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On the Identity of the Weedy Bittercresses (*Cardamine* : Brassicaceae) in United States Nurseries: Evidence from Molecules and Morphology

Angela R. Post, Regina Ali, Alexander Krings, Jenny Xiang, Brian R. Sosinski, and Joseph C. Neal*

Bittercress (Brassicaceae) is one of the most prolific and costly weeds of the container nursery industry. Bittercress accessions from container nurseries throughout the major production zones in the United States were examined and compared with herbarium specimens. The identity of these weedy bittercress species were further explored using sequences of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region and the nrDNA region for the COP1-interacting protein 7 (*CIP7*). Four species of bittercress were detected in the nursery industry of the United States, including New Zealand bittercress, hairy bittercress, flexuous bittercress, and little bittercress. The taxon referred to here as *Cardamine flexuosa* With. (flexuous bittercress) likely contains two genotypes previously reported as European *C. flexuosa* and Asian *C. flexuosa*. Phylogenetic relationships between the four species we examined, particularly in relationship to flexuous bittercress, were not fully resolved by the molecular evidence generated for this study. New Zealand bittercress is nonnative and does not appear in current keys to the species for the United States. Flexuous bittercress is also an alien species, which appears in some U.S. keys but not in all. To aid nurserymen and botanists in identification of these four closely related bittercress species, a key was developed and is accompanied by detailed descriptions and illustrations.

Nomenclature: Flexuous bittercress, *Cardamine flexuosa* With. CARFL; hairy bittercress, *Cardamine hirsuta* L. CARHI; Japanese bittercress, *Cardamine scutata* Thunb. CARSC; little bittercress, *Cardamine oligosperma* Nutt. CAROL; New Zealand bittercress, *Cardamine corymbosa* Hook *f.*

Key words: *Cardamine*, bittercress, *CIP7*, ITS, molecular genetics, taxonomy, key.

Bittercress, *Cardamine* (Brassicaceae), is a cosmopolitan genus with about 220 species worldwide, and they occur on every continent except Antarctica (Al-Shehbaz 1988). In North America, approximately 70 taxa from this genus are represented including accepted subspecies (USDA, NRCS 2008). The genus was first described by Linnaeus (1753), but the earliest complete treatment of the genus was the O. E. Schulz monograph in 1903. Schulz (1903) placed *Cardamine* in the tribe Arabideae, but there have been criticisms of the artificial nature of this placement and of his system of classification in general (Janchen 1942; Lihová et al. 2006). Al-Shehbaz (2006) recognized 25 tribes within Brassicaceae and moved *Cardamine* out of the tribe Arabideae, placing it in the tribe Cardamineae with 10 other genera. This Cardaminine alliance (about 340 species) includes amoracia (*Armoracia* spp. P. G. Gaertn., B. Mey. & Scherb.), rocket (*Barbarea* spp. W. T. Aiton), bittercress (*Cardamine* spp. L.), toothwort (*Dentaria* L.), iti (*Iti* spp. Garn.-Jones et. P.N. Johnson), iodanthus [*Iodanthus* spp. (Torr. & A. Gray) Steud.], gladecress (*Leavenworthia* spp. Torr.), watercress (*Nasturtium* spp. R.Br.), Virginia cress (*Planodes* Greene), yellowcress (*Rorippa* spp. Scop.), and selenia (*Selenia* spp. Nutt.). Despite extensive work on certain members of Brassicaceae, such as mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.] and cabbage (*Brassica oleracea* L.), studies are lacking on several important alliances within the family, including the Cardaminine alliance (Koch et al. 2003). Among these genera, *Cardamine* is a morphologically variable genus containing many weedy members that are self-compatible (Appel and Al-Shehbaz 2003; Kimata 1983) but may also hybridize readily with one another (Urbanska et al. 1997).

Hybridization and polyploidy have been reported extensively in *Cardamine* (Bleeker et al. 2002; Franzke et al. 1998; Franzke and Mummenhoff 1999; Franzke and Hurka 2000; Lihová et al. 2000; Marhold et al. 2002a, 2002b, 2004; Neuffer and Jancke 1997; Urbanska et al. 1997) and several natural hybrids exist. The genus possesses chromosome numbers ranging from $n = 8$ to $n = 128$—crinkleroot [*Cardamine diphylla* (Michx.) Alph. Wood] and cutleaf toothwort [*Cardamine concatenata* (Michx.) D. Schwarz], respectively. The latter is the highest chromosome number known for the family (Appel and Al-Shehbaz 2003). Morphologically, the genus *Cardamine* has several complexes that are difficult to distinguish, and at least one of these complexes, the cutleaf toothwort alliance, exists in North America (Sweeney and Price 2001). It is probable, based on hybridization and polyploidy among bittercress, that other complexes exist on the North American continent. Polyploid complexes are well documented within *Cardamine*, including cuckoo bittercress (*Cardamine pratensis* L.)(Lovkvist 1957), large bittercress (*Cardamine amara* L.)(Lovkvist 1957), and *C. concatenata* (Sweeney and Price 2001) groups. One study has reported that up to 58% of all known *Cardamine* species are exclusively polyploid and that 8% of the taxa include both diploid and polyploid populations (Lihová and Marhold 2003; Marhold et al. 2004). In cases where multiple cytotypes have been reported for a single species, it is possible that the taxonomy is unresolved or that these complexes represent dynamic systems that may eventually produce stable hybrids (Lihová and Marhold 2003).

The vegetative characters among *Cardamine* species are extremely plastic and vary according to their environment, making identification of these members difficult. The basic characters of weedy bittercress include a basal rosette of pinnately compound leaves that vary in size and shape, inflorescences in a raceme with flowers having four white petals and typically six stamens. Fruits are a forcefully dehiscent silique with yellowish to brown seeds > 1 mm in

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diameter. Currently, there is no widely followed sectional classification within the genus. Although Schulz (1903) divided the group into 13 sections, 6 of the sections are monotypic, and this sectional classification has not been upheld by phylogenetic studies (Lihová and Marhold 2003).

In *Cardamine*, several molecular approaches have been used to distinguish taxa at the species level. Nuclear ribosomal ITS sequences have been used most commonly across the family (Al-Shehbaz et al. 2006), including in studies on bittercress (Bleeker et al. 2002; Fain et al. 2005). It is important to note that ITS sequences frequently have multiple motifs within the same individual and require cloning to obtain homogeneous data sets (Al-Shehbaz et al. 2006). Noncoding regions of chloroplast DNA, including *trnT*, *trnL*, *trnF*, the *trnL* intron, and intergeneric spacers—*trnT–trnL*, *trnL–trnF*, and *psbA–trnH*—are also commonly used as markers for phylogenetic studies of Brassicaceae (Al-Shehbaz et al. 2006). Other studies have used the nuclear-encoded chalcone synthase gene (*Chs*) and the chloroplast gene *matK* (Koch et al. 2001). Relationships in the genus have been partially resolved using chloroplast DNA sequence data from the *trnL* intron and the *ndhF* gene (Bleeker et al. 2002; Sweeney and Price 2000). Franzke et al. (1998) partially resolved relationships among about 20 taxa within *Cardamine* using ITS sequences, chloroplast *trnT–L* gene, and the *trnL* intron. Franzke and Hurka (2000) also used a combined approach with allozymes, ITS sequencing, noncoding chloroplast DNA (cpDNA), and random amplification of polymorphic DNA (RAPD) to resolve relationships in the *C. pratensis* complex. Treatment of the *C. amara* complex was also supported using RAPD (Lihová et al. 2000). Most recently, Marhold used ITS and amplified fragment length polymorphism (AFLP) analysis to elucidate the relationships between groups using diploid taxa from polyploidy complexes (Marhold et al. 2004).

The most recent treatments of the genus have been in New Zealand (Pritchard 1957), Central and South America (Sjostedt 1975), the Carpathians and Pannonia, as described by Marhold (1994, 1995a,b), the Iberian Peninsula (Lihová et al. 2000), and China (Zhou et al. 2001). Several complex groups within bittercress have been addressed separately, including the *C. pratensis* complex (Franzke and Hurka 2000; Marhold 1994; Marhold 1996), and the *C. amara* complex, both of Eastern Europe (Lihová et al. 2000; Marhold 1995a; Marhold et al. 2002a), the *C. concatenata* alliance of Eastern North America (Sweeney and Price 2001), and the *C. flexuosa* complex (Lihová et al. 2006). There have been no comprehensive taxonomic studies of the genus for North America in the recent past. For this study, we focus on the identity of weedy *Cardamine* present in the United States container nursery trade.

