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Phylogenetics of the Caprifolieae and *Lonicera* (Dipsacales) Based on Nuclear and Chloroplast DNA Sequences

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***Abstract*—**Recent phylogenetic analyses of the Dipsacales strongly support a Caprifolieae clade within Caprifoliaceae including *Leycesteria*, *Triosteum*, *Symphoricarpos*, and *Lonicera*. Relationships within Caprifolieae, however, remain quite uncertain, and the monophyly of *Lonicera*, the most species-rich of the traditional genera, and its subdivisions, need to be evaluated. In this study we used sequences of the ITS region of nuclear ribosomal DNA and five chloroplast non-coding regions (*rpoB–trnC* spacer, *atpB–rbcL* spacer, *trnS–trnG* spacer, *petN–psbM* spacer, and *psbM–trnD* spacer) to address these problems. Our results indicate that *Heptacodium* is sister to Caprifolieae, *Triosteum* is sister to the remaining genera within the tribe, and *Leycesteria* and *Symphoricarpos* form a clade that is sister to a monophyletic *Lonicera*. Within *Lonicera*, the major split is between subgenus *Caprifolium* and subgenus *Lonicera*. Within subgenus *Lonicera*, sections *Coeloxylosteum*, *Isoxylosteum*, and *Nintooa* are nested within the paraphyletic section *Isika.* Section *Nintooa* may also be non-monophyletic. Our analysis of the genus *Lonicera* highlights instances of homoplasy in several morphological characters (e.g. hollow stems in subgenus *Caprifolium* and section *Coeloxylosteum* in subgenus *Lonicera*). Furthermore, our data indicate possible instances of hybridization in section Nintooa and biogeographic disjuctions between the Old and New Worlds (e.g. subsections *Distegiae and Alpigenae*).

***Keywords*—**chloroplast non-coding region, *Heptacodium*, honeysuckle, *Kolkwitzia, Weigela, Zabelia.*

Recent phylogenetic studies have resolved many relationships within Dipsacales (Donoghue et al. 2003; Bell and Donoghue 2005), the most important exception being within tribe Caprifolieae of Caprifoliaceae (sensu Donoghue et al. 2001, 2003; = Caprifoliaceae s. str. sensu Backlund and Pyck 1998; APG 1998). This clade contains *Leycesteria* Wallich. (6 species), *Lonicera* L. (ca. 200 species), *Symphoricarpos* Duhamel. (ca. 15 species), and *Triosteum* L. (6 species), and is well-supported in all recent analyses based on morphology (Judd et al. 1994) and DNA sequences (reviewed in Donoghue et al. 2003). However, none of the previous analyses have adequately tested the monophyly of each of the included genera, or confidently resolved relationships among them.

Caprifolieae includes genera that are among the most speciose in the Northern Hemisphere (i.e. *Lonicera*) and genera that contain few species (i.e. *Triosteum* and *Leycesteria*, Hsu and Wang 1988). With the exception of *Leycesteria,* the genera span the New and Old Worlds. *Triosteum,* with just six species, has both North American and Asian representatives (China and Japan) while *Symphoricarpos* has just one species in China, the remainder being native to North and Central North America. *Lonicera* is mainly distributed in temperate and subtropical areas, with several species extending their range into tropical areas of India, Malaysia, and the Philippines (Rehder 1903; van Steenis 1946). Many species within Caprifolieae have medicinal properties. Species of *Symphoricarpos* contain alkaloids and saponins used medicinally by several Native American tribes, while extracts from species of *Lonicera* have long been used in some traditional Chinese medicines for inflammation relief. A number of species have been bred as ornamentals worldwide, including several *Lonicera* species which have escaped from cultivation in the United States and have become costly invasives, including Japanese honeysuckle (*L. japonica* Thunb.) and the bush honeysuckles Amur (*L. maackii* Rupr.), Tartarian (*L. tatarica* L.), and Morrow's honeysuckle (*L. morrowii* Gray), and their hybrid Bell's honeysuckle (*L.* × *bella* Zabel).

Historically, *Lonicera* has received the most extensive taxonomic evaluation. Rehder (1903) published a synopsis of *Lonicera*, wherein he reviewed the taxonomic literature in detail and proposed a classification system. This was the first and most comprehensive taxonomic published treatment of *Lonicera* and has since been adopted widely with only minor changes. Rehder (1903) recognized two subgenera in *Lonicera*, the first of which, *Chamaecerasus* (= *Lonicera*), with more than 150 species, consisted of four sections (*Coeloxylosteum, Isika, Isoxylosteum*, and *Nintooa*) and 20 subsections. Later, Rehder combined subsections within *Chlamydocarpi* and *Vesicariae* (Rehder 1909, 1913). The second subgenus, *Periclymenum* (= *Caprifolium*), with approximately 22 species, was composed of four subsections. Subgenus *Lonicera* is characterized by two-flowered cymes and free leaves, whereas subgenus *Caprifolium* has three-flowered cymes in whorls and perfoliate leaves subtending the inflorescences.

In his treatment of the Japanese species of *Lonicera*, Nakai (1938) proposed many sections and subsections, including sections *Bracteatae*, *Fragrantissimae, Monanthae*, *Pararhodanthae*, *Praeflorentes*, *Ramosissimae*, *Rhodanthae*, *Rotatae*, and subsections *Cerasinae, Euchranthae*, *Subsessiliforae*, and *Tetramerae*. However, these groups have rarely been accepted with the exception of section *Monanthae*, which has a single species endemic to Japan (*L. gracilipes*) and was treated as a subsection within section *Isika* by Hara (1983). In their treatment of Chinese *Lonicera* species, Hsu and Wang (1988) largely adopted Rehder's classification system, but rejected several of Rehder's subsections, including *Spinosae* in section *Isoxylosteum*, *Tataricae* in section *Coelxylosteum*, *Distegiae* in section *Isika*, and *Breviflorae* and *Longiflorae* in section *Nintooa.* Rehder's four sections, therefore, have been generally agreed upon by most botanists who have considered the problem. Nevertheless, phylogenetic relationships among and within

these sections have never been explicitly addressed. Furthermore, the circumscriptions and evolutionary relationships of the numerous subsections have not been subjected to phylogenetic analysis.

The primary objectives of the present study were to resolve relationships within Caprifolieae, and test the monophyly of *Lonicera* and its subgenera, sections, and subsections using sequences from nuclear and chloroplast DNA regions that have been used widely in resolving relationships of closely related genera, including the ITS region of nuclear ribosomal DNA (Baldwin et al. 1995) and five chloroplast non-coding regions (*rpoB–trnC* spacer, *atpB–rbcL* spacer, *trnS–trnG* spacer, *petN–psbM* spacer, and *psbM–trnD* intergenic spacer; Shaw et al. 2005).

