	7	8	9	10
1. <i>B. henryi</i>	–									
2. <i>B. henryi</i>	0.0	–								
3. <i>B. henryi</i>	0.1	0.0	–							
4. <i>B. lanceolata</i>	3.5	3.7	3.7	–						
5. <i>B. lanceolata</i>	3.5	3.7	3.7	0.0	–					
6. <i>B. lanceolata</i>	3.5	3.7	3.7	0.0	0.0	–				
7. <i>B. graebneriana</i>	13.3	13.3	13.3	13.7	13.7	13.7	–			
8. <i>B. graebneriana</i>	12.4	12.5	12.5	12.9	12.9	12.9	0.6	–		
9. <i>B. distichophylla</i>	13.9	13.9	13.9	13.5	13.5	13.5	7.1	6.5	–	
10. <i>Thesium impeditum</i>	28.8	28.8	28.8	30.9	30.9	30.9	32.3	31.6	31.2	–

each species was used in the MLR test and several models were applied to the reduced data set, including F81, F84, and HKY (Baum et al. 1998). There have been several estimated evolutionary rates for the ITS region. We used both the lowest (3.2×10^{-10} base substitution per site per year; Suh et al. 1993) and highest (7.8×10^{-9} base substitution per site per year; Sang et al. 1995) of these rates.

RESULTS

Sequences of all accessions varied in length between 668 and 732 base pairs (bp), and within *Buckleya* from 719 to 732 bp. Alignment of the ten sequences produced a data matrix of 755 characters, requiring 120 gaps, 20 of which were parsimony informative. There were 233 variable sites, of which 100 were parsimony informative. There was little sequence variation ($< 0.6\%$) within each species, while sequence divergence between species ranged from 3.5 to 13.9% (Table 2). Twelve informative indels, ranging from one to six bases, were found in the ITS sequences of *Buckleya* (Table 3). Two indels marked *B. graebneriana* (6 and 8 in Table 3), three marked *B. distichophylla* (3, 7, and 9), and six marked *B. lanceolata* and *B. henryi* (1–2, 4–5, and 10–12). GenBank accession numbers are listed in Table 1 and the matrix of aligned sequences is available from TreeBASE (<http://phylogeny.harvard.edu/treebase>; accession # M753).

A parsimony analysis generated a single shortest tree of 276 steps (Figure 1, CI = 0.96, RI = 0.95). Individuals of each species are united with strong bootstrap and decay support. Also well

Table 3. Informative indels in the ITS sequences of *Buckleya*.

Indel Site	1	2	3	4	5	6	7	8	9	10	11	12
	5	100	128	136	176	212	316	488	494	541	544	591
				-141		-213	-317	-489	-497		-545	

<i>B. distichophylla</i>												
	-	-	-	GAT-G	-	--	--	GT	----	-	--	-
<i>B. graebneriana</i>												
	-	-	-T	GACGAC	-	TG	GA	--	--CA	-	--	-
<i>B. henryi</i>												
	G	T	G	-----	A	--	CG	GT	AAGC	G	TG	A
<i>B. lanceolata</i>												
	G	T	G	-----	A	--	CG	GT	-ACA	G	TG	A

