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NECTAR SPUR EVOLUTION IN THE MEXICAN LOBELIAS (CAMPANULACEAE: LOBELIOIDEAE)[1]

MARGARET M. KOOPMAN[2] AND TINA J. AYERS[3]

Department of Biological Sciences, P.O. Box 5640, Northern Arizona University, Flagstaff, Arizona 86011-5640 USA

Phylogenetic studies are often hampered by the independent evolution of characters that may potentially obscure relationships. The adaptive significance of the nectar spur and its evolution within the Mexican lobeliads (Campanulaceae) is considered here. The taxonomic delimitations of *Heterotoma* from the Mexican species within the genera *Lobelia* and *Calcaratolobelia* were tested. Independent molecular data were gathered to determine whether the Mexican spurred lobeliads should be treated as distinct genera. The internal transcribed spacer (ITS) region from 18–26S nuclear rDNA and chloroplast DNA from the 3′ *trnK* intron were sequenced from 14 representative species. Our data suggest that *Heterotoma*, as originally conceived, is a good evolutionary unit within *Lobelia* and that the presence of a nectar spur is an important morphological character that can be used in defining phylogenetic position. This study also suggests that morphological changes associated with hummingbird pollination have evolved more than once in the Mexican lobeliads, from small blue-flowered, insect-pollinated relatives.

Key words: *Heterotoma*; ITS; *Lobelia*; nectar spur; pollination; systematics; 3′ *trnK* intron.

A fundamental objective in evolutionary biology is the examination and discovery of the causes and effects of species origin. The use of phylogenetic data to study the evolution of floral morphology has proven to be valuable in the reconstruction of trait origin (Armbruster, 1992, 1993; Johnson et al., 1998; Dodd et al., 1999). This paper specifically focuses on the origin of the nectar spur within the Mexican lobeliads and the concomitant changes in pollination mode. Mapping traits onto phylogenies is considered to be an effective method to study adaptive evolution and is useful because the historical origins of such adaptations are often not available from the fossil record. The evolution of floral traits affects rates of speciation and extinction. These traits can also influence pollinator behavior, body shape, or the pollination mode altogether (Hodges and Arnold, 1995). One such well-studied character is the nectar spur: a hollow, slender, saclike outgrowth of the perianth. This trait is found in at least 15 families of angiosperms (Hodges, 1997), and nectar is stored at or near the base of the spur, acting as a reward for visiting pollinators. The spur is undeniably tied to reproduction because spurs can create an effective barrier to nectar reward. This physical barrier increases the possibility of reproductive isolation and may be responsible for increased speciation rates. Hodges and Arnold (1995) show that the evolution of the nectar spur in *Aquilegia* L. represents a key innovation, a novel adaptation that allows for diversification.

Nectar spurs have a great deal of morphological plasticity. In some genera, spurs are simple structures involving a single perianth whorl (as in *Aquilegia*). In other genera, spurs are complex structures involving both perianth whorls and associated portions of the androecium and gynoecium (as in *Delphinium* L. and the spurred lobeliads investigated here). In order for successful mutualisms to occur, pollinators need to be both proficient in nectar harvesting and effective in pollination. In 1862, Darwin hypothesized that the evolution of ''deep'' flowers could be a response to a kind of ''race'' with pollinators. Studies illustrate that nectar spur morphology often correlates to the mouth parts and foraging habits of pollinators (Nilsson, 1988; Temeles et al., 2002). When the length of a nectar spur is experimentally reduced, such as in *Platanthera* L. C. Rich. (Orchidaceae), occurrences of pollinia removal by pollinators and fruit set are altered (Nilsson, 1988).

Great diversity in floral morphology and color exists within the subfamily Lobelioideae of the Campanulaceae. Typically, gardeners think of lobelias as having blue corollas, a short tube, and two lips. However, lobeliad corollas range from burgundy, one-lipped, and rotate (*Lysipomia vinosa* Ayers, T. Ayers, unpublished manuscript), to cream or yellow and salverform, with a cylindrical tube up to 12 cm long that are nearly regular in symmetry (*Brighamia insignis* A. Gray). Even within the large, cosmopolitan genus *Lobelia* L. (ca. 400 spp.), the corolla limb can be one- or two-lipped.