Materials and Methods

***Plant Material*—**Fifty-one individuals of *Lonicera* were sampled, representing 47 species, 21 of the 24 subsections, all four sections, and both subgenera (Appendix 1). From other Caprifolieae we included two species of *Leycesteria* and three species each of *Symphoricarpos* and *Triosteum* and the monospecific genus *Heptacodium.* From Caprifoliaceae, two species of *Weigela* (Diervilleae) and one species each of *Kolkwitzia* and *Zabelia* (Linnaeae) were included in the analysis. The latter were used as outgroups for rooting purposes based on Donoghue et al. (2003) and Zhang et al. (2003). ITS sequences of *Leycesteria and Triosteum* were obtained from GenBank (Gould and Donoghue 2000); all others were newly obtained for this study.

***Molecular Techniques*—**DNAs were extracted from silica-gel dried leaves using a DNeasy Plant Extract Mini kit following the manufacturer's protocol (Qiagen, Valencia, California). All amplification was performed using either Eppendorf or MJ Research thermal cyclers in 25–50 μL volumes with the following reaction components: 1–5 μL template DNA (10–100 ng), 1–2 μL primer, 10× buffer (Invitrogen Corporation, Carlsbad, California), 200 μmol/L each dNTP, 1.5–2.0 mmol/L $MgCl_2$, 0.4–0.6 μmol/L each primers, and 2.5 units *Taq*. Some reactions included bovine serum albumin with a final concentration of 0.2 μg/μL to improve amplification. ITS reactions included 10% dimethylsulfoxide. The sequencing primers and parameters for amplification are given in Table 1.

For most of the accessions, PCR products for ITS were cloned using the T-A tail cloning technique, and P^{gem} plamid system (Promega, Maddison, Wisconsin) and XLI blue competent *E. coli* cells (Stratagene, La Jolla, California) following manufacturer's instructions. Two to five clones were sequenced for representative species to check for sequence heterogeneity among ITS repeats. Cycle sequencing reactions were carried out using a BigDye Terminator V3.1 kit (Applied Biosystems Inc., Foster City, California). Sequences were analyzed using an ABI automated DNA Sequencer 377, ABI PRISM 3100 or 3730 (Perkin-Elmer/ABI), and edited using Sequencher (version 4.1, Gene codes Corporation, Ann Arbor, Michigan).

***Phylogenetic Analysis*—**The edited sequences were aligned by eye using Se-Al v2.0a8 (Rambaut 1996). Matrices have been submitted to TreeBASE (study number S2161). Sequence limits of the ITS-1, 5.8S, and ITS-2 were determined by comparing them to a published sequence in GenBank (*Diervilla lonicera*, AF078722, Kim and Kim 1999). Character states were equally weighted and unordered. Gaps were treated as missing data and new character states. Phylogenetic analyses were conducted in PAUP* 4.0b10 (Swofford 2001) and Mr. Bayes v3.0b4 (Huelsenbeck and Ronquist 2001). Options for maximum parsimony (MP) analyses included heuristic searches with 1000 random sequence addition replicates, with tree bisection-reconnection (TBR) branch swapping, MULPARS in effect, and deepest descent off. In all other cases a two step search was performed with random sequence addition and TBR branch swapping, with MAXTREES set to autoincrease, keeping 5 trees per 1,000 replicates, followed by an exhaustive search on all of the most parsimonious trees with MAXTREES set to 15,000. Bootstrap analyses (BP) of 1,000 replicates were conducted using heuristic searches to estimate support for individual clades (Felsenstein 1985). Decay indices (DI) were obtained using the PAUP decay index command in MacClade 4.0 (Maddison and Maddison 2000), which generated a file of trees with single resolved branches which was then executed in PAUP* using the heuristic search option to find the shortest trees consistent with each constraint.

Prior to combining the data sets, conflict between data partitions was explored. Congruence of the data sets was evaluated by two methods: through visual inspection of individual bootstrap values and the incongruence length difference test (ILD; Farris et al. 1995). We considered data sets as incongruent if clades with BP ≥ 84% were in conflict (e.g. Hansen et al. 2005). The ILD test was implemented in PAUP* using the partition homogeneity test using one thousand heuristic partition homogeneity replicates, each with 10 random addition sequence replicates, TBR branch swapping, MulTrees off, gaps treated as missing data, and constant characters included. In addition, where specific predictions (monophyly of sections) about topology broke down we used the Shimodaira-Hasegawa test as implemented in PAUP* (Shimodaira and Hasegawa 1999).

The best model of nucleotide substitution for each data set was determined by performing hierarchical likelihood ratio tests using MrModeltest 2.2 (Nylander 2004). A general time reversible (GTR) model with unequal base frequencies and among site variation that was gamma distributed was selected for all six data sets: nrITS, *rpoB–trnC* spacer, *atpB–rbcL* spacer, *trnS–trnG* spacer, *petN–psbM* spacer, and *psbM–trnD* spacer. Bayesian analyses were performed using Mr. Bayes version 3.0b4 (Huelsenbeck and Ronquist 2001). Four simultaneous chains of Markov chain Monte Carlo were run starting from random trees for 5 million generations, sampling every 100 generations. A burn-in of 100,000 generations was implemented due to stabilization of the likelihood scores prior to this point. Posterior probabilities (PP) from the analysis are shown on the majority rule consensus tree.

Results

***Nuclear and Chloroplast Data Sets*—**Complete sequences were obtained for the nrDNA ITS and five cpDNA spacer regions (*rpoB–trnC* spacer, *atpB–rbcL* spacer, *trnS–trnG* spacer, *petN–psbM* spacer, *psbM–trnD* intergenic spacer) from all 64 accessions (Appendix 1). Details on variation in sequence lengths, alignment length, the number of variable sites, and parsimony informative sites as well as the range of pairwise distances and the consistency indices are listed in Table 2. No sequence heterogeneity of the nrDNA ITS region was found among 2–5 clones within each species. There were many large indels particularly in the *trnS–trnG* and *rpoB–trnC* spacer regions. Many of these indels supported relationships amongst Caprifolieae genera and within *Lonicera,* however the parsimony analyses resulted in identical trees whether indels were included or not and have therefore been excluded from the analysis presented here. Maximum pairwise distances within *Lonicera* measured 6.4% for the ITS region, 2.8% for the *rpoB–trnC* spacer, 3.0% for the *atpB–rbcL* spacer, 3.7% for the *trnS–trnG* spacer, 2.8% for the *petN–psbM*

Table 1. PCR parameters for the ITS and five chloroplast noncoding regions.