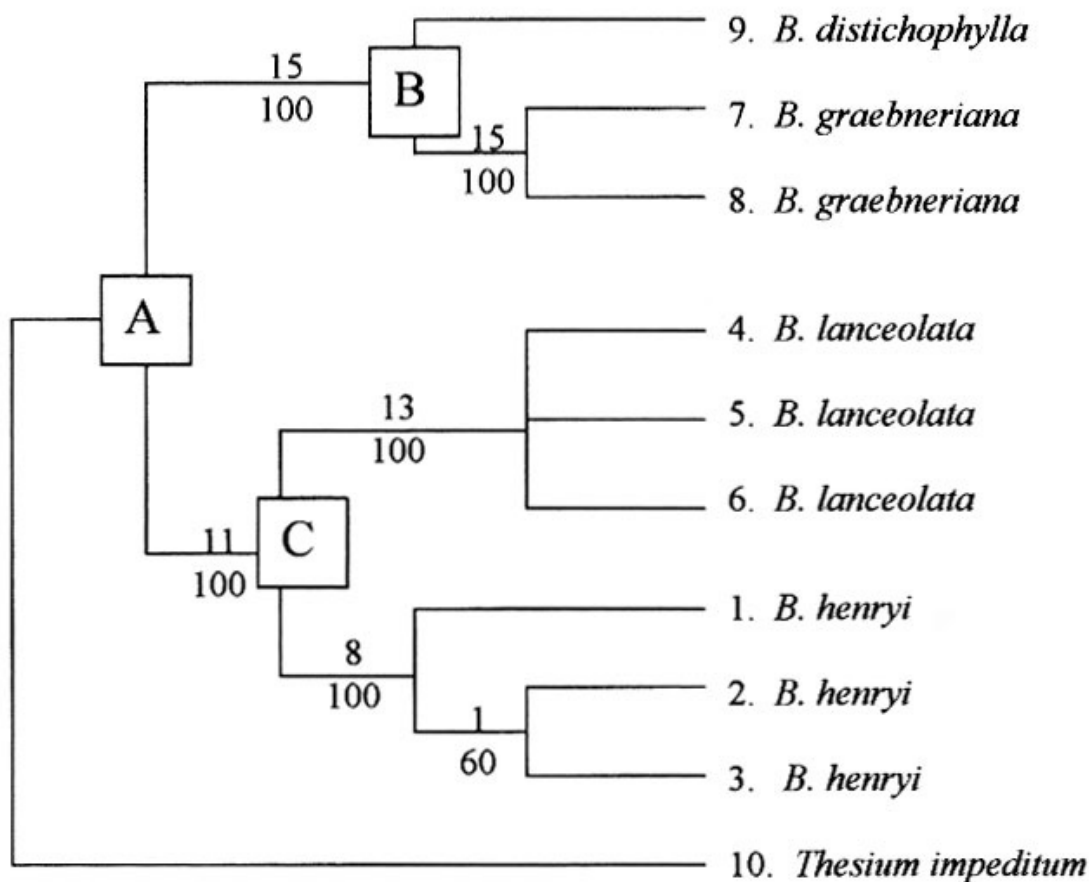


Figure 1. The single tree of 276 steps for *Buckleya* (CI = 0.96, RI = 0.95). Estimated times of divergence are as follows: A. 8.5 MYA, B. 5 MYA, and C. 2.4 MYA. Numbers above and below branches are decay values and bootstrap percentages, respectively.

supported are the species pairs of *Buckleya distichophylla*–*B. graebneriana* and *B. lanceolata*–*B. henryi*.

The maximum likelihood ratio test using different models did not reject the null hypothesis that the ITS region in *Buckleya* evolved in a clocklike fashion ($P > 0.05$). Using the rate of 3.2×10^{-10} base substitutions per site per year, the estimated time of divergence of the two species pairs, *B. distichophylla*–*B. graebneriana* and *B. lanceolata*–*B. henryi*, was 207 MYA. This seems unlikely as it predates the presumed radiation of angiosperms (Doyle 1998). Using the rate of 7.8×10^{-9} base substitution per site per year, the two species pairs, *B. distichophylla*–*B. graebneriana* and *B. lanceolata*–*B. henryi*, diverged from one another at around 8.5 MYA, and the estimated times of divergence between *B. distichophylla* and *B. graebneriana*, and between *B. lanceolata* and *B. henryi* were 5 and 2.4 MYA, respectively. These estimates seem more likely in view of the observation that rates of nucleotide substitution may be elevated in parasitic and hemi-parasitic plants (Nickrent et al. 1998).

DISCUSSION

ITS sequence divergence. Several studies have shown that the ITS region is not variable enough to resolve relationships among morphologically similar woody plants (e.g., *Hamamelis*, Li et al. 2000; Wen and Shi 1999; *Cercis*, Davis et al., unpubl. data). This does not appear to be the case for parasitic or hemi-parasitic plant groups, although the data are still limited. For example, Nickrent and Starr (1994) found that high substitution rates in ITS sequences of *Viscum* (Santalaceae) created difficulties in sequence alignment. In *Buckleya*, interspecific variation can be as high as about 14%, two to three times higher than in non-parasitic woody angiosperms (Baldwin et al. 1995; Wen and Shi 1999).