Historically, the taxonomic delimitation of the genus *Heterotoma* Zucc. from *Lobelia* was based upon the presence of a nectar spur. *Heterotoma* was originally described in 1832 as a monotypic genus. The type was a large, red-flowered, Mexican perennial species that possessed a complex spur. In 1852, small, blue-flowered species were added to *Heterotoma* using the nectar spur as the defining character. In 1943, McVaugh included all New World, spurred lobeliad taxa in *Heterotoma* (McVaugh, 1943, p. 30–35). By 1953, Wimmer recognized 11 spurred species within *Heterotoma*. The spurred species occur primarily throughout the pine–oak regions of the Sierra Madre Occidental and Sierra Madre del Sur in western and south-central Mexico south into the mountains of northern Costa Rica. In a monograph by Ayers (1990), *Heterotoma* was shown to be polyphyletic and hence was dismantled. All *Heterotoma* species (14 taxa and 11 species), with the exception of *H. lobelioides* Zucc., were put into *Lobelia*, thus returning *Heterotoma* to its original monotypic status. This realignment was based upon overall gross morphology, anatomical differences in the spurs, and evidence that one small, blue-flowered spurred species (*H. pringlii* B.L. Robinson) was sister to a

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[2] Current address: Department of Botany, University of Wisconsin, Madison, Wisconsin 53706 USA.

[3] Author for correspondence (tina.ayers@nau.edu).

nonspurred species in the genus *Lobelia*) (*L. margarita* Wimmer). In 1997, Wilbur suggested the recognition of two spurred genera of lobeliads, *Heterotoma* and *Calcaratolobelia*. He agreed with Ayers' resurrection of a monotypic *Heterotoma*, but transferred the remaining 11 spurred species and one nonspurred species (*L. margarita*) to a new genus, *Calcaratolobelia* Wilbur. *Heterotoma* is currently considered monotypic and is differentiated from *Lobelia* by its crescent-shaped, laterally inflated floral spurs (Ayers, 1990), while floral spur morphology in *Calcaratolobelia*, if present, is conical or cylindrical.

Floral variation in the Mexican lobeliads is correlated with different pollination modes. In general flowers have been predicted to be ornithophilous when, together with a suite of traits, they have a characteristic red with bright yellow or orange coloration known to attract birds (Grant, 1993). Hummingbirds (Trochilidae) are attracted to the large red-and-yellow, sickle-shaped nectar spur of *Heterotoma lobelioides* and pollinate the flowers; insects completely ignore them and have not been seen visiting the flowers (T. Ayers, unpublished data). Nonspurred lobeliad species with coloration similar to that of *H. lobelioides* include *Lobelia cardinalis* L., *L. laxiflora* Kunth and *L. tupa* L. All are hummingbird-pollinated (Meehan, 1902; Devlin and Stephenson, 1984, 1985, 1987; Bernardello et al., 2000; Arizmendi, 2001). The 11 spurred species in the genus *Calcaratolobelia* are small, white-, blue- or pink-flowered herbs with gibbous hypanthia or conical-cylindrical nectar spurs. Bee flies (Bombyliidae) actively visit these flowers and are the most probable pollinator (T. Ayers, unpublished data).

Phylogenetic studies based upon morphology may be hampered by the independent evolution of characters in two or more lineages that potentially obscure relationships. One such trait is the nectar spur and its role in the evolution of Mexican lobeliads. Also in question is how ornithophilous species have evolved from insect-pollinated ancestors. Has ornithophily evolved a minimum number of times (are spurred and nonspurred species sister?) or has ornithophily evolved numerous times from insect-pollinated relatives? It is imperative to use robust phylogenetic data to study pollination modes. Here we examined recent generic circumscriptions and the question of pollination evolution by gathering molecular evidence to reconstruct the evolutionary history of spurred and nonspurred Mexican lobeliads. Is *Calcarotolobelia* monophyletic? Do independent molecular data sets support the recognition of two genera of spurred taxa separate from *Lobelia*? Can a robust phylogenetic framework allow us to understand the evolution of hummingbird pollination in a predominantly insect-pollinated lineage?