Regions	Initial denaturing step, time	reps	Denaturing temperature, time	Primer annealing temperature, time	Chain extension temperature, time	Final extension step, time	Primer source
ITS	94°C, 3 min	34x	94°C, 30 sec	50°C, 1 min	72°C, 1 min	72°C, 10 min	Baldwin et al. (1995)
rpoB–trnC	80°C, 5 min	35x	96°C, 1 min	55°C, 2 min	72°C, 3 min	72°C, 5 min	Shaw et al. (2005)
atpB–rbcL	94°C, 2 min	34x	94°C, 1 min	53°C, 1 min	72°C, 90 sec	72°C, 5 min	Zurawski et al. (1984), Crayn and Quinn (2000)
trnS–trnG	94°C, 3 min	34x	94°C, 45 sec	59°C, 1 min	72°C, 90 sec	72°C, 7 min	Hamilton (1999)
petN–psbM	94°C, 3 min	34x	94°C, 1 min	55°C, 1 min	72°C, 2 min	72°C, 7 min	Lee and Wen (2004)
psbM–trnD	94°C, 3 min	34x	94°C, 1 min	55°C, 1 min	72°C, 2 min	72°C, 7 min	Lee and Wen (2004)

TABLE 2. Comparison of the variation in the nuclear ribosomal region, the five chloroplast non-coding regions, and the combined data set. PI = parsimony informative characters, CI = consistency index, and RI = retention index.

Statistic	All	ITS	*rpoB–trnC*	*atpB–rbcL*	*petN–psbM*	*trnS–trnG*	*psbM–trnD*
Range of raw length		546–621	1074–1296	590–730	578–1253	557–668	602–1178
Aligned length	6536	653	1479	798	1449	878	1279
Variable sites (percent)	1630 (25)	220 (34)	384 (26)	156 (20)	291 (21)	202 (24)	377 (30)
PI sites (percent)	872 (13)	132 (20)	195 (13)	92 (12)	197 (14)	119 (14)	137 (11)
Range of pairwise distance	0–0.11	0–0.13	0–0.15	0–0.074	0–0.082	0–0.12	0–0.23
CI, RI	0.77, 0.82	0.6, 0.71	0.83, 0.87	0.86, 0.91	0.79, 0.87	0.9, 0.94	0.89, 0.88

spacer, and 2.5% for the *psbM–trnD* intergenic spacer region. Pairwise distances are reported between *Lonicera* and representatives of the other included genera in Table 2.

Congruence of Data Sets—Results of the ILD test comparing ITS to all 5 cpDNA data sets taken together suggests that they are incongruent ($p < 0.01$). Comparison of tree topologies indicated that the incongruence was evident in two clades. In the *Nintooa* clade of the chloroplast tree, *Lonicera henryi* is sister to *L. japonica* (BP = 100%), while in the ITS tree, *L. henryi* is sister to *L. giraldii* (BP = 84%). From section *Isika, L. fragrantissima* is sister to *L. standishii* (BP = 98%) in the chloroplast tree rather than to *L. hemsleyana* as is the case in the ITS tree (BP = 97%). The ILD tests were repeated with these taxa excluded, but the partitions remained incongruent ($p < 0.01$). The cause of the remaining heterogeneity is unclear, though this could reflect differences in the number of parsimony informative (PI) characters (Table 2) and substitution rates between markers (Dolphin et al. 2000; Darlu and Lecointre 2002). Therefore, we proceeded to combine the data sets because the conflicts appear to be localized (Yoder et al. 2001, Hipp et al. 2004, Levin and Miller 2005).

Phylogenetic Relationships—The data set from the chloroplast DNA had 5,883 characters, 740 of which were parsimony informative, over 200,000 trees were retained with 1,882 steps. The nuclear DNA data set contained 653 characters of which 132 were parsimony informative. This analysis yielded 1,915 trees with 504 steps. The combined data set of chloroplast and nuclear DNA regions had 6,536 characters, 872 of which were parsimony informative. MP analyses generated 2,136 trees of 2,433 steps; the strict consensus tree is shown in Figs. 1, 2 (CI = 0.77, RI = 0.82). *Heptacodium* was sister to the Caprifolieae clade and *Triosteum* species formed a clade that split from the remaining Caprifolieae (BP = 95%; DI = 6). *Symphoricarpos* and *Leycesteria* formed a clade (BP = 84%, DI = 2) that was sister to the monophyletic *Lonicera* (BP = 100%; DI = 20). Within *Lonicera* there were two major clades corresponding to the two subgenera: *Caprifolium* and *Lonicera*. Within *Lonicera,* section *Coeloxylosteum* formed a clade as did section *Isoxylosteum*. However, species of section *Nintooa* were in two separate clades and section *Isika* was paraphyletic, within subgenus *Lonicera*. All subsections within section *Isika* formed monophyletic clades except for subsections *Pileatae*, and *Vesicariae*.

Bayesian analyses of the partitioned data set resulted in stabilization of likelihood scores by 100,000 generations; all prior generations were discarded as burn-in. Many nodes were resolved with posterior probabilities of 100% (Fig. 1). As in the MP analyses, *Lonicera* sections *Coeloxylosteum* and *Isoxylosteum* were monophyletic, whereas sections *Isika* and *Nintooa* were not. However in the MP analyses, *L. etrusca* and *L. hispidula* were not sister species within subgenus *Caprifolium*; although, bootstrap support for this topology was less than 50%. Overall there was not strong disagreement between the Bayesian and MP analyses for the placement of any taxa, but there was higher support for the topology of the tree from the Bayesian analysis. Most notably there was support for many of the deeper nodes in the tree. However, there was relatively weak support for the placement of *Triosteum* as the basal taxa to Caprifoliaeae (PP = 75).

DISCUSSION

Intergeneric Relationships Within Caprifolieae—In previous phylogenetic results of Dipsacales there is a well-supported but poorly resolved Caprifolieae clade containing *Lonicera*, *Leycesteria*, *Symphoricarpos*, and *Triosteum* (e.g. Backlund and Donoghue 1996; Pyck et al. 1999; Zhang et al. 2003; Donoghue et al. 2003). The precise relationships of this clade to other Caprifoliaceae s. l. have not been strongly supported. For rooting purposes we included *Zabelia* and *Kolkwitzia* from Linnaeeae and two species of *Weigela* from Diervilleae. Recent molecular analyses have suggested that the monospecific *Heptacodium* may be the sister group of Caprifolieae (Pyck and Smets 2000; Bell et al. 2001; Donoghue et al. 2003). Although our sampling of the remainder of Dispacales is limited, our analyses add support to the placement of *Heptacodium* with Caprifolieae (Figs. 1, 2). Nevertheless, *Heptacodium* is morphologically unique with its strongly 3-nerved leaves and dense cymes. This uniqueness is reflected in the molecular data also, with an exceptionally long branch leading to *Heptacodium* (Fig. 2).