Phylogenetic relationships. Both Pilger (1935) and Carvell and Eshbaugh (1982) recognized four species in *Buckleya*, whereas Kiu and Lin (1988) treated *B. henryi* as a synonym of *B. lanceolata*. As pointed out by Carvell and Eshbaugh (1982), however, *B. henryi* is easily distinguished from *B. lanceolata*. In *B. henryi* the bracts are elliptic and twice as long as the fruit (vs. oblanceolate, and three times as long as the fruit in *B. lanceolata*);

the leaf tips are acuminate and are not markedly caudate (vs. lanceolate-acuminate and notably caudate in *B. lanceolata*); and the flowers are both terminal and axillary (vs. terminal, rarely axillary male inflorescences in *B. lanceolata*). Because *B. henryi* is restricted to China and *B. lanceolata* is endemic to Japan, the two species do not occur sympatrically, as reported by Kiu and Lin (1988). Our results show that there are few base substitutions within the same species (< 0.6% sequence divergence), but between different species sequence divergences range from 3.5 to 13.9%. The molecular evidence, which shows a 3.5–3.7% sequence divergence between *B. henryi* and *B. lanceolata*, provides further support for the recognition of the two species as distinct.

Pilger (1935) noted that the bracts and sepals of the Asiatic species of *Buckleya* remained closely attached to the fruit before fruit maturity, and as a result he recognized two sections: the North American *B. distichophylla* in section *Eubuckleya*, and all three Asiatic species in section *Quadriala*. Carvell and Eshbaugh (1982), however, noted that *B. distichophylla* and *B. graebneriana* share the attribute of non-striate ovaries, reflective of weaker vascular connections between the floral tube and the bracts (Smith and Smith 1943). Further, deciduous bracts occur not only in *B. distichophylla* but also in *B. graebneriana*, suggesting a close relationship of these two species. Additional characters supporting the close relationship of the two species pairs (*B. henryi*–*B. lanceolata* and *B. distichophylla*–*B. graebneriana*) include inflorescence position, and the coloration of the buds, branchlets, and scale tip of the leaves (Table 4). These observations led Carvell and Eshbaugh (1982) to reject Pilger's sectional classification of *Buckleya*. Nonetheless, they recognized the close relationship of *B. henryi* and *B. lanceolata*, as suggested by Pilger (1935).

In this study, the sequence divergence between *Buckleya distichophylla* and *B. graebneriana* is from 6.5–7.1%, which is much less than that between *B. distichophylla* and either *B. henryi* or *B. lanceolata* (13.5–13.9%). The two species pairs are also comparable in terms of number of base substitutions and indels supporting each pair. There are 30 base changes and 9 indels supporting *B. distichophylla*–*B. graebneriana*, and 38 and 8 for *B. henryi*–*B. lanceolata*. Thus, our results support Carvell and Eshbaugh's (1982) hypothesis on the interspecific relationships in *Buckleya*. It should be noted, however, that *B. henryi* and *B. distichophylla* are remarkably similar in overall aspect and ecolog-

Table 4. Morphological variation of species of *Buckleya* (Carvell and Eshbaugh 1982 and our observations).

Character	<i>B. distichophylla</i>	<i>B. graebneriana</i>	<i>B. lanceolata</i>	<i>B. henryi</i>
Habit	Shrub to 4 m tall, stems arching	Shrub to 2 m tall, stems erect	Shrub 2 m tall, stems erect	Shrub 2.5 m tall, stems arching
Trichomes	Unicellular, conical to papillate, sparse	Unicellular, soft needle-shaped, conical to papillate, dense	Multicellular, filiform, or unicellular, conical to papillate, dense	Unicellular, conical to papillate, sparse
Bark	Gray	Gray	Pale gray	Grayish brown
Bud color	Light tan	Light tan	Red-brown	Red-brown
Bud shape	Lanceoloid	Ovoid	Ovate	Ovoid
Upper leaves on branchlet	Lanceolate, apex often slightly curved, shortly attenuate	Elliptic to ovate, acuminate to acute, shortly attenuate	Lanceolate-acuminate, apex slightly curved, markedly caudate	Lanceolate, acuminate, tip slightly curved, attenuate to shortly attenuate
Lower leaves on branchlet	Elliptic, acute, shortly attenuate	Obovate, cuspidate, shortly attenuate	Elliptic, acuminate, caudate, shortly attenuate	Elliptic, acute, shortly attenuate, puberulent above
Leaf scale tip	Whitish-yellow	Whitish-yellow	Reddish-brown	Reddish-brown
Flowers	Male and female terminal	Male and female terminal	Male terminal and rarely axillary; female terminal	Male terminal and axillary; female terminal
Bract	Narrowly elliptic to lanceolate, deciduous, as long as or slightly longer than fruit	Lanceolate, deciduous, as long as or slightly longer than fruit	Oblanceolate to narrowly elliptic, persistent, three times as long as fruit	Narrowly elliptic, persistent, two times as long as fruit
Fruit	Not striate, ellipsoid to ovoid, puberulent	Not striate, fusiform, glabrous	8-striate, ellipsoid to ovoid, glabrous to densely puberulent	8-striate, ellipsoid to obovoid, glabrous to densely puberulent