MATERIALS AND METHODS

Fourteen taxa representing the range in morphological variation and pollination mode within the Mexican lobeliads were available for investigation (Appendix, see Supplemental Data accompanying online version of this article). Five spurred species and nine nonspurred species were sampled. Three of the taxa are hummingbird-pollinated; the remaining species possess characteristics that attract insect pollinators (Fig. 1). DNA samples were collected from herbarium specimens, greenhouse-grown seeds, or in the wild. Total DNA was extracted and isolated from leaf tissues using a modified CTAB extraction method (Doyle and Doyle, 1987). *Lobelia angulata* Forst., *Downingia* (Lobelioideae), and *Codonopsis* (Campanuloideae) were chosen as outgroups because they represent closely related, yet very distinct taxa from the group in question.

Two independent molecular data sets were gathered to address the questions outlined earlier. Nuclear ribosomal DNA from the internal transcribed spacer (ITS) region and the 3′ *trnK* intron of *matK* chloroplast DNA were amplified via polymerase chain reaction (PCR). Each 50-μL reaction contained 5.0 μL Invitrogen (Carlsbad, California, USA) 10× PCR buffer, 2.0 μL 5 mmol/L dNTPs, 2.0 μL 2 mmol/L $MgCl_2$, 0.5 μL 5U/μL Invitrogen Platinum *Taq* DNA polymerase, 4.0 μL each of 10 mmol/L primer and 6 μL genomic DNA. Five percent dimethyl sulfoxide was added to each PCR reaction to reduce the possibility of secondary structure (Soltis et al., 1998). The ITS region was generated using ITS4 and ITS5 primers as outlined in Baldwin et al. (1995). Double-stranded DNAs were generated using the following amplification conditions: initial denaturation (94°C, 5 min) followed by 29 cycles of annealing (60°C, 30 s) and extension (72°C, 1 min), concluding with a final extension (72°C, 3 min) and a holding temperature of 4°C. The 3′ *trnK* intron was amplified with *matK* 8 and *trnK* 2R (Steele and Vilgalys, 1994; Schultheis, 2001) primers. Cycling parameters for the amplification of the 3′ *trnK* intron are the following: initial denaturation (95°C, 1 min), followed by 25 cycles of annealing (56°C, 30 s), extension (72°C, 1 min) and denaturation (95°C, 30 s), concluding with a final extension (72°C, 5 min) and a holding temperature at 4°C. Amplified PCR products were cleaned using the QIAquick PCR purification protocol (Qiagen Inc., Valencia, California, USA). PCR amplification concentration was checked with gel electrophoresis on a 1% agarose gel using a low molecular mass DNA ladder. Purified double-stranded products were sequenced in Tucson at the Arizona Research Laboratory on a polyacrylamide gel using a Big Dye terminator chemistry kit, version 2 and an ABI 377 (Applied Biosystems, Foster City, California, USA) sequencer.

Sequences were initially viewed and corrected using DNA STAR-Seqman II version 5.01, and were aligned in DNA STAR-Megalign, version 5.01 (Lasergene Navigator, 1999, Madison, Wisconsin, USA) using a Clustal W alignment. After subsequent manual alignment, data sets were analyzed using PAUP*, version 4.0 b010 (Swofford, 2002) and a heuristic search. Indels were treated as missing data. All parsimony uninformative characters were excluded from all analyses. Tree bisection and reconnection (TBR) branch swapping and 10 random addition sequences were performed. The searches described were performed separately for each region and then combined. Statistical support for individual clades was generated using the bootstrap (10 000 replicates) and decay indices. Sequences have been deposited in GenBank (Appendix). ITS sequence for the outgroup, *Codonopsis nervosa* Nannf., came from GenBank accession AF136237.

RESULTS

Sequence analysis—Boundaries for the ITS region were determined by comparisons with Baldwin et al. (1995). The total aligned length of the ITS1, 5.8S, and the ITS2 was 792 base pairs (bp). A total of 208 potentially informative sites were within this region (127 variable sites within ITS1 [40%], four informative sites within the 5.8S [2.2%], 77 variable sites within ITS2 [26%]). Within the ingroup, pairwise sequence divergence ranged from 1.3 to 19.9. GC content varied from 57.9 to 65.1%. The total aligned length of the 3′ *trnK* intron was 492 bp. Twenty-three potentially informative characters existed. Pairwise sequence divergence within the ingroup ranged from 0.0 to 6.4 and GC content from 33.9 to 36%.