Container nurseries have often been a foothold for introduced weed species and bittercress includes some of the most prolific weed species in the container nursery industry. Despite the availability of numerous herbicides labeled for bittercress control, it remains one of the most common and costly weeds in container nurseries (Bond and Turner 2006; Mathers 1996). In 2007, the U.S. Department of Agriculture (USDA) Interregional Research Project 4 (IR-4) Ornamental Horticulture survey (USDA 2007) reported bittercress to be the most prominent weed across the United States in greenhouse and nursery production, and the third most prominent weed in landscapes. A single plant is capable of producing thousands of seeds and projecting them 0.5 to 2.5 m from the mother plant. Seeds also become sticky when wet, which aids in their dispersal (Salisbury 1961). Bittercress is problematic for herbaceous annual and perennial nursery crops because it can complete a life cycle in about 5 wk and is reported to fruit between 8 and 12 mo yr^{-1} in irrigated environments (Bachman and Whitwell 1995; Bond and Turner 2006).

The efficacy of herbicides applied for bittercress control in nurseries is often unsatisfactory (Altland et al. 1998; J. E. Altland, personal communication; J. C. Neal, personal communication). Smith et al. (1997) reported 85% control of bittercress with isoxaben in gravel beds surrounding production areas up to 12 wk after application. Similarly, Altland et al. (1999) observed 92% control of small bittercress plants in containerized 'Nachez' crapemyrtle (*Lagerstroemia indica* L.) and 'Carousel' Japanese barberry (*Berberis thunbergii* DC.). However, only 71% control was observed for intermediate-sized bittercress and 48% for large bittercress plants over the same period. The reasons for variable or unsatisfactory control have not been resolved. However, resistance to isoxaben has been reported for certain populations of flexuous bittercress in Europe (Eelen and Bulcke 1997).

Most bittercress in U.S. container nurseries is thought to be hairy bittercress. However, because of the morphological diversity and variable responses to herbicides, we and other researchers have questioned this assumption. Fain et al. (2005) reported three other species in the nursery trade, including *C. flexuosa* native to Asia, *C. oligosperma* Nutt. native to the United States, and *C. scutata* native to Japan. However, the Fain et al. (2005) research included only 12 populations of bittercress, depended on genetic data from unconfirmed sources for identification, and did not relate genetic data to morphological evidence. Therefore, it is important to extend this research to include more populations from throughout the United States and to correlate morphological evidence with molecular data to accurately identify the bittercress species in U.S. nurseries. Many authors have cited phenotypic plasticity and morphological overlap among weedy bittercress species around the world (Bond and Turner 2006; Eelen and Bulcke 1997; Fain et al. 2005; Lihová et al. 2006), and a previous study has shown many of these species to be very closely related (Lihová et al. 2006). To date, there have been no studies, to our knowledge, focusing specifically on the weedy species occurring in United States container nurseries; however, Lihová et al. (2006) did include these weedy species in a worldwide phylogeny for the *C. flexuosa* complex. As a result, current keys to bittercress in North America do not include all species present and may result in frequent misidentification (Detling 1936; Hickman 1993; Hitchcock et al. 1964; Radford et al. 1968; Rollins 1993; Weakley 2007).

With the recent development of several nuclear gene regions suitable for the study of speciation in Brassicaceae (Schranz et al. 2007), it may be possible to further resolve phylogenetic relationships among the closely related species of *Cardamine* occurring in United States nursery crops. Our main objectives were to (1) increase sampling of bittercress populations from nurseries throughout the major production zones in the United States, (2) employ molecular and morphological data to accurately identify the encountered species, and (3) provide appropriate keys, descriptions, and illustrations to facilitate the identification of the encountered species.

Materials and Methods

Taxa Sampling. Bittercress accessions were collected from 21 nurseries in six major nursery stock producing states, including California, Missouri, Mississippi, North Carolina, New York, and Oregon. At each nursery, collections were made of the most common bittercress phenotype present as well as any phenotypes that differed from the most common one. This sampling methodology allowed us to capture a representative sample of bittercress species occurring in United States nurseries.

Morphological Analysis. On-site examinations of *Cardamine* herbarium specimens at two major U.S. herbaria (New York Botanical Garden, Bronx, NY [NY]; Missouri Botanical Garden, St. Louis, MO [MO]) and three European herbaria (Royal Botanical Gardens, Kew, Richmond, Surrey, U.K. [K]; Linnaean Herbarium, London, U.K. [LINN]; Natural History Museum, London, U.K. [BM]) were undertaken to ensure a thorough survey of *Cardamine* species and to investigate morphological variation. Additional specimens were examined through loans from five other institutions: the California Academy of Sciences, San Francisco, CA (CAS); the Gray Herbarium, Cambridge, MA (GH); the University of Texas Herbarium, Austin, TX (TEX); the University of Washington Burke Museum, Seattle, WA (WTU); and the United States National Herbarium, Washington, DC (US) (acronyms follow Holmgren et al. 1990). Type specimens were examined for weedy taxa already reported for the United States and other closely related taxa. For all herbarium specimens, we examined leaf, stem, and root morphology; surface hairs; floral parts; and fruiting characters. Suitable specimens were also selected for molecular analysis.

Molecular Analysis. A total of 37 accessions were included in the analysis, including specimens from 21 container nurseries in six states (previously mentioned above) and from selected herbarium material (listed below). *Rorippa* Scop. and *Nasturtium* R. Br. were selected as outgroups following Franzke et al. (1998). Total genomic DNA was extracted from freshly collected and herbarium material using the DNeasy Plant Mini Kit.[1] Sequence data were obtained from nuclear gene region *CIP7* using primers designed for rockcress (*Arabidopsis* Heynh.)(A. Lawton-Rauh, unpublished data). Primers ITS4 and ITS5 (White et al. 1990) were used to amplify ITS in a single piece. Polymerase chain reaction (PCR)(50 µl) consisted of the following components: 2 µl template DNA, 10× PCR buffer,[2] 2.5 mM each deoxynucleotide triphosphate, 25 mM $MgCl_2$, 1 µl each primer, and 1 unit of *Taq* polymerase (+1.5 µl 10 mg/mL dimethyl sulfoxide [DMSO] for amplification of ITS sequence). PCR cycling parameters for *CIP7* were the following: initial denaturation step at 95 C for 5 min, then 35 cycles of denaturation at 95 C for 1 min, primer annealing at 50 C for 30 s, and extension at 72 C for 2 min, with a final extension cycle at 72 C for 6 min. PCR reactions for *CIP7* were performed in a Bio-Rad iCycler thermal cycler.[3] PCR reactions for ITS sequences were run on a Stratagene Robocycler gradiant 96.[4] Before sequencing, PCR reactions for *CIP7* were run on a 1% Tris–acetate–ethylenediaminetetraacetic acid (TAE) agarose gel, and bands were extracted using the 15-min UltraClean DNA purification kit[5] following the manufacturer's protocol. PCR reactions for the ITS sequence were cleaned up using an enzymatic cleanup reaction of Exonuclease I[6] and Antarctic Phosphatase.[7] The enzymes were added to the PCR product and run on the Bio-Rad iCycler thermal cycler at 37 C for 15 min, followed by 80 C for 15 min. The ITS sequence was cloned using the TOPO TA cloning kit,[8] and 5 clones from each of 6 samples were sequenced. DNA sequencing was performed using the ABI prism BigDye Terminator cycle sequencing ready reaction kit[9] v.3.1, and sequencing products were detected on a 3730xl Sequencer.[10] DNA sequences generated from this study have been deposited in GenBank.

Phylogenetic Analysis. Sequences were aligned using ClustalW (Larkin et al. 2007) and adjusted by eye in Vector NTI 10.0.[11] Modeltest 3.7 (Posada 2005) was used to determine the optimal substitution model for each region using the Akaike's Information Criterion (AIC)(Posada and Buckley 2004). Three matrices were analyzed (see below).

Matrix 1—CIP7. The *CIP7* matrix consisted of newly generated sequences from 33 *Cardamine* accessions and includes the outgroups *Nasturtium* and *Rorippa*. Because a gap of ~40 bp was detected when trying to align these sequences in Vector NTI software,[11] only 186 bp of the forward sequence was used to construct the matrix. The length of the gap was determined based on comparison with previously published *Arabidopsis CIP7* sequence in GenBank (NM_118877.3).