Conflicting topologies and poor resolution have characterized analyses of the relationships within Caprifolieae. Donoghue et al. (2001) found *Leycesteria* to be sister to a clade containing *Triosteum* and the sister taxa *Lonicera* and *Symphoricarpos*, but with bootstrap support <50%. Zhang et al. (2003) obtained the following relationships: (*Triosteum* (*Symphoricarpos* (*Leycesteria Lonicera*))), all with bootstrap support <65%. In our trees (Fig. 1), *Triosteum* (BP = 96%) is sister to a clade containing the remaining genera, followed by a clade (BP=88%) of *Leycesteria* and *Symphoricarpos*, which is sister to *Lonicera* (BP = 100%). *Triosteum* is the only herbaceous group and has been considered to be in its own tribe (Hsu 1983). A direct connection between *Leycesteria* and *Symphoricarpos* was suggested in an early phenetic analysis of morphological characters by Hsu (1983). Both genera have terminal inflorescences, but we know of no clear synapomorphies for this clade. While this topology is consistent with the Bayesian result (Fig. 1), support for the placement of *Triosteum* is not as high (PP = 75%). Although our analyses provide stronger results for relationships within Caprifolieae than previous studies, additional studies, including a broader sample of other Dipsacales, are necessary to assess the placement of *Triosteum* and the relationship between *Leycesteria* and *Sym-*

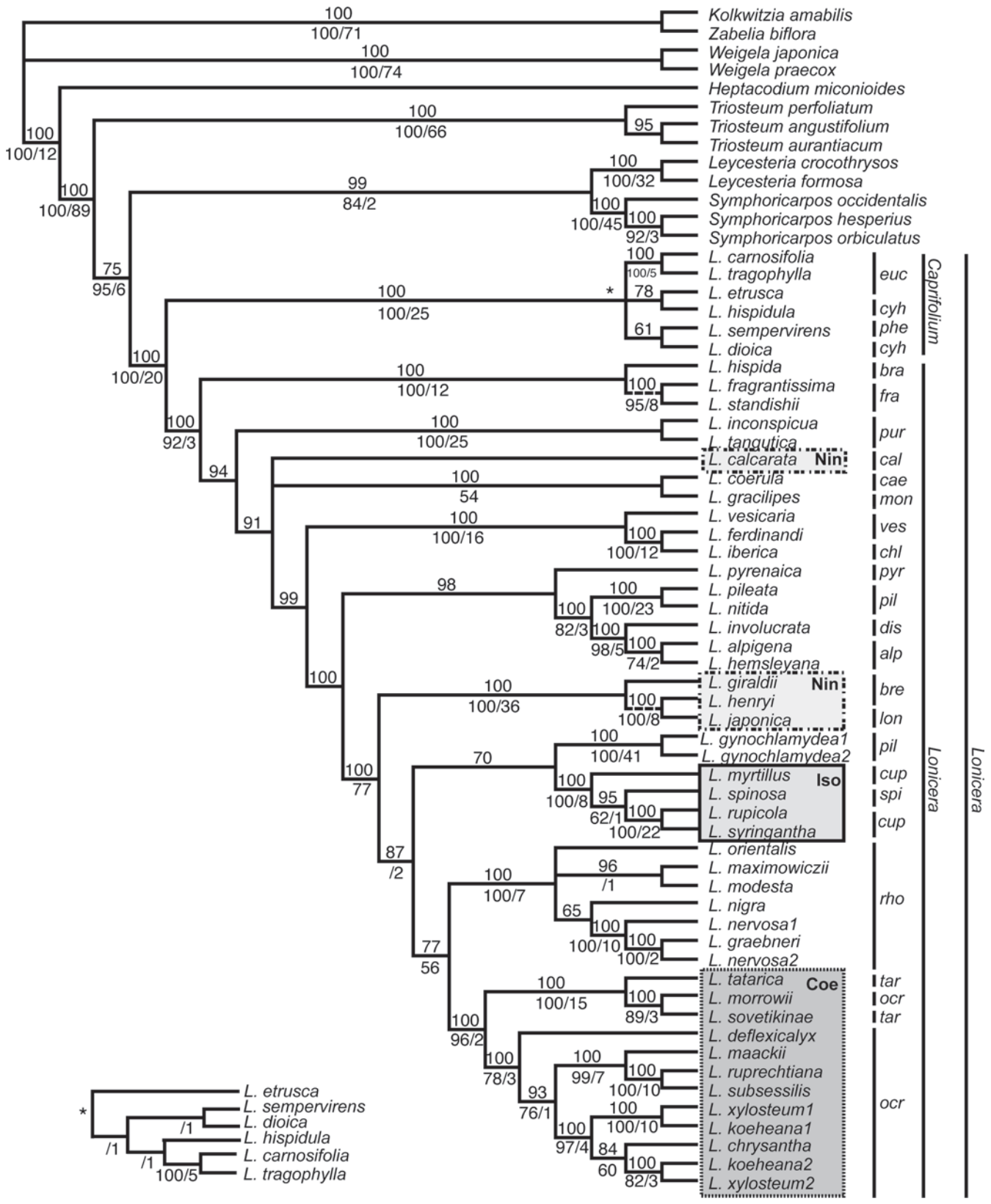

FIG. 1. The combined majority rule consensus tree from the Bayesian analysis and the strict consensus tree from the parsimony analysis based on sequences of nrDNA (ITS), and cpDNA (*rpoB–trnC* spacer, *atpB–rbcL* spacer, *trnS–trnG* spacer, *petN–psbM* spacer, and *psbM–trnD* spacer). Dashed lines represent conflict between the chloroplast and nuclear data (BS > 84%) in the parsimony analysis. Numbers above the branches are posterior probabilities and below the branches are bootstrap percentages/decay indices. The asterisk indicates the different topology of the *Caprifolium* subgenus as determined by the parsimony analysis (left corner). Acronyms on the right indicate taxonomic groups following Rehder (1903, 1909, 1913), Hara (1983) and Hsu and Wang (1988). Genus: *Lonicera*. Subgenera: *Caprifolium* and *Lonicera*. Sections: *Nintooa* (**Nin**), *Isoxylosteum* (**Iso**), and *Coeloxylosteum* (**Coe**) indicated in shaded boxes all others in the section *Isika*. Subsections: *Alpigenae (alp), Bracteatae (bra), Breviflorae (bre), Calcaratae (cal), Chlamydocarpi (chl), Coeruleae (cae), Cupulae* (*cup*), *Cyhpeolae (cyh), Distegiae (dis), Eucaprifolium (euc), Fragrantissimae (fra), Longiflorae (lon), Monanthae* (*mon*), *Ochranthae (ocr), Phenianthi (phe), Pileatae (pil), Purpurascentes (pur), Pyrenaicae (pyr), Rhodanthae (rho), Spinosae (spi), Tataricae (tar), Vesicariae (ves).*