Phylogenetic analysis—All cladograms generated from the phylogenetic analysis of ITS, the 3′ *trnK* intron and the combined data placed all spurred taxa in a single well-supported lineage, which was sister to one clade of Mexican lobelias. Analysis of the ITS region generated one most parsimonious tree (MPT) with a length of 527 (CI = 0.71; RI = 0.73). The tree placed the four insect-pollinated, nonspurred Mexican species (*L. nana* Kunth, *L. anatina* E. Wimm., *L. divaricata* Hook. and Arn., *L. irasuensis* Planch. and Oerst.) in a clade with the spurred taxa. *Calcaratolobelia flexuosa* (C. Presl.) Wilbur was basal to the remaining spurred taxa and *Hetero-*

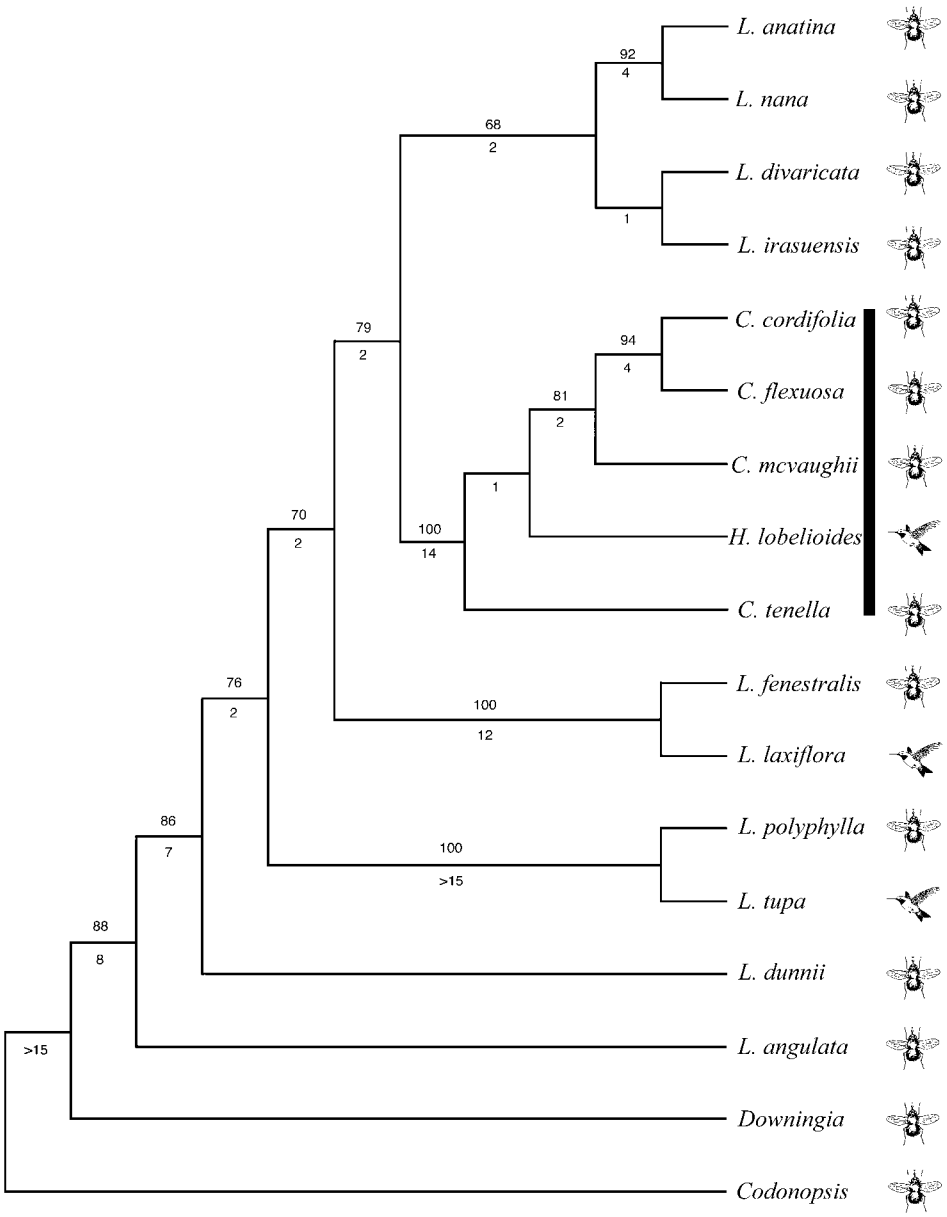

Fig. 1. The most parsimonious tree from analysis of combined ITS and 3′ *trnK* intron sequence data. Tree length = 577 steps, CI = 0.67, RI = 0.68, RC = 0.45. Numbers above nodes indicate bootstrap values; those below are decay indices. The image of the bee fly represents insect pollination; the hummingbird is representative of bird pollination. The bar highlights the spurred taxa. *L.* = *Lobelia*, *C.* = *Calcarotolobelia*, *H.* = *Heterotoma.*