Matrix 2—ITS. The ITS matrix consists of newly generated sequences of seven *Cardamine* accessions from container nurseries and herbarium specimens, as well as a previously published sequence for *Rorippa* (GenBank X98638). There were two to four clones for each of the four *C. flexuosa* accessions, three clones of one *C. corymbosa* accession, two clones of one *C. hirsuta* accession, and three clones of one *Cardamine oligosperma* Nutt. var. *kamtschaticai* (Regal) Detling. We also included *Cardamine* accessions from Lihová et al. (2006), including three of the Asian *C. flexuosa* accessions, three of the European *C. flexuosa* accessions, two of *C. hirsuta* accessions, two of *C. oligosperma* accessions, two of *C. scutata* accessions, and one *C. corymbosa* accession. The previous accessions of *C. flexuosa*, *C. hirsuta*, and *C. oligosperma* were chosen because they were collected in the United States and are appropriate to the scope of this research. There were no U.S. accessions of *C. scutata* or *C. corymbosa* available. Japanese *C. scutata* accessions were included because this species has been reported for the United States by previous authors, and a Canadian accession of *C. corymbosa* was included because we identified *C. corymbosa* in the United States during the course of morphological studies. The ITS matrix was constructed to verify research by previous authors (Lihová et al. 2006) and is representative of the taxa included in the *CIP7* matrix (above). It included 631 bp with no significant indels.

Matrix 3.—Combined. The combined matrix includes both sets of data described above and is 817 characters long. The data was entered in PAUP*[12] as two data partitions ITS + *CIP7*.

Each matrix was subjected to maximum parsimony using PAUP* v. 4.0b10 (Swofford 2002) and Bayesian inference

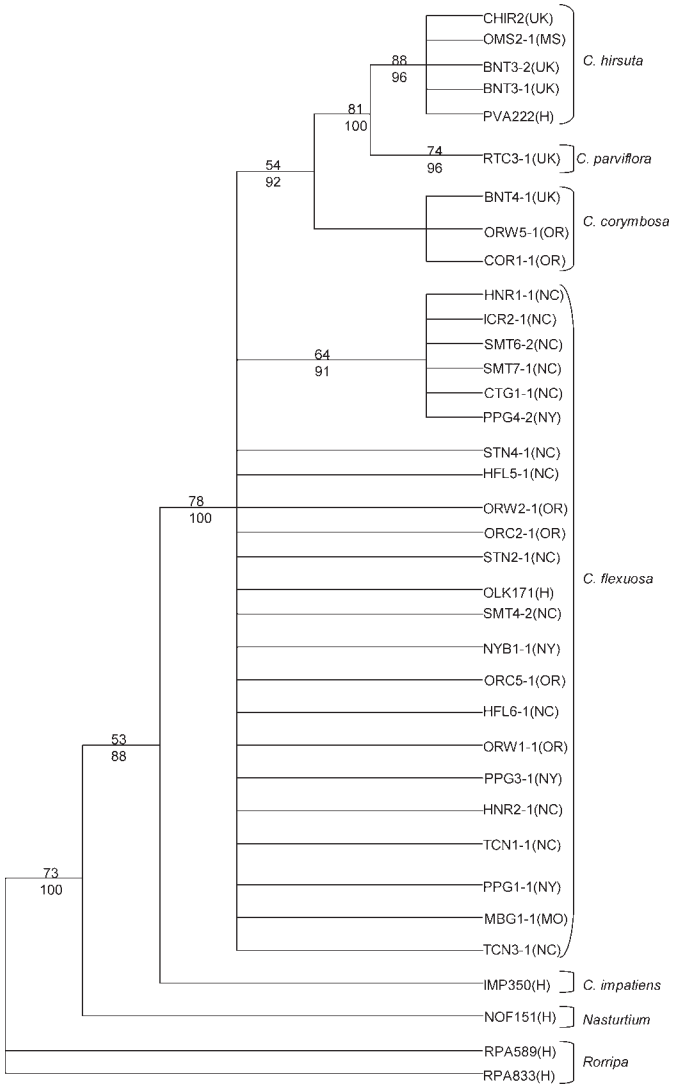

Figure 1. Strict consensus of 700,000 most parsimonious trees resulting from parsimony analysis of nuclear gene region *CIP7* for *Cardamine* species in United States nurseries (length = 59; consistency index [CI] = 0.814; retention index [RI] = 0.875). Bootstrap support (BS) is given above branches, and Bayesian posterior probabilities (PP) are given below branches. Species identifications shown are based on morphological examinations. State abbreviations are given in parenthesis next to accessions. UK denotes fresh accessions from England, and H denotes herbarium specimens.

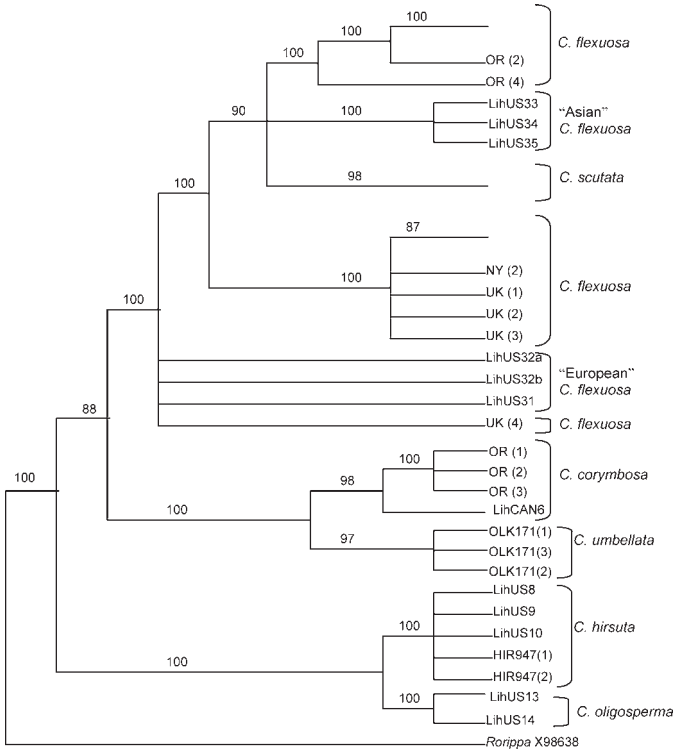

Figure 2. Majority-rule consensus for internal transcribed spacer (ITS) for bittercress (*Cardamine*) species in U.S. nurseries, including our fresh accessions, labeled with state acronyms, and selected ones, labeled Lih, from Lihova et al. (2006). UK denotes fresh accessions from England, and OLK and HIR are herbarium sheets. Bayesian posterior probabilities (PP) are given above branches. Species identifications shown are based on morphological examinations.

using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). For the ITS and combined matrices, we used a heuristic search with 1,000 random-addition-sequence replicates, tree-bisection-reconnection branch swapping, and MULtrees option in effect. The resulting most-parsimonious trees were summarized in a strict consensus. For the *CIP7* matrix, phylogenetic trees were generated first with the MULtrees option off. The most parsimonious trees, which were used as the starting trees for a second parsimony analysis, were executed in the same way, except with MULtrees option on (Krings et al. 2008; Liede-Schumann et al. 2005). The second search was stopped after 700,000 trees because of memory limitations and summarized in a strict consensus tree. For all matrices, support for each clade was estimated using 10,000 bootstrap (BP) replicates in a fast heuristic search (Felsenstein 1985).

Each data set was also subjected to Bayesian inference in MrBayes v. 3.1.2. AIC was used to determine DNA substitution models in ModelTest v. 3.7. Four independent Bayesian analyses were run for 1 million generations sampling trees every 100 generations. Burn-in was estimated by plotting likelihood scores using Tracer (Rambaut and Drummond 2004). After excluding 1,100 trees from the burn-in phase a majority-rule consensus tree was generated to estimate the posterior probabilities (PPs) of clades.

The *CIP7* alignment included 37 accessions and 186 characters, of which, 45 were variable (23 autapomorphic; 22 parsimony informative)(Figure 1). The ITS matrix included 8 accessions from our collections, 14 accessions from Lihová et al. (2006), and one out-group. There were 631 characters, of which, 68 were variable (39 autapomorphic; 24 parsimony informative). The ITS topology differed in placing *C. corymbosa* as sister to *C. flexuosa*; however, that relationship was not well supported (Figure 2). *Cardamine hirsuta* accessions from our study grouped with three *C. hirsuta* accessions from Lihová et al. (2006) with 100% PP, and *Cardamine oligosperma* accessions resolved as a sister to *C. hirsuta* with 100% PP. This topology is congruent with the majority-rule consensus reported by Lihová et al. (2006) for their reduced ITS data set. One Lihová et al. (2006) accession of *C. corymbosa* formed a group with three clones of *C. corymbosa* in our study with 98% PP. Accessions of *Cardamine flexuosa* did not fully resolve in a single clade. The Asian *C. flexuosa* accessions of Lihová et al. (2006) resolved as a polytomy with two accessions from our study and the *C. scutata* with high PP (90%). The remainder of our accessions formed a second clade with high PP (100%), and the Lihová et al. (2006) European *C. flexuosa* formed a