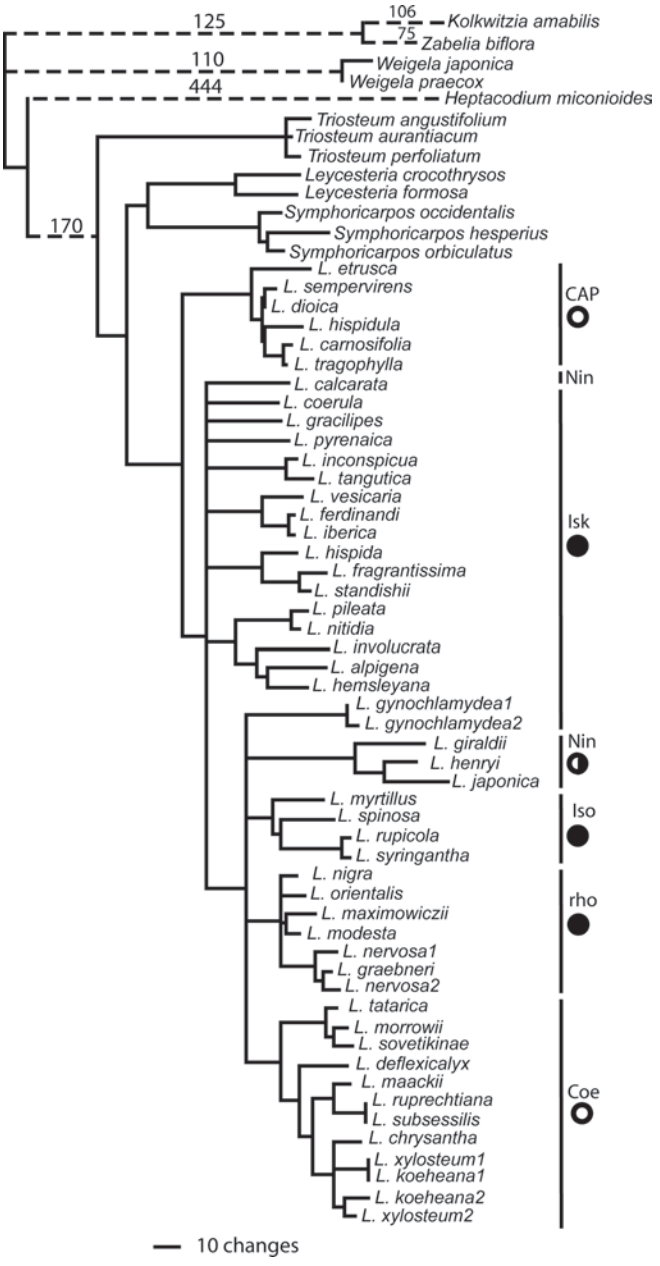

FIG. 2. Phylogram of the strict consensus tree based on a parsimony analysis of the combined data of chloroplast and nuclear DNA regions, showing branch lengths. Dashed lines indicate shortened lines to improve visualization of the remaining branches; above those branches is the numerical branch length. Hollow branches (circle), solid (filled circle) or representatives of both have been mapped onto the tree for sections of *Lonicera*.

phoricarpos suggested here. All of the species of *Lonicera* sampled in our study form a well supported clade in both MP and Bayesian analyses.

Subgenus Delimitations—Rehder (1903) divided *Lonicera* into two subgenera: *Lonicera* and *Caprifolium*. With just over 20 species, *Caprifolium* is the smaller subgenus in the genus *Lonicera,* though its geographical distribution is broad with a range throughout Europe and North America, Afghanistan, and Central China. Morphologically, the subgenera are distinctive, most significantly by the inflorescence, with the former having two-flowered cymes, while the latter has three-flowered cymes. In both MP and Bayesian trees (Fig. 1), species of subgenus *Caprifolium* form a clade that is sister to subgenus *Lonicera*. This provides the first phylogenetic support for the recognition of these two taxa.

Sections of Subgenus Caprifolium—Based on the corolla symmetry and the fusion of bractlets, Rehder (1903) recognized four subsections in subgenus *Caprifolium*: *Phenianthi* (5 spp.), *Cyhpeolae* (10 spp.), *Eucaprifolia* (7 spp.), and *Thoracianthae* (1 sp.) However, in our phylogenetic trees (Fig. 1), subgroups with 2 or more species sampled (*L. carnosifolia*, *L. hispidula* and *L. dioica* of *Cypheolae*, and *L. tragophylla* and *L. etrusca* of *Eucaprifolia*) do not form monophyletic groups, indicating that it may be inappropriate to separate the subgenus into smaller groups. Nevertheless, more species of the subgenus are needed to further test the monophyly of subsections.

Sections of Subgenus Lonicera—Four sections are generally recognized in subgenus *Lonicera*: *Coeloxylosteum*, *Isoxylosteum*, *Nintooa*, and *Isika* (Rehder 1903; Hara 1983; Hsu and Wang 1988). Each of these sections is marked by a unique combination of morphological characters. For example, section *Isoxylosteum* is characterized by branches with solid pith, five nectaries, and the absence of accessory buds. Section *Coeloxylosteum* is characterized by flowers with two-lipped corolla and hollow branches. Both of these characters are shared with *Nintooa* and we can infer from our trees that hollow branches evolved more than once within *Lonicera* (Fig. 2). Section *Nintooa* differs from others in its twining or creeping habits, combined with cymes forming terminal panicles, and corollas with long, slender tubes. Section *Isika* is the largest and most diverse section. These plants have solid branches, as in section *Isoxylosteum*, but differ in having both zygomorphic and nearly actinomorphic corollas with 1–3 nectaries, and in having accessory buds. In our trees (Fig. 1), *Coeloxylosteum* and *Isoxylosteum* each form a clade, supporting their monophyly, whereas *Nintooa* is divided into two separate clades, suggesting that it is non-monophyletic. Furthermore, all three of these sections are nested within section *Isika*. Therefore, section *Isika*, as traditionally circumscribed, is paraphyletic.

