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## Phylogenetic Relationships of *Silene multinervia* and *Silene* Section *Conoimorpha* (Caryophyllaceae)

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**Abstract**—The Californian *Silene multinervia* (Caryophyllaceae) and Eurasian members of section *Conoimorpha* in subgenus *Behenantha* are the only *Silene* species that have calyces with 15 or more prominent parallel, unbranched veins. We show that *S. multinervia*, which has been considered a recent introduction of the Asian *S. coniflora* (section *Conoimorpha*) to North America, is clearly not synonymous with the latter species based on morphological or molecular data. We present a chromosome count of *S. multinervia* ( $2n = 24$ ), which is different from the base number  $x = 10$ , a putative synapomorphy for section *Conoimorpha*. Gene trees based on sequences from three different genomes fail to group *S. multinervia* with the European section *Conoimorpha* species. The *S. multinervia* sequences form a monophyletic group placed in an unresolved position within subgenus *Behenantha*.

**Keywords**—\*BEAST, cpDNA, chromosome count, coalescent, mitochondrial DNA, RNA polymerase genes.

Intercontinental disjunctions of plant species or species-pairs have received considerable interest from biogeographers (e.g. Raven 1972; Thorne 1972; Lee et al. 1996; Wen 1999; Milne 2006). Classical explanations often include vicariance or anthropogenic introduction. However, recent studies based on molecular data suggest that the most likely explanation for some Eurasia-North America disjunctions is pre-human dispersal events [e.g. *Plantago ovata* Forssk. (Meyers and Liston 2008), *Oligomeris linifolia* (Vahl) J. F. Macbr. (Martín-Bravo et al. 2009), and *Senecio mohavensis* A. Gray (Coleman et al. 2003)]. In other cases, species which previously have been regarded by some botanists as native to North America have been found to have been introduced by humans [e.g. *Cakile edentula* (Bigelow) Hook. (Raven and Axelrod 1978; Sauer 1988, p. 34) and *Vulpia myuros* (L.) C. C. Gmel. (Raven and Axelrod 1978)].

*Silene* L. (Caryophyllaceae) is a genus of approximately 700 species, most of which have their natural distribution in Eurasia (Oxelman et al. 2001). There are, however, also native *Silene* species in North and South America as well as in Africa, and species that have spread as weeds throughout the world. *Silene* is divided into the subgenera *Silene* and *Behenantha* (Otth) Endl. [syn. *S.* subgenus *Behen* (Dumort.) Rohrb.] (Popp and Oxelman 2004). *Silene* subgenus *Silene* includes the well-known species *S. acaulis* L. and *S. gallica* L., whereas major groups in *Silene* subgenus *Behenantha* include section *Melandrium* (Röhl.) R. K. Rabeler (containing the familiar *S. latifolia* Poir.), sections *Physolychnis* (Bentham) Bocquet and *Conoimorpha* Otth, the *Silene vulgaris* group, and *S. noctiflora* L. (with the closely related *S. turkestanica* Regel, Sloan et al. 2009). *The Flora of North America* lists 52 native and 18 introduced or naturalized North American *Silene* species (Morton 2005). Most of the North American species belong to subgenus *Behenantha*, either to the section *Physolychnis* s. l. (Popp et al. 2005; Popp and Oxelman 2007) or to the *S. menziesii* group (Popp and Oxelman 2007), while *S. antirrhina* L. and *S. repens* Patr. belong to *Silene* subgenus *Silene* (Eggens et al. 2007; Popp and Oxelman 2007).

Considerable attention has been given to the phylogenetic position of the section *Melandrium* to facilitate understanding of the evolution of dioecy (e.g. Atanassov et al. 2001;

Filatov and Charlesworth 2002; Filatov 2005; Nicolas et al. 2005; Rautenberg et al. 2010), and in several cases section *Conoimorpha* has been suggested to be the sister group to these dioecious species (e.g. Desfeux and Lejeune 1996; Erixon and Oxelman 