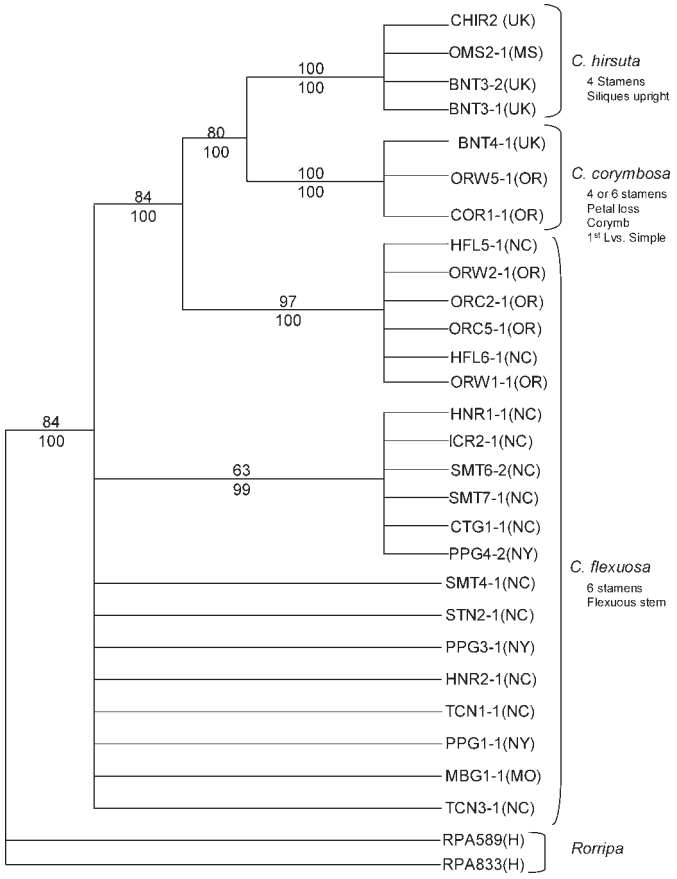

Figure 3. Strict consensus of 13 of the most parsimonious trees resulting from parsimony analysis for the combined matrix of *CIP7* and internal transcribed spacer (ITS) for bittercress (*Cardamine*) species in U.S. nurseries (length = 73; consistency index [CI] = 0.922; retention index [RI] = 0.978). Bootstrap support (BS) is given above branches, and Bayesian posterior probabilities (PP) are given below branches. Species identifications shown are based on morphological examinations. Morphological characters are given under species identification. State abbreviations are given in parenthesis after each accession. UK denotes fresh accessions from England, and H denotes herbarium specimens.

polytomy with these accessions and with one accession from the U.K. (Figure 2). One accession of *C. umbellata* was included in this study, which resolved as sister to *C. corymbosa* with 100% PP. This relationship is congruent with that reported by Lihová et al. (2006). Accessions of *C. scutata* included from Lihová et al. (2006) fell within the *C. flexuosa* group on the tree, and this is also congruent with the majority-rule consensus reported for their reduced ITS data set (2006).

The combined alignment includes 29 taxa and 817 characters (ITS: 631 bp; *CIP7*: 186 bp), of which, 70 were variable (2 autapomorphic; 68 parsimony-informative). This matrix resolved relationships among the *Cardamine* accessions with higher BS support and PP than either of the separate matrices alone (Figure 3). In the combined matrix, the *Cardamine hirsuta* and *Cardamine corymbosa* clades have strong BS support (100%) and strong PP (100%). These two species were resolved as sisters among the taxa samples. There were two distinct groups of *Cardamine flexuosa*. One group contained six accessions and was sister to *C. hirsuta* and *C. corymbosa* with strong BS (84%) and PP support (100%). A second group with six accessions had weak BS support (63%), but strong PP support (99%), which forms a polytomy with the remaining eight *C. flexuosa* accessions.

Cardamine hirsuta and *Cardamine corymbosa* resolved as sister to one another based on the molecular data, which are congruent with morphological evidence. Both these species exhibit at least partial loss of two stamens. *Cardamine hirsuta* exhibits complete loss of the two outside stamens, and *C. corymbosa* exhibits six stamens in early petaliferous flowers and only four later in the season, when it begins to produce cleistogamous flowers. Along with the loss of stamens, *C. corymbosa* also exhibits an apparent loss of petals in flowers with only four stamens. Even in early flowers producing six stamens, petals are sometimes fewer than four either because of fusion or loss. *Cardamine corymbosa* also has the ability to form stolons and a corymbose inflorescence, which is atypical of the family (Figure 3).

Based on molecular data, only six accessions of *C. flexuosa* formed a well-defined group that was sister to the previous taxa (*C. corymbosa* and *C. hirsuta*). Morphology supports *C. flexuosa* as a distinct species. exhibiting six stamens and stems, which flex at the nodes.

Based on molecular evidence alone, we identified at least three bittercress species occurring in the United States container nursery industry, including New Zealand bittercress, flexuous bittercress, and hairy bittercress. A fourth species, little bittercress, native to the United States, also occurs; however, sequence data for this species (*Cardamine oligosperma* Nutt.) was incomplete and not useful for analysis. The previously reported *Cardamine scutata* (Fain et al. 2005) was not encountered in this study. The phylogeny constructed in this study failed to fully resolve species-level relationships within this closely related weedy complex. However, paired with data from Lihová et al. (2006), we were able to support the identity of species we encountered. The topology of our ITS tree was congruent with Lihová et al (2006). Previous studies have stressed the need for a sequence of single-copy nuclear gene regions to help resolve these relationships (Lihová et. al. 2006). However, it is apparent that several regions must be screened for utility when trying to resolve interspecific relationships of these closely related taxa, even when that utility has been demonstrated for other groups in the same family. It is possible that *C. flexuosa* is a polyphyletic taxon because other species were well resolved. It is also possible that previous reports (Lihová et al. 2006) of two cytotypes within *C. flexuosa* are accurate. Lihová et al. (2006) reported both tetraploid populations and putatively octoploid populations within *C. flexuosa*, although we did not explore this aspect.

The gene regions explored in this study included the COP-1 Interactive Protein 7, which is a single-copy nuclear gene believed to positively regulate light-regulated genes in *Arabidopsis* (Yamamoto et al. 1998). Low-copy and single-copy nuclear regions such as this are often highly variable between species and have demonstrated utility on species level phylogeny reconstruction. However, many times, these regions exhibit fewer parsimony-informative characters than the more commonly used ITS region (Hughes et al. 2006), as is the case here. The noncoding ITS region has been used when constructing many angiosperm phylogenies because it evolves more rapidly than cpDNA regions. However, ITS often exhibits paralogy problems and may have insufficient variable characters for resolving low-level relationships (Bailey and Doyle 1999). Data generated for this study shed some light on relationships among closely related taxa in *Cardamine*; however, molecular work on this group is far from

complete. Because these taxa are so closely related and, in some cases, have polyploid origins, it will require the exploration of further nuclear gene regions to fully resolve these interspecific relationships.

We sampled *Cardamine* from six states important to U.S. nursery production, including California, Mississippi, Missouri, New York, North Carolina, and Oregon. Two major nursery producing states, Florida and Texas, are not represented in our molecular data. Two Texas nurseries were surveyed late in this study and, therefore, were not included in the molecular analysis, but morphologically, the only species we encountered in Texas was *C. flexuosa*. Florida is missing completely from our sampling of major production areas, but, anecdotally, it is unlikely to harbor any species not encountered elsewhere in the country because many Florida container nurseries ship their stock, and likewise their weeds, nationwide.

Most accessions collected from United States container nurseries resolved in the phylogeny as flexuous bittercress (90%), although historically, most were thought to be hairy bittercress. The earliest report of flexuous bittercress in the United States is from the 1933 [Voucher: Fernald, Long & Fogg 1721 (NY)], and because of its current widespread distribution in the U.S., it has likely been frequently misidentified as *C. hirsuta*. A common mistake when examining floral characters is to count the four long stamens and assign an identification of hairy bittercress not taking into account that two stamens are shorter and not easily seen without dissecting the flowers under magnification. A previous study (Lihová et al. 2006) indicates flexuous bittercress has at least two distinctive genotypes in the United States. Lihová et. al. (2006) reported a difference between Asian and European populations of *Cardamine flexuosa* citing ''unequivocal evidence'' that they are distinct taxa. However, this evidence is purely molecular in nature, and the authors concede that morphological variability overlaps the two groups and that they are difficult to differentiate using morphology alone. Based on our molecular evidence, it is likely, but not definitive, that individuals from both regions have been introduced as weeds to North America and occur in United States nurseries. It remains unclear, especially through morphological evidence, whether they should be considered as distinct taxa. Morphologically, we placed these two genotypes in the same category under the description of *C. flexuosa*. Based on morphology, hairy bittercress comprised 6% of our accessions, and New Zealand bittercress and little bittercress comprised only 2% each. The previously reported Japanese bittercress (Fain et al. 2005) was not encountered in this study. If the species occurs, it is only present in very low abundance because the nature of our sampling method was biased toward encountering species that occurred at higher densities.