LONICERA *SECTION ISOXYLOSTEUM*—This section consists of eight species that are distributed in eastern Himalayas and southwestern China. These plants are compact shrubs with solid, white pith, small leaves, and flowers with nearly actinomorphic corollas that are non-gibbous at the base. Rehder (1903) recognized two subsections: *Microstylae* (= *Cupulae* Hsu and Wang 1988) and *Spinosae*. These differ in whether the stamens and styles are exerted from the corolla tube or not. Hsu and Wang (1988), however, combined the two subsections and treated *L. spinosa*, the only species of subsection *Spinosae*, as a variety of *L. myrtillus*. In our combined tree, *L. spinosa* is embedded within section *Microstylae*, supporting Hsu and Wang's merge of these two sections.

LONICERA *SECTION NINTOOA*—All 30 species in this section are vines and their upper leaves are reduced or even bractlike. There are three subsections, including the monotypic subsection *Calcaratae*, which is unique in having a long nectar spur, connate ovaries, and bracteoles. Interestingly, although support for the placement of *L. calcarata* is low, it does not form a clade with other representative species of *Nintooa*: *L. japonica* (subsection *Breviflorae)*, *L. giraldii*, and *L. henryi* (subsection *Longiflorae)*. When we forced all four species of *Nintooa* to form a clade, six more steps were needed. This is not significantly longer than the unconstrained phylogeny as

judged by the one-tailed nonparametric Shimodaira-Hasegawa test ($P > 0.5$). Thus, more data are needed to test the monophyly of *Nintooa*. Within *Nintooa* our chloroplast data offer strong support for the sister relationship of *L. henryi* and *L. japonica*; however, the nuclear rDNA data recognize the closer relationship of *L. henryi* with *L. giraldii*. *Lonicera henryi* is a hexaploid ($2n = 54$), while the other two species are diploid species (Rüdenberg and Green 1966). Thus, hybridization may be the cause of the phylogenetic incongruence among the species. Therefore, it is desirable to further examine the evolutionary history of the section with an extensive taxon sampling.

It has been postulated that *Nintooa* links the two subgenera because its members have hollow branches and climbing habit, traits that are also found in subgenus *Caprifolium*, though the leaves subtending the inflorescence are not fused (Rehder 1903). However, in our phylogenetic trees section *Nintooa* appears to have arisen from within section *Isika* of subgenus *Lonicera*. Therefore, twining habit and hollow branches appear to have arisen more than once in *Lonicera*.

LONICERA *SECTION COELOXYLOSTEUM*—This section contains about 14 species from eastern and Central Asia, north and west of the Himalayas, Europe, and North Africa. It is a rather homogeneous group, characterized by evanescent pith, distinctly two-lipped corollas, and the tendency to have distinct ovaries, an upright habit, and red fruits. The two subsections, *Ochranthae* and *Tataricae*, are distinguished only by minor morphological differences. Members of *Tataricae* are more glabrous throughout, and their flowers are pink to white, and not fading to yellow. *Ochranthae* species, in contrast, are rarely glabrous, and their flowers are white or yellowish white, rarely tinged with red, and fading to yellow. In addition, there are numerous hybrids between their members, though all species are diploid ($2n = 18$; Rehder 1903; Green 1966). Sequences from both chloroplast and nrDNA suggest that neither of the two subsections formed a clade, owing to the placement of *L. morrowii* (subsection *Ochranthae*) within *Tataricae*, supporting the merger of the two subsections (Hsu and Wang 1988). It is also noteworthy that accessions of *L. xylosteum* and of *L. koeheana* do not form clades in either the chloroplast or nuclear tree. Instead, we recovered two clades containing one accession of each species (Fig. 1); clearly these species are closely related, and might even be con-specific. Hybridization, as an explanation for the topology we found is unlikely since trees based on nrDNA and cpDNA were concordant. However, more accessions are needed to critically evaluate this result.

LONICERA *SECTION ISIKA*—This is the largest section in *Lonicera*, with about 75 species, and it is distributed throughout the range of the genus. This group is also most diverse in habit, in the size and shape of the bracts and bracteoles, in the outer scales of the winter buds, and in the shape of the corolla. Accordingly, Rehder (1903) recognized 12 subsections within *Isika*, half of these with only one or two species. When he proposed *Isika*, Rehder pointed out that some species in this section were related to section *Isoxylosteum* and others to section *Coeloxylosteum*, while still others formed clearly defined groups that exhibited no clear affinity to other *Lonicera* species (Rehder 1903). Our trees support the paraphyly of *Isika*–species groups assigned to this section form a grade of lineages, with some being closely allied with the other sections (Fig. 1). For example, subsection *Rhodanthae* as a well supported clade appears to be more closely related to sections *Nintooa*, *Isoxylosteum*, and *Coeloxylosteum* than it is to other species of *Isika.* Similarly, *L. gymnochlamydea* of subsection *Pileatae* forms a clade with the other three sections and is distantly related to other species of the subsection.

Other subsections within section *Isika* each form their own clades. The proposed merge of *Chlamydocarpi* with *Vesicariae* (Rehder 1909, 1913) is upheld by our data and would result in yet another well supported subsection. *L. hispida* of subsection Bracteatae forms a robust clade with species of subsection *Fragrantissimae*. This is supported by morphology; plants of the subsections are often setosely hispid and have branches terminated by two axillary winter buds (Rehder 1903). In the ITS tree, however, *L. hemsleyana* of subsection *Alpigenae* is grouped with subsection *Fragrantissimae*, while our chloroplast data is consistent with morphology in placing *L. hemsleyana* together with other species of subsection *Alpigenae*. We suspect that sequence heterogeneity might play a role here, but it is necessary to do a thorough analysis of the DNA region to test this speculation. Subsections *Distegiae* and *Alpigenae* are grouped in a well supported clade, supporting Rehder's (1903) observations that *Distegiae* is similar to *Alpigenae* in general habit and pubescence. *Distegiae* has two species, represented in our analysis by *L. involucrata*, native to northern and western North America, which is sister to members of subsection *Alpigenae,* represented here by two of the 14 species (*L. alpigena* and *L. hemsleyana*) is restricted to Europe and Asia. Therefore, our data indicate an interesting biogeographic disjunction between the New and the Old Worlds. A critical appraisal of the status of these subsections as well as their biogeography will require the inclusion of additional species. Our results for section *Isika*, along with other sections set the stage for more detailed phylogenetic analyses within the major clades that we have identified, which will surely provide many further insights into character evolution, hybridization, and the biogeographic history of *Lonicera*.