toma lobelioides and *C. tenella* (Turcz.) Wilbur were supported as sister taxa. Results using only unambiguously aligned sequences (deleting a 33-bp section of ITS1) were identical to results utilizing the entire sequence. The 3′ *trnK* intron data produced a single MPT with a length of 39 (CI = 0.74; RI = 0.84) and show *L. anatina*, *L. nana*, and *L. divaricata* as sister to the spurred taxa. *Calcaratolobelia tenella* was supported as the basal species in the spurred lineage. Although a partition homogeneity test (Michevich-Farris index of incongruence; Huelsenbeck et al., 1996), as implemented in PAUP*, had significant conflict ($P = 0.04$) between the two data sets, analysis of bootstrap support showed that the only clade with high support that was not recovered in both data sets was a subset of the clade of Mexican lobelias (i.e., the placement of *Lobelia irasuensis*) and *L. dunnii*. The combined ITS and 3′ *trnK* intron sequence data generated one MPT (Fig. 1). The tree of the combined data showed excellent support for a lineage of spurred lobelias (bootstrap = 100, decay = 14). A sister relationship was supported (bootstrap = 79, decay = 2) between one clade of insect-pollinated Mexican lobelias (*L. nana*, *L. anatina*, *L. divaricata*, and *L. irasuensis*) and all of the spurred species.

DISCUSSION

The results of this study support the hypothesis that complex nectar spurs evolved only once in the Campanulaceae and have more value in classification than has been recently hypothesized. The nectar spur appears to supersede in importance flower color and other morphological characters as an indi-

cator of evolutionary relationship within the Mexican lobeliads. *Calcaratolobelia* as delimited by Wilbur is paraphyletic because it does not include *Heterotoma lobelioides* and thus should not be recognized. The resurrection of *Heterotoma* sensu lato (s.l.) at the generic level would delineate a monophyletic group, and was the outcome originally proposed (Koopman and Ayers, unpublished data). However, recognition of *Heterotoma* s.l. would obscure the evolutionary origins of the spurred species from their Mexican relatives. This action cannot be supported unless a total dismantling of *Lobelia* occurs in the future and the genus *Lobelia* is then restricted to only those species in the type section (sect. *Lobelia*). The molecular evidence supports Ayers' (1990) inclusion of the small, spurred, blue-flowered, insect-pollinated species within *Lobelia*. To accurately portray the lineage of spurred lobeliads as a monophyletic group, the data presented here necessitates the inclusion of *Heterotoma lobelioides* within *Lobelia* as *L. lobelioides* (Zucc.) Koopman and T. Ayers comb. nov.

The coevolution between plant and pollinator has the potential to be an important force in species origin and diversification. Hodges and Arnold (1994) proposed that the evolution of a nectar spur in *Aquilegia* was a key innovation that promoted pollination specialization and diversification. This species richness is seen in other groups that have evolved nectar spurs (Hodges, 1997; Hodges and Arnold, 1995). A more accurate phylogenetic estimate encompassing more species is needed within the Mexican lobelias before this kind of comparison can be made within *Lobelia*. The results suggest that there may be distinct lineages within the Mexican lobelias and only one (containing *L. anatina*, *L. nana*, *L. divaricata*, and their relatives) is sister to the spurred taxa. The small number of spurred taxa suggests that the nectar spur is of recent origin or that the spur may not be acting as a key innovation with respect to diversification within *Lobelia*. However, the nectar spur does have considerable variation that may be associated with pollinator specialization. Within *Lobelia*, there are relatively few innovations in hypanthium or corolla tube. Hundreds of species have the common bilabiate condition, but there have been independent changes to the limb, possibly driven by pollination variation, resulting in a single lip, nectar guides, and changes in the size, shape, and color of the corolla lobes. Morphological changes in length and shape of the nectar spur can be easily seen within the spurred lobelias (Fig. 2). Although the complex spurs involve the same tissues in each species, no two species have identical spurs. Within the insect-pollinated spurred species, the two groups as defined by Ayers (1990) have a range in spur length. Each group contains species with minuscule spurs (e.g., *Lobelia cordifolia* and *L. knoblochii*) and at least one species with a long, cylindrical spur (*L. goldmannii* and *L. villaregalis*). This independent lengthening of the spur may be occurring as pollinators change, although pollination biology within this group is in need of study.