These data demonstrate that, in contrast to prior assumptions, *C. flexuosa* is the most common bittercress encountered in container nursery crop production. Furthermore, there is significant morphological and genetic diversity within this species. Inconsistent control of bittercress with common nursery herbicides has been reported (Altland et al. 1998). Although this research has not addressed that issue, it is possible differential control reported by nurseries is, at least in part, due to the presence of multiple species, and significant genetic diversity within the most common species.

Taxonomic Treatment. *Cardamine L. Sp. Pl. 2: 654. 1753. Type: "Habitat in Europae pascuis aquosis." [lectotype: no. 835.15 LINN non. vidi. designated by Britton and Brown 1913].*

Each of these four *Cardamine* species have odd, pinnately compound leaves, which can be extremely variable in size and shape. Basal leaves typically have three to seven pairs of lobed leaflets; the terminal leaflet is up to two times larger than the lateral ones. Lateral leaflets get progressively smaller closer to the stem. Cauline leaves are also odd pinnately compound with fewer leaflet pairs than basal leaves. Inflorescences are mostly ebracteate. Petals are white and 2 to 4 mm long. All four species arise from fibrous root systems or a weak taproot, and all have forcefully dehiscent siliques. We provide a key and detailed illustration for each species known or reported to occur in the nursery industry (Figures 4–8), designed to aid in the identification of these species in several stages of growth.

Key to *Cardamine* in U.S. Container Nurseries

1. Basal leaves many forming a tight persistent rosette; stems one to many, erect.
   2. Cauline leaves nearly ovate to obovate. . . . . ***C. oligosperma***
   2. Cauline leaves lanceolate to oblanceolate with or without slight lobing.
      3. Stamens four; siliques < 1 mm wide, upright and parallel to rachis; stem straight < 20 cm tall . . . . . ***C. hirsuta***
      3. Stamens six; siliques > 1 mm wide, upright but held at an angle to rachis; stem straight to flexuous > 20 cm tall . . . . . ***C. flexuosa***
1. Basal leaves rosetted upon germination, then deciduous and few, or open, wiry rosette; stems one to many, erect or trailing.
   4. Plants forming stolons rooting at nodes; wiry decumbent habit; many stems arising from fibrous root system . . . . . ***C. corymbosa***
   4. Plants without stolons; erect habit; one to many stems arising from fibrous roots or weak taproot.
      5. Sepals present; petals present; stamens four or six
         6. Petals four, 3.0 to 4.0 mm long.
            7. Petals cuneate–obovate to obovate, held open; stamens six. . . . . ***C. flexuosa***
            7. Petals spatulate held upright to slightly open; stamens four . . . . . ***C. hirsuta***
         6. Petals two to four, or four with one or more appearing fused, 3.0 to 4.0 mm long . . . . . ***C. corymbosa***
      5. Sepals present; petals absent; stamens four . . . . . ***C. corymbosa***

Descriptions

***Cardamine corymbosa* Hook. *f.*, Ic. Pl. t. 686. 1844. Type: "Campbell's Island" (fl & fr). [holotype: *Hook f. s.n.*, K!].**

(Figure 4) Decumbent annual 15 to 20 cm tall. *Stems* wiry, unbranched, creeping, and rooting at the nodes to produce daughter plants. *Basal leaves* alternate, compound, leaflets

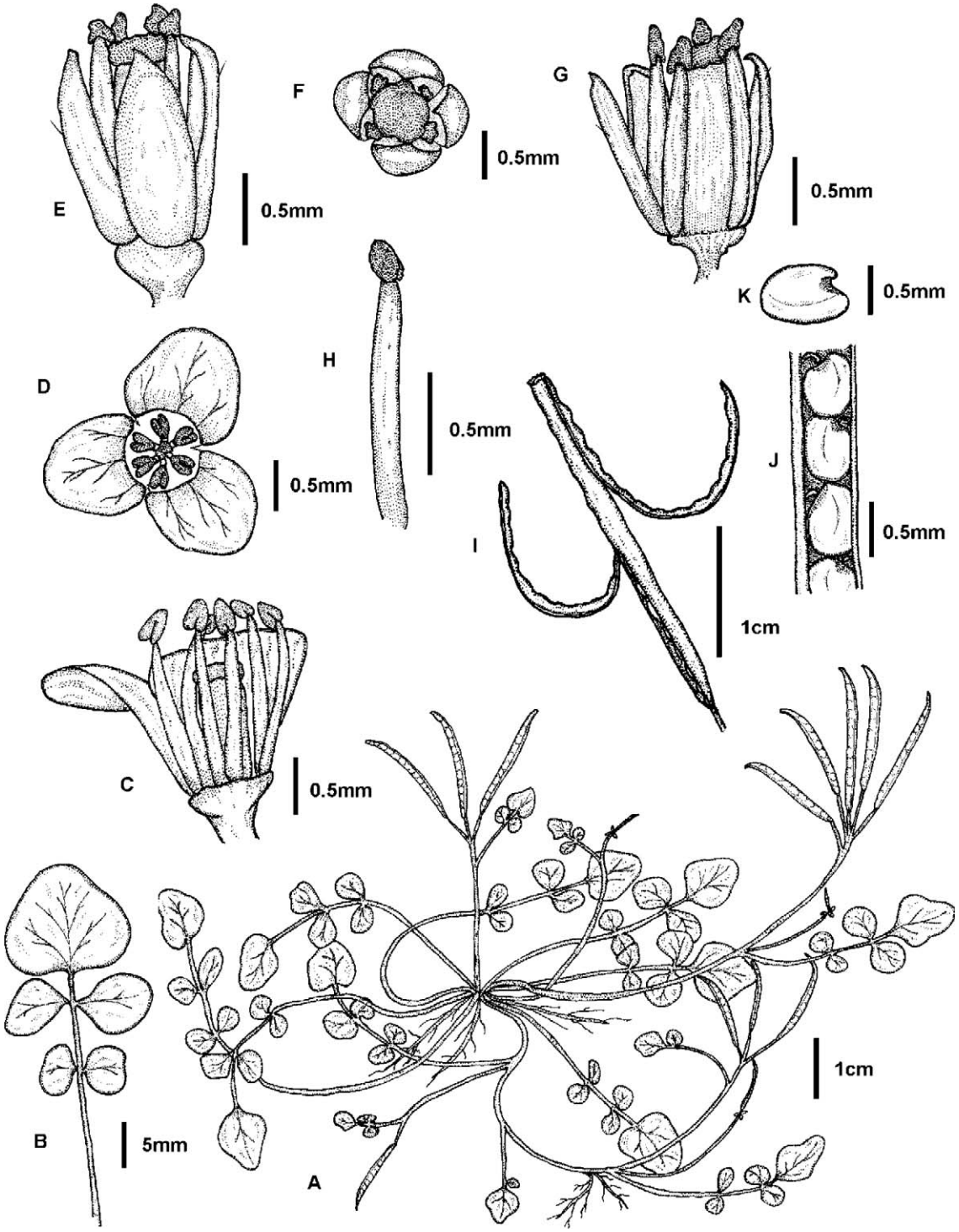

Figure 4. Illustration of New Zealand bittercress (*Cardamine corymbosa* Hook. *f*), showing (a) habit, (b) basal leaf, (c) petaliferous flower cutout, (d) petaliferous flower-top view, (e) apetalous flower, (f) apetalous flower-top view, (g) apetalous flower cutout, (h) stamen, (i) silique, (j) silique cutout, and (k) seed. [Illustrations by Nancy C. Routh.]

three to five, sessile or nearly so, and ovate to orbicular terminal leaflet up to two times larger than lateral ones. *Flowers* dimorphic (see notes below), four sepals alternating with four white petals, one or more petals fused. *Stamens* six, four long and two short. *Inflorescence* indeterminate corymb, rather than the typical cruciferous raceme; pedicels 0.8 to 1.5 cm long. *Siliques* 1.8 to 2.7 cm, six to seven mature seed in each valve (or 12 to14 fruit^{-1}).

Notes. Later in the life cycle, flowers are produced having no petals and only four long stamens. These flowers are likely equivalent to the cleistogamous flowers described for *C. corymbosa* by Schulz (1903). Fruit is a two-valved silique, which dehisces forcefully, though not as explosively as close relatives *C. flexuosa* or *C. hirsuta.*

Representative specimens examined. NEW ZEALAND: *Laird s.n* (K); *Philipson 10145* (K).