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Appendix 1. Taxa and their source, voucher, and GenBank accession numbers (in the order of ITS, *trnS–trnG* spacer, *psbM–trnD* spacer, *rpoB–trnC* spacer, *atpB–rbcL* spacer, and *petN–psbM* spacer; Boldface indicates sequences obtained for this study). Rehder, Hara and Hsu and Wang's systems are used as frameworks for taxon sampling (Rehder 1903, 1909, 1913; Hara 1983; Hsu and Wang 1988).

Heptacodium miconioides Rehd. (ITS only), cultivated at Arnold Arboretum, *anonymous 1549-80G*, China (A), **EU240665**. *H. miconioides* Rehd. (petN-psbM, *atpB–rbcL*, rpoB-trnC), cultivated at Arnold Arboretum, *anonymous 1549-80B*, China (A), **EU265454, EU265518, EU265582**. *H. miconioides* Rehd. (*trnS–trnG*, psbM-trnD), cultivated at Arnold Arboretum, *anonymous 1549-80A*, China (A), **EU265326, EU265390**. *Kolkwitzia amabilis* Graebn., cultivated at Arnold Arboretum, *Youngerman and Driskill 20447B* (A), **EU240666, EU265327, EU265391, EU265455, EU265519, EU265583**. *Leycesteria crocothyrsos* Airy Shaw, cultivated at Kew Gardens, *Del Tredici s.n.* (A), AF265277, **EU265328, EU265392, EU265456, EU265520, EU265584**. *L. formosa* Wall, *Boufford et al. 29341*, China (A), AF265276, **EU265329, EU265393, EU265457, EU265521, EU265585**. *Symphoricarpos hesperius* G.N.Jones, cultivated at Arnold Arboretum, *Youngerman and Driskill 786-83Mass* (A), **EU240667, EU265330, EU265394, EU265458, EU265522, EU265586**. *S. occidentalis* Hook, cultivated at Arnold Arboretum, *Gilsdorf and Warren 1078-81Mass* (A), **EU240668, EU265331, EU265395, EU265459, EU265523, EU265587**. *S. orbiculatus* Moench, cultivated at Arnold Arboretum, *Gilsdorf and Warren 431* (A), **EU240669, EU265332, EU265396, EU265460, EU265524, EU265588**. *Triosteum angustifolium* L., *Cantino 1407* (BHO), AF265292, **EU265333, EU265397, EU265461, EU265525, EU265589**. *T. aurantiacum* E.P.Bicknell, *Walters et al., s.n.* (BHO), AF265290, **EU265334, EU265398, EU265462, EU265526, EU265590**. *T. perfoliatum* L., *Cantino 1408* (BHO), AF265291, **EU265335, EU265399, EU265463, EU265527, EU265591**. *Weigela japonica* Thunb., cultivated at Arnold Arboretum, *anonymous 1104-89B*, China (A), **EU240670, EU265336, EU265400, EU265464, EU265528, EU265592**. *W. praecox* L.H.Bailey, cultivated at Arnold Arboretum, *anonymous 843-84Mass* (A), **EU240671, EU265337, EU265401, EU265465, EU265529, EU265593**. *Zabelia biflora* (Turcz.) Makino, cultivated at Arnold Arboretum, *Youngerman and Driskill 94-23B*, China (A), **EU240672, EU265338, EU265402, EU265466, EU265530, EU265594**. *Lonicera alpigena* L., cultivated at Arnold Arboretum, *Elsik and Zinman 947-67-B* (A), **EU240673, EU265339, EU265403, EU265467, EU265531, EU265595**. *L. calcarata* Hemsl., *Li 3304*, China (A), **EU240674, EU265340, EU265404, EU265468, EU265532, EU265596**. *L. carnosifolia* C.Y.Wu, *Li XY27*, China (A), **EU240675, EU265341, EU265405, EU265469, EU265533, EU265597**. *L. chrysantha* Turcz., cultivated at Arnold Arboretum, *Hardy-Brown, Heja, and Heffner 973-81A*, Russia (A), **EU240676, EU265342, EU265406, EU265470, EU265534, EU265598**. *L. coerulea* Regel, cultivated at Arnold Arboretum, *anonymous 1010-86B*, Tien Shan (A), **EU240677, EU265343, EU265407, EU265471, EU265535, EU265599**. *L. deflexicalyx* Batalin., cultivated at Quarryhill Botanical Garden, *Higson 88.101*, China (A), **EU240678, EU265344, EU265408, EU265472, EU265536, EU265600**. *L. etrusca* Santi, cultivated at Arnold