The data presented here suggest that the ancestral state within the Mexican spurred lobelias and their sister species is insect pollination and that hummingbird pollination has evolved multiple times (Fig. 1). The derivation of ornithophily from entomophily has been recognized across several other lineages, and according to Grant (1994), the number of ornithophilous species in a genus reflects the age of the bird–plant relationship. *Lobelia*, with only three ornithophilous species (*L. lobelioides*, *L. cardinalis*, and *L. laxiflora*) in western North America presumably has a more recent affiliation with hummingbirds in that region than a genus rich in ornithophilous flowers, such as *Castilleja* Mutis. The presence of only one spurred, ornithophilous species suggests that the evolution of the nectar spur in Campanulaceae may be relatively recent (Grant, 1994; Hodges and Arnold, 1995). The recent evolution of the entire spurred lineage is also supported by the long external branch leading to the spurred lineage coupled with the short internal branches (Fig. 2), which is consistent with rapid radiation (Richardson et al., 2001).

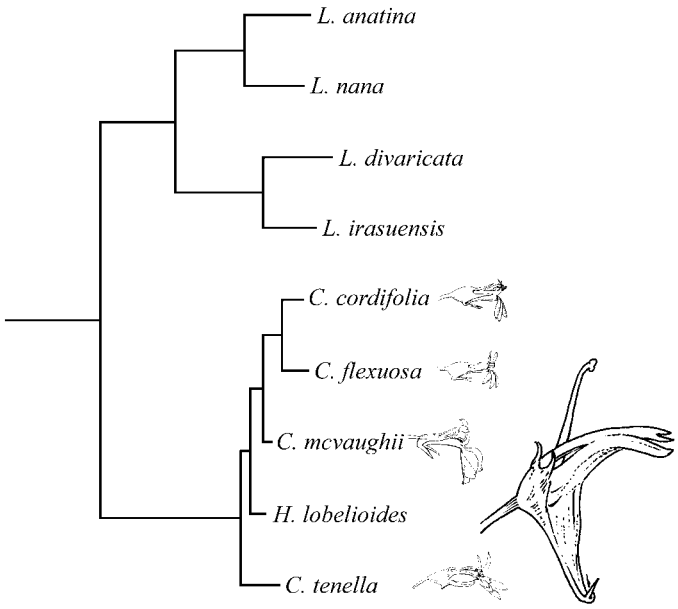

Fig. 2. Phylogram of the most parsimonious tree from combined ITS and 3′*trnK* intron sequence data. *Calcarotolobelia cordifolia*, *C. flexuosa*, and *C. tenella* are magnified two times. The remaining species are life size. Insect-pollinated species are redrawn from Ayers (1987, 1990). *L.* = *Lobelia*, *C.* = *Calcarotolobelia*, *H.* = *Heterotoma*.

The importance of the nectar spur within Mexican lobeliad evolution has been overlooked in recent years. More detailed sampling is necessary in this large group before the effect of the nectar spur on the evolution of Mexican lobelias can be fully resolved. Further research could answer inquiries into whether the nectar spur is a key innovation and if the trait will stimulate diversification within the lobelias. Developmental genetics could identify regulatory genes of floral traits that would help to clarify and reconstruct the evolution of pollination syndromes within the Mexican lobelias and better assess the role of pollinator interaction in species origin. Detailed pollination studies could resolve questions regarding the roles of spur morphology and pollinator in reproductive isolation and speciation.

Taxonomic treatment—Follows Ayers (1990) with the following addition:

LOBELIA LOBELIOIDES (Zucc.) Koopman and T. Ayers comb. nov. *Heterotoma lobelioides* Zucc., Flora 15, Beibl. 2: 101. 1832. *Myopsia mexicana* K. Presl, Prodr. Monogr. Lobel. 8. 1836. TYPE: Mexico, Oaxaca(?), La Cumbre de San Antonio, 8000 ft. no date, *Karwinski s.n.* (Holotype: M; isoptyes: JE, M, W [fragment]).

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