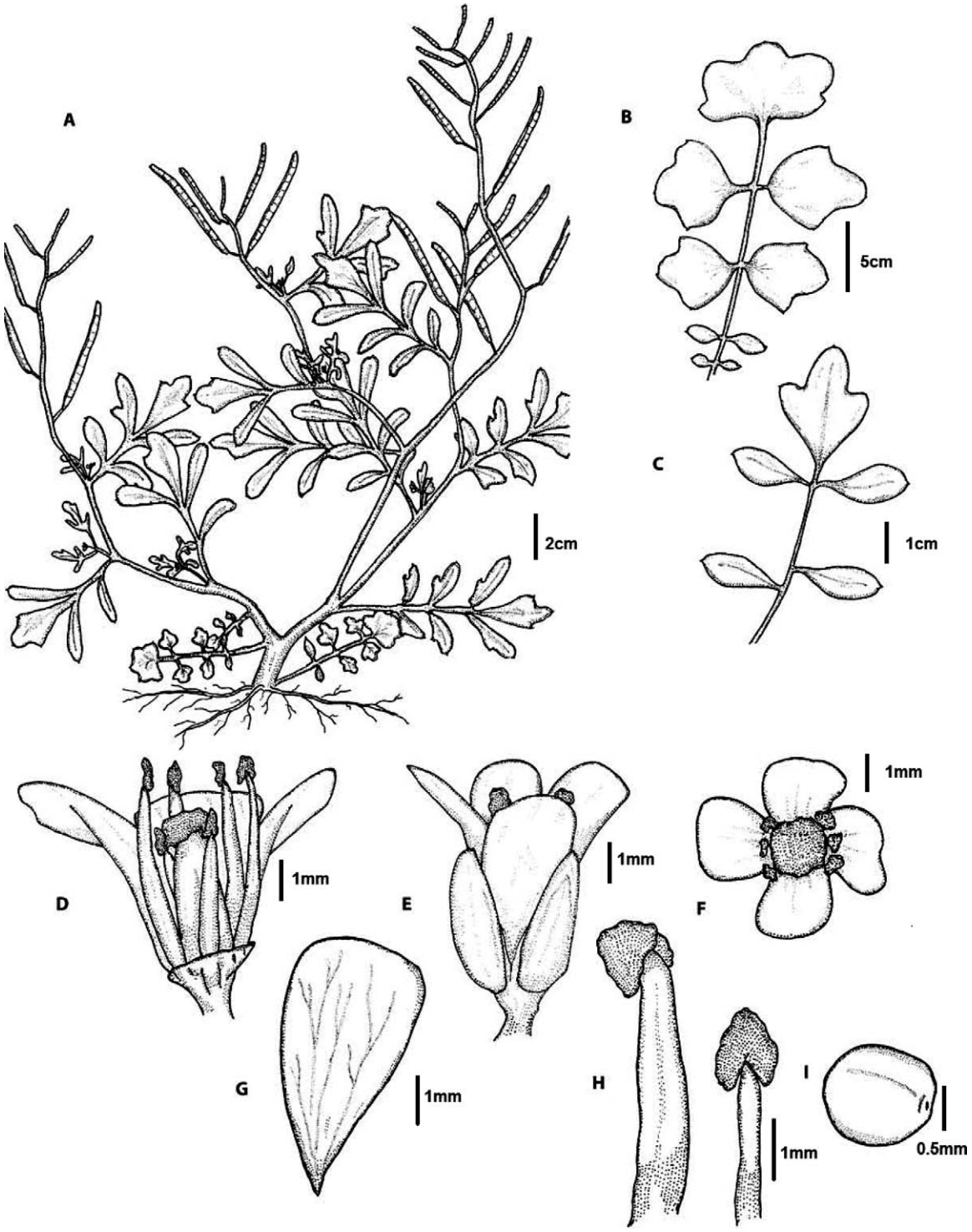

Figure 5. Illustration of flexuous bittercress (*Cardamine flexuosa* With.), showing (a) habit, (b) basal leaf, (c) cauline leaf, (d) flower cutout, (e) flower-side view, (f) flower-top view, (g) stamens, (h) petal, (i) silique, and (j) seed. [Illustrations by Nancy C. Routh.]

***Cardamine flexuosa* With., Arr. Brit. Pl., ed. 3: 578. 1796.—Type: "Rookery at Edgebaston" (fl & fr). [Lectotype, designated by Post et al., *in review*: Curt. 277!]).**

(Figure 5) Erect annual to 30 cm tall. *Stems* one to many, angular, ribbed, flexing at the nodes (zigzag). *Basal rosette* upon germination. *Leaves* odd pinnately compound, 7 to 13 leaflets with the terminal one up to two times larger than the lateral leaflets. *Basal leaflets* ovate to orbicular; petiolulate, rarely sessile, terminal leaflet may approach a reniform shape. *Cauline leaves* odd, pinnately compound; leaflets lanceolate to oblanceolate. All leaflets are irregularly lobed with three to five lobes, sometimes terminating in a short tooth. At onset of flowering, many basal leaves die back, and the rosette is no longer visible. *Flowers* four green (sometimes purplish); sepals half the length of petals. *Petals* four white, cuneate to 4 mm long. *Stamens* six (four long and two short); the four long ones are generally

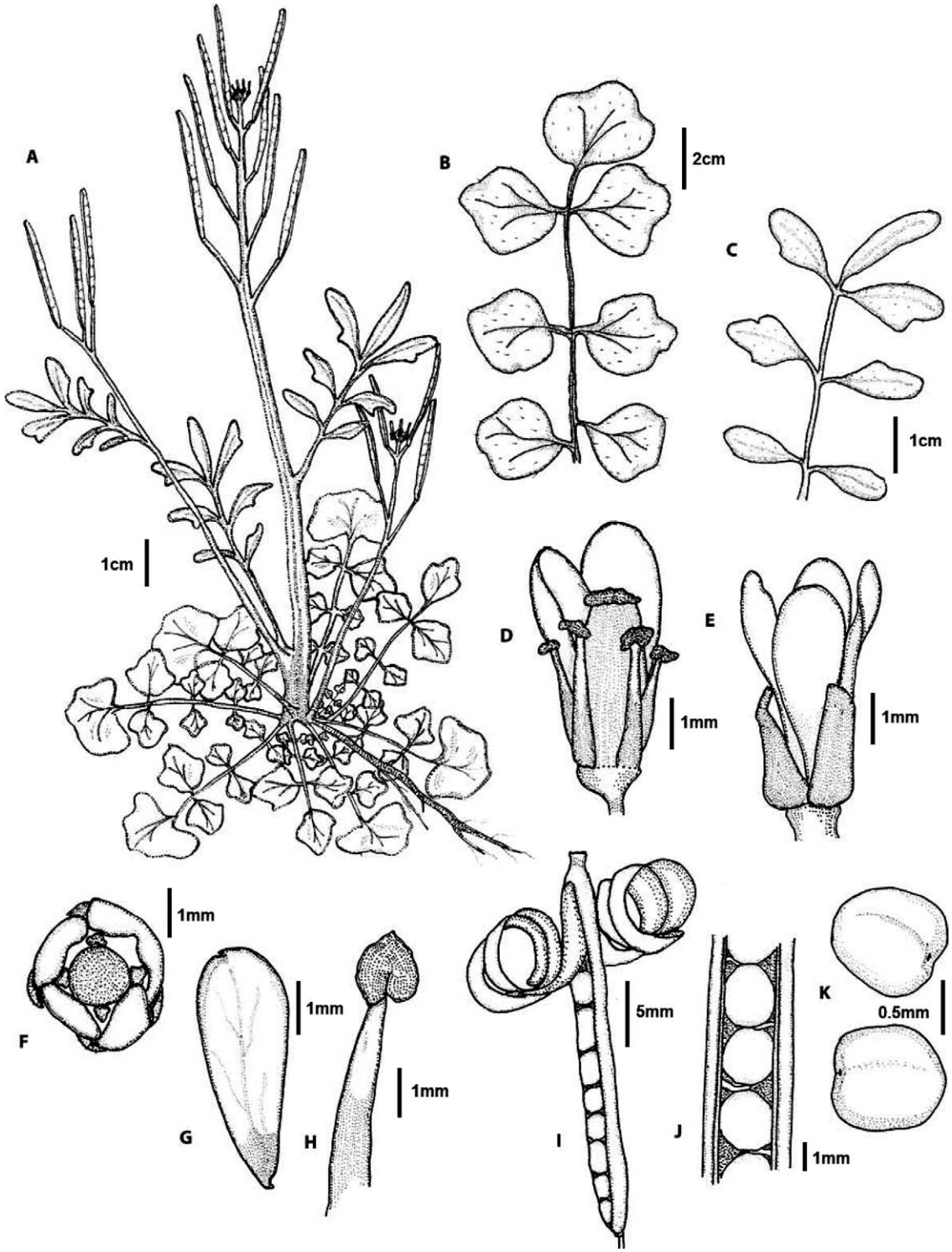

Figure 6. Illustration of hairy bittercress (*Cardamine hirsuta* L.), showing (a) habit, (b) basal leaf, (c) cauline leaf, (d) flower-cutout, (e) flower-side view, (f) flower-top view, (g) petal, (h) stamens, (i) silique, (j) silique-cutout, and (k) seeds. [Illustrations by Nancy C. Routh.]

longer than the blossom. *Inflorescence* a raceme. *Fruit* a two-valved capsule, 1.5 to 2.5 cm long, held at an angle from the rachis; dehisces forcefully releasing 20 to 24 seeds.