Arboretum, *Weissman and Mackenzie 345-94C,* Spain (A), **EU240679, EU265345, EU265409, EU265473, EU265537, EU265601**. *L. ferdinandi* Franch, cultivated at Arnold Arboretum, *Elsik, Erwin and Hofstetter 18360-A* (A), **EU240680, EU265346, EU265410, EU265474, EU265538, EU265602**. *L. fragrantissima* Lindl. & Paxt., cultivated at Arnold Arboretum, *anonymous 696-86C* (A), **EU240681, EU265347, EU265411, EU265475, EU265539, EU265603**. *L. gracilipes* Miq., *Murata s.n.*, Japan (A), **EU240682, EU265348, EU265412, EU265476, EU265540, EU265604**. *L. graebneri* Rehd., *Boufford et al. 28354,* China (A), **EU240683, EU265349, EU265413, EU265477, EU265541, EU265605**. *L. gynochlamydea* Hemsl. (1), cultivated at Arnold Arboretum, *Youngerman and Driskill 1723-80E,* China (A), **EU240684, EU265350, EU265414, EU265478, EU265542, EU265606**. *L. gynochlamydea* Hemsl. (2), cultivated at Arnold Arboretum, *Youngerman and Driskill 1776-80A,* China (A), **EU240685, EU265351, EU265415, EU265479, EU265543, EU265607**. *L. hemsleyana* Rehd., *Del Tredici 77694,* China (A), **EU240686, EU265352, EU265416, EU265480, EU265544, EU265608**. *L. henryi* Hemsl., cultivated at Arnold Arboretum, *Youngerman and Driskill 1792-80Mass,* China (A), **EU240687, EU265353, EU265417, EU265481, EU265545, EU265609**. *L. hispida* Pall, *Boufford et al. 29096,* China (A), **EU240688, EU265354, EU265418, EU265482, EU265546, EU265610**. *L. hispidula* A. Gray, cultivated at Rancho Santo Botanical Garden, *Michael Wall 8523,* California, USA (A), **EU240689, EU265355, EU265419, EU265483, EU265547, EU265611**. *L. iberica* M.Bieb., cultivated at Arnold Arboretum, *anonymous 955-1A* (A), **EU240690, EU265356, EU265420, EU265484, EU265548, EU265612**. *L. inconspicua* Batal., *Boufford et al. 28458,* China (A), **EU240691, EU265357, EU265421, EU265485, EU265549, EU265613**. *L. involucrata* Banks, cultivated at Arnold Arboretum, Kopf, *Hardy-Brown and Jones 485-82B* (A), **EU240692, EU265358, EU265422, EU265486, EU265550, EU265614**. *L. japonica* Thunb., cultivated at Arnold Arboretum, *Li 271* (A), **EU240693, EU265359, EU265423, EU265487, EU265551, EU265615**. *L. giraldii* Rehd., cultivated at Quarryhill Botanical Garden, *Higson 92.327,* China (A), **EU240694, EU265360, EU265424, EU265488, EU265552, EU265616**. *L. maackii* Rupr., cultivated at Arnold Arboretum, *Hardy-Brown, Richardson, Unger and Thompson 178-83D,* China (A), **EU240695, EU265361, EU265425, EU265489, EU265553, EU265617**. *L. maximowiczii* (Rupr.) Regel, cultivated at Arnold Arboretum, *anonymous 381-83Mass,* Korea (A), **EU240696, EU265362, EU265426, EU265490, EU265554, EU265618**. *L. morrowii* A Gray, cultivated at Arnold Arboretum, *anonymous 1323-84A* (A), **EU240697, EU265363, EU265427, EU265491, EU265555, EU265619**. *L. myrtillus* Hook.f. & Thoms., *Boufford et al. 28875,* China (A), **EU240698, EU265364, EU265428, EU265492, EU265556, EU265620**. *L. nervosa* Maxim. (2), *Boufford et al. 29295,* China (A), **EU240699, EU265365, EU265429, EU265493, EU265557, EU265621**. *L. nervosa* Maxim. (1), *Boufford et al. 28660,* China (A), **EU240700, EU265366, EU265430, EU265494, EU265558, EU265622**. *L. nigra* L., *Z. Chen, 990011* (PE), **EU240701, EU265367, EU265431, EU265495, EU265559, EU265623**. *L. pileata* Oliver, cultivated at Arnold Arboretum, *Youngerman and Driskill 18-92F* (A), **EU240702, EU265368, EU265432, EU265496, EU265560, EU265624**. *L. pyrenaica* L., cultivated at Arnold Arboretum, *anonymous 346-94A,* Spain (A), **EU240703, EU265369, EU265433, EU265497, EU265561, EU265625**. *L. rupicola* Hook.f. & Thoms., *Boufford et al. 28126,* China (A), **EU240704, EU265370, EU265434, EU265498, EU265562, EU265626**. *L. sempervirens* L., cultivated at Univ. of Texas, Austin, *Gould 160* (TEX), **EU240705, EU265371, EU265435, EU265499, EU265563, EU265627**. *L. sovetkinae* Tkatsch., cultivated at Arnold Arboretum, *Elsik, Dumaine and Groves 796-74B* (A), **EU240706, EU265372, EU265436, EU265500, EU265564, EU265628**. *L. spinosa* Jacques., *Z. Chen,* Tibet, June 1999 (PE), **EU240707, EU265373, EU265437, EU265501, EU265565, EU265629**. *L. standishii* Hock, cultivated at Arnold Arboretum, *Youngerman and Driskill 861-82A,* China (A), **EU240708, EU265374, EU265438, EU265502, EU265566, EU265630**. *L. tangutica* Maxim., *Boufford et al. 28914,* China (A), **EU240709, EU265375, EU265439, EU265503, EU265567, EU265631**. *L. tatarica* L., cultivated at Arnold Arboretum, *Elsik, Fich and Carey 299-78B,* Tajikistan (A), **EU240710, EU265376, EU265440, EU265504, EU265568, EU265632**. *L. tragophylla* Hemsl., *Li and Ren 4014,* China (A), **EU240711, EU265377, EU265441, EU265505, EU265569, EU265633**. *L. vesicaria* Kom., cultivated at Arnold Arboretum, *anonymous 239-75D* (A), **EU240712, EU265378, EU265442, EU265506, EU265570, EU265634**. *L. dioica* L., cultivated at Smith College, *Theis 001,* Vermont, USA (A), **EU240713, EU265379, EU265443, EU265507, EU265571, EU265635**. *L. xylosteum* L. (1), cultivated at Arnold Arboretum, *anonymous 838-76D,* Austria (A), **EU240714, EU265380, EU265444, EU265508, EU265572, EU265636**. *L. koehneana* Rehd. (1), cultivated at Arnold Arboretum, *Youngerman and Driskill 815-84A,* China (A), **EU240715, EU265381, EU265445, EU265509, EU265573, EU265637**. *L. modesta* Rehd., cultivated at Arnold Arboretum, *anonymous 1002-86A* (A), **EU240716, EU265382, EU265446, EU265510, EU265574, EU265638**. *L. nitida* E.H. Wilson, cultivated at Arnold Arboretum, *Youngerman and Driskill 469-97A,* China (A), **EU240717, EU265383, EU265447, EU265511, EU265575, EU265639**. *L. orientalis* Lam., cultivated at Arnold Arboretum, *Heffner, Hardy-Brown, and Heja 607-87A* (A), **EU240718, EU265384, EU265448, EU265512, EU265576, EU265640**. *L. koehneana* Rehd. (2), cultivated at Arnold Arboretum, *anonymous 1059-65A* (A), **EU240719, EU265385, EU265449, EU265513, EU265577, EU265641**. *L. ruprechtiana* Regel, cultivated at Arnold Arboretum, *Youngerman and Driskill 694-88C* (A), **EU240720, EU265386, EU265450, EU265514, EU265578, EU265642**. *L. subsessilis* Rehd., cultivated Arnold Arboretum, *Michener and Lovejoy 1652-77E,* Korea (A), **EU240721, EU265387, EU265451, EU265515, EU265579, EU265643**. *L. syringantha* Maxim, cultivated at Quarryhill Botanical Garden, no voucher, China (A), **EU240722, EU265388, EU265452, EU265516, EU265580, EU265644**. *L. xylosteum* L. (2), cultivated at Arnold Arboretum, *Youngerman and Driskill 856-84A* (A), **EU240723, EU265389, EU265453, EU265517, EU265581, EU265645**.