ical preference. Both grow on moist slopes along streams, both attain roughly the same height, and both exhibit the same overall gestalt as they arch gracefully over rocky boulders amongst the surrounding vegetation. In contrast, *B. graebneriana* is a low, stiffly erect, densely colonial shrub adapted to more xeric conditions along the crests of ridges and on well drained slopes. *Buckleya lanceolata* appears more similar to *B. distichophylla* and *B. henryi* in general habit and habitat preference; we presume this is a retained ancestral condition.

Biogeography. Floristic affinities between eastern North America and eastern Asia have long been known, as exemplified by disjunct distributions of species in many genera (Boufford and Spongberg 1984; Hong 1993; Wen 1999). A general phylogenetic pattern has recently been identified based on a comparison of molecular phylogenies of seven disjunct plant genera (Wen 1999; Xiang et al. 1998, 2000). In these groups it appears that the eastern and western North American species are more closely related to one another than they are to Asian species. Several other recent phylogenetic studies indicate that the Xiang et al. (1998) pattern is part of several broader patterns when areas of endemism within Asia are taken into account. For example, the Japanese and North American species of *Hamamelis* are more closely related to one another than they are to the Chinese species (Li et al. 2000; Wen and Shi 1999). This is also true for species of *Viburnum* section *Pseudotinus* (Adoxaceae; Donoghue and Li, unpubl. data), and is consistent with an analysis of *Weigela* and *Diervilla* (Caprifoliaceae; Kim and Kim 1999). In contrast, in *Triosteum* (Caprifoliaceae) the Chinese and North American species are more closely related to one another than they are to the Japanese species (Gould and Donoghue 2000).

The results for *Buckleya* reveal yet another pattern, which is perhaps most similar to that seen in *Triosteum*. Here the single North American species, *B. distichophylla*, is linked with one of the Chinese species, *B. graebneriana*, while the Japanese species, *B. lanceolata*, is most closely related to the second Chinese species, *B. henryi*. The difference between the *Buckleya* and the *Triosteum* patterns is that in *Buckleya* the Japanese species, *B. lanceolata*, and the Chinese species, *B. henryi*, are differentiated both on the basis of morphological and ITS data, and generally, as here, are recognized as separate species. In contrast, popula-

tions of *T. sinuatum* Maxim. from Japan and from northeastern China do not appear to be differentiated and have been treated as conspecific.

The biogeographic history of *Buckleya* is difficult to understand without fossil evidence. We note, however, that estimated divergence times, based on several ITS nucleotide substitution rates, are in the late Miocene and Pliocene, when migration between Europe and eastern North America was difficult via the North Atlantic (Tiffney 1985), though perhaps still possible for fleshy-fruited, potentially bird-dispersed plants such as *Buckleya*. Exchange between the Old and New World was possible more recently across Beringia. Phylogenetic analyses of many additional disjunct taxa are needed in order to uncover general patterns that may implicate particular pathways and vicarious events.

KEY TO SPECIES

1. Scale tip of leaf whitish-yellow; fruits without distinct vascular strands, without longitudinal striations on ovary; bracts deciduous (2)
1. Scale tip of leaf reddish-brown; fruits with vascular strands evident as longitudinal striations on ovary; bracts persistent (3)
 2. Lower leaves elliptic, acute; upper leaves lanceolate with slightly curved apex; trichomes in inflorescence conical to papillate, ca. 0.1 mm long; plants solitary, stems arching *B. distichophylla*
 2. Lower leaves obovate, cuspidate; upper leaves elliptic to ovate, acuminate; trichomes in inflorescence elongate, ca. 0.5 mm long; plants colonial, stems erect *B. graebneriana*
 3. Bracts elliptic, 2 times as long as fruit; leaves not markedly caudate; trichomes sparse *B. henryi*
 3. Bracts oblanceolate, 3 times as long as fruit, leaves markedly caudate, trichomes dense *B. lanceolata*

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