Representative specimens examined. AUSTRALIA: *Lepschi 1936* (K). CHINA: *Hu 209* (K); *Hu 9417* (K); *Yao 8885* (K). JAPAN: *coll. ign 2320/51* (K); *Furuse 10770* (K). USA: CT *Tucker & Tucker 13815* (MO); GA *Duncan 23704* (NCSC); *Adams 17603* (NCSC); *Cusick 32229* (MO); FL *Correll & Popenoe 51473* (NY); *Correll & Popenoe 51504* (NY); *Burch 6502* (NY); IL *Cusick 35226* (NY); *Cusick 35228* (MO); NJ *Mackenzie 3129* (MO); OH *Cusick 33305* (MO); *Cusick 32647* (NY); MO MBG1-2 *Post 73* (NCSC); MBG2-1 *Post 74* (NCSC); NC *Post 45* (NCSC); *Post 46* (NCSC); *Post 47* (NCSC); *Post 48* (NCSC); *Post 49* (NCSC); *Post 50* (NCSC); *Post & Adkins 84* (NCSC); *Post & Adkins 84* (NCSC); *Post & Adkins 75* (NCSC); *Post & Adkins 75* (NCSC); *Post & Adkins*

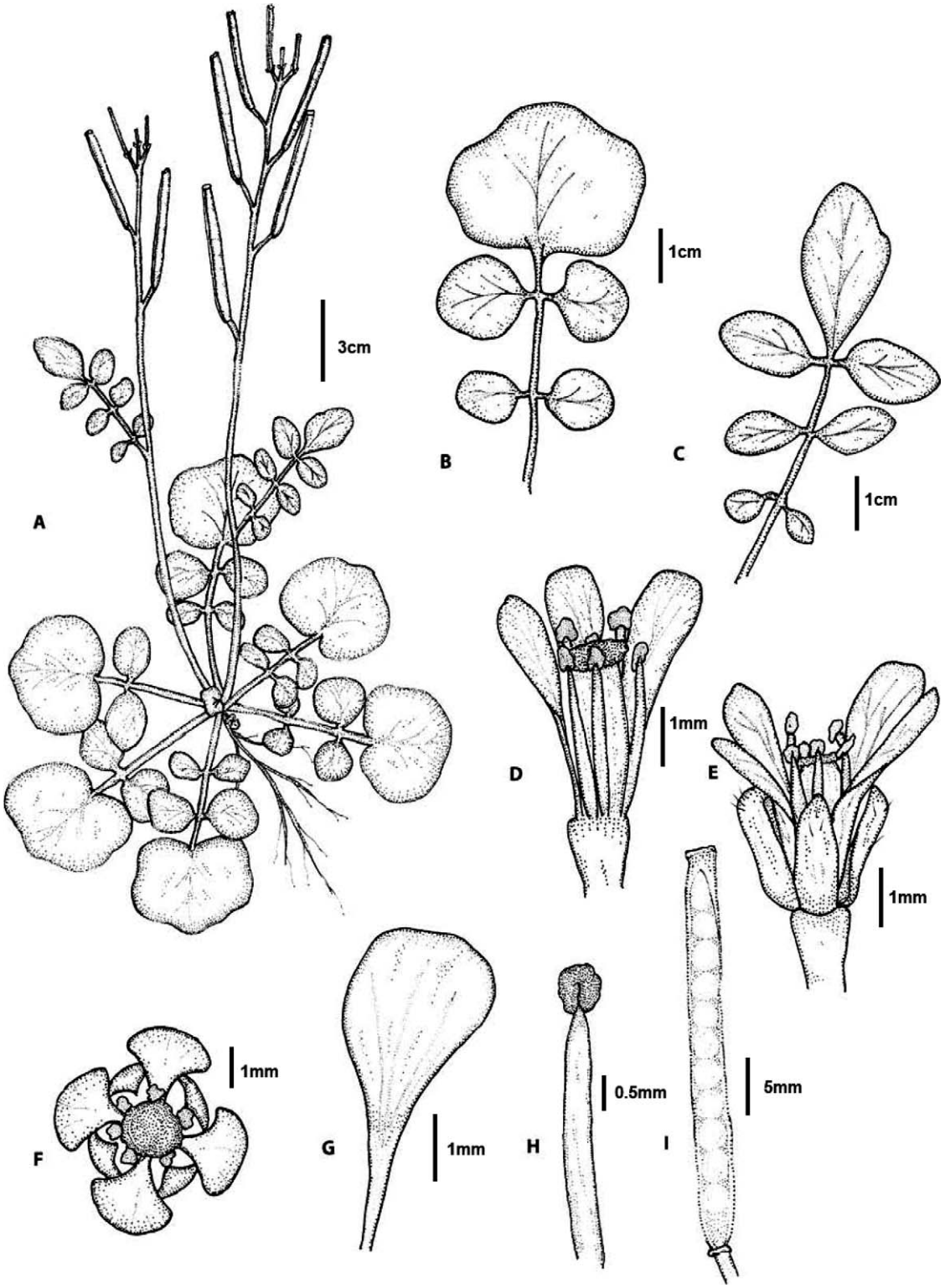

Figure 7. Illustration of little bittercress (*Cardamine oligosperma* L.), showing (a) habit, (b) basal leaf, (c) cauline leaf, (d) flower cutout, (e) flower-side view, (f) flower-top view, (g) petal, (h) stamens, (i) silique, (j) silique-cutout, and (k) seeds. [Illustrations by Nancy C. Routh.] Images for illustration provided by Carol W. Witham and Gary McDonald.

76 (NCSC); *Post & Adkins 78* (NCSC); *Post 58* (NCSC); *Post 59* (NCSC); *Post 60* (NCSC); *Post 61* (NCSC); *Post 62* (NCSC); *Post 63* (NCSC); *Post 64* (NCSC); *Post 65* (NCSC); *Post 66* (NCSC); *Post 67* (NCSC); *Post 56* (NCSC); *Post 56* (NCSC); *Post 56a* (NCSC); *Post 52* (NCSC); *Post 53* (NCSC); NY *Post 87* (NCSC); *Post 88* (NCSC); OR *Altland s.n.* (NCSC); PA *Cusick 31707* (MO); SC *Barkley 23* (MO).

***Cardamine hirsuta* L., Sp. Pl.: 655, 1753. Type: "*In Europae areis, hortis, arvis*". (fl & fr) [Lectotype, designated by Fawcett and Rendle 1914; LINN, no. 835.13!; isolectotype: LINN, no. 835.14!]).**

(Figure 6) Erect annual 10 to 17 cm tall. *Stem* usually only one. *Basal rosette* persists throughout the season. *Basal leaves* odd, pinnately compound; 9 to 13 leaflets with terminal one

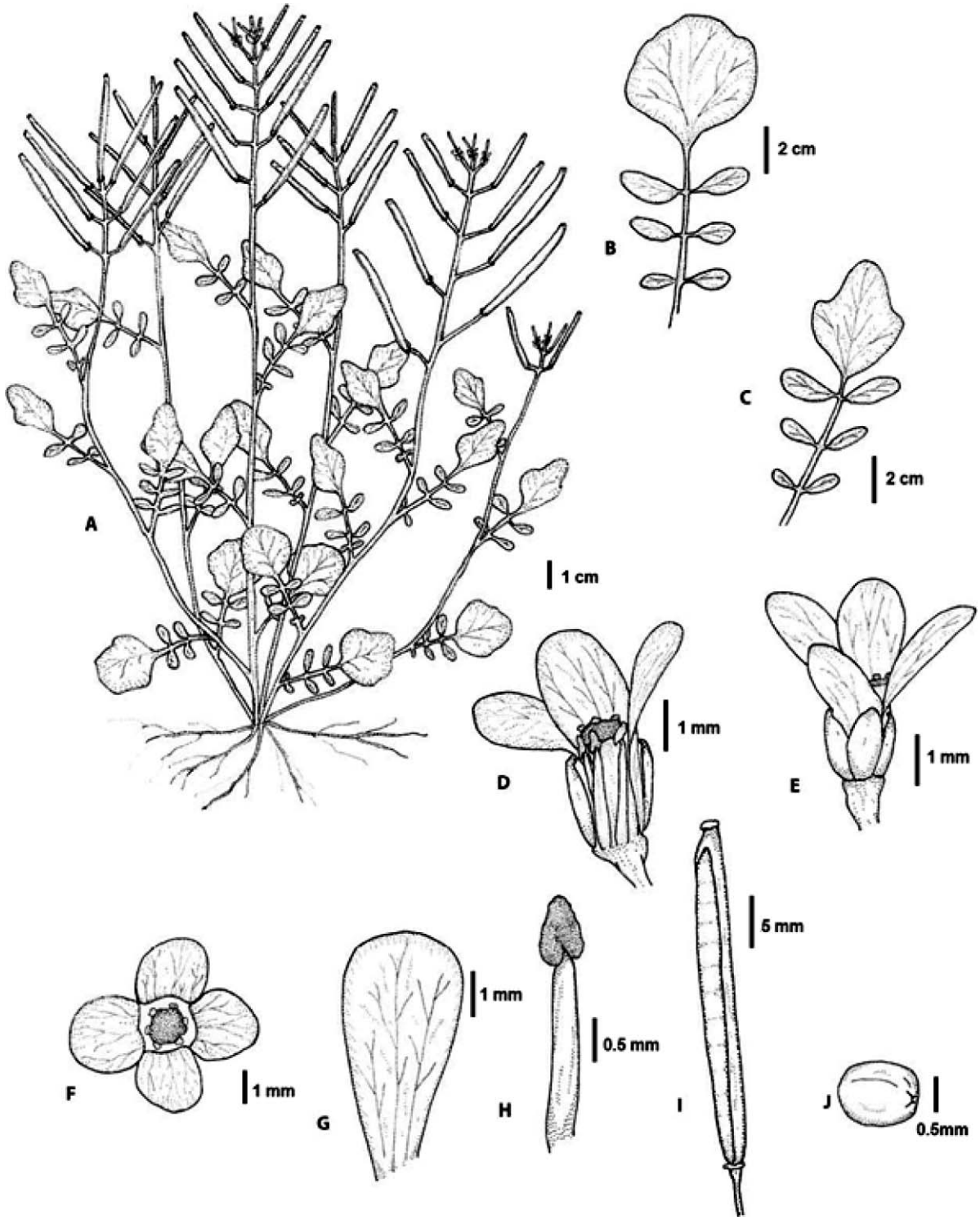

Figure 8. Illustration of Japanese bittercress (*Cardamine scutata* Thunb.), showing (a) habit, (b) basal leaf, (c) cauline leaf, (d) flower cutout, (e) flower-side view, (f) flower-top view, (g) petal, (h) stamens, (i) silique, (j) silique cutout, and (k) seeds. [Illustrations by Nancy C. Routh.]

up to two times larger than the lateral ones. *Basal leaflets* ovate to orbicular; petiolulate three to five lobes, sometimes terminating in a short tooth. *Terminal leaflets* may approach a reniform shape. *Cauline leaves* odd, pinnately compound, five to seven leaflets, lanceolate to oblanceolate. *Cauline leaflets* are irregularly lobed, except for the terminal leaflet that is rarely lobed. *Flowers* four green sepals alternating with four white, spatulate petals. *Stamens* four and generally longer than the blossom. *Inflorescence* a raceme. *Fruit* a two-valved capsule, 1.8 to 2.5 cm long, held upright and nearly parallel to the rachis. *Fruits* dehisce forcefully releasing 16 to 24 seeds.

Representative specimens examined. POLAND: *Zelazny s.n.* (NCSC). USA: AL *Kral 88031* (MO); KY *Athey 537* (MO); MS *Bryson 20610* (NCSC); *Bryson 20611* (NCSC); *Bryson 20615* (NCSC); NC *DeLoach & Dukes 61* (NCSC); *Godfrey 49011* (NCSC); *Ingle 23* (NCSC); *Ittenbach 17* (NCSC);

Jones 41 (NCSC); *Pultorak 692* (NCSC); *Post 51* (NCSC); *Smith 169* (NCSC); *Thomas & Spell 165086* (NY); OR *coll. ign. s.n.* (K); SC *Angerman s.n.* (NCSC); *Nelson & Chicone 21022* (MO); *Wooten 3* (MO); TN *Kelly 83* (NCSC); *McNeilus 99-47* (NY); *Phillippe 2520* (NCSC); VA *Churchill s.n.* (NCSC); *Selden 25* (NCSC).

***Cardamine oligosperma* Nutt., Fl. N. Amer. i. 85. 1838. Type: "Oregon woods" (fr). [holotype: *Nuttall s.n.*, NY!; Isotype: *Nuttall s.n.*, GH!, K!].**

(Figure 7) Erect annual 20 to 30 cm tall. *Stem* usually single. *Basal rosette* persists throughout the season. *Basal leaves* odd, pinnately compound; five to nine leaflets with the terminal one up to two times larger than the lateral ones. *Basal leaflets* are ovate to orbicular, petiolulate, with three to five lobes sometimes terminating in a short tooth. *Cauline leaves* odd, pinnately compound; three to five leaflets, ovate to obovate. *Cauline leaflets* irregularly lobed and may terminate in a short tooth. *Flowers* four green sepals alternating with four white, spatulate petals. *Stamens* six (four long and two short). *Inflorescence* a raceme. *Rachis* typically straight, sometimes curving. *Fruit* a two-valved capsule, 2.2 to 2.8 cm long, and 1.5 to 2.0 mm wide, held upright; dehisce forcefully releasing 12 to 16 seeds.

Representative specimens examined. AK *Zika 16967* (MO); *Zika 16971* (MO); *Zika 16990* (MO); NV Morefield 3087 (NY); CA *Hansen 94* (K); OR *Constance & Rollins 2966* (K); *coll. ign s.n.* (Isotype–NY); UT *Goodrich 16272* (NY); *Holmgren 274* (NY); WA *Eyerdam s. n.* (MO); *Halse 3576* (MO); *Suksdorf s.n.* (Isotype–MO, US); *Suksdorf 723* (Isotype–MO, NY); *Suksdorf 7452* (Isotype–CAS, GH, MO, NY, WTU).

***Cardamine scutata* Thunb., Trans. Linn. Soc. London 2: 339. 1794. Type: "Japan". (fr) [holotype: *Thunberg s.n.*, UPS–Thunb.!].**

(Figure 8) Erect annual 15 to 50 cm tall. *Stem* usually single. *Basal leaves* not rosulate. *Basal leaflets* odd, pinnately compound, three to nine with the terminal one up to two times larger than the lateral ones, and obovate to reniform. *Cauline leaves* odd, pinnately compound, three to five leaflets. *Cauline leaflets* with three to five lobes; terminal leaflet sometimes unlobed. *Flowers* four green sepals alternating with four white, spatulate petals to 4.5 mm long. *Stamens* six (four long and two short). *Inflorescence* a raceme. *Rachis* straight. *Fruit* a two-valved capsule, 1.5 to 2.8 cm long, 1.0 to 1.5 mm wide, held upright; dehisce forcefully releasing 20 to 36 seeds.

Representative specimens examined. AUSTRALIA: *Kissane s.n.* (K). JAPAN: *Furuse 9050* (K); *Furuse 11112* (K); *Ohba 71505* (K); *Taquet 563* (K); *Tsugaru 12964* (MO); *Tsugaru 13005* (MO); *Tsugaru 22220* (MO). RUSSIA: *Gage 2176* (NY).

Sources of Materials

[1] DNeasy Plant Minikit, Qiagen Inc., Valencia, CA 91355.

[2] 10× PCR Buffer, Promega Corporation, Madison, WI 53711.

[3] Bio-Rad iCycler, Bio-Rad Laboratories, Hercules, CA 94547.

[4] Robocycler gradient 96, (formerly Stratagene), now Agilent Technologies, Santa Clara. CA 95051.

[5] Ultraclean DNA purification kit, MoBio laboratories, Carlsbad, CA 92010.

[6] Exonuclease I, Molecular Cloning Laboratories (MCLAB), South San Francisco, CA 94080.

[7] Antarctic Phosphatase, Molecular Cloning Laboratories (MCLAB), South San Francisco, CA 94080.

[8] TOPO TA cloning kit, Invitrogen, Carlsbad, CA 92008.

[9] ABI Prism BigDye Terminator cycle sequencing ready reaction kit v.3.1, Applied Biosystems, Carlsbad, CA 92008.

[10] 3730xl Sequencer, Applied Biosystems, Carlsbad, CA 92008

[11] Vector NTI 10.0, Invitrogen, Carlsbad, CA 92008.

[12] PAUP* Phylogenetic Analysis Using Parsimony (and Other Methods) software, Sinauer Publishers, Inc., Publishers, Sunderland, MA 01375-0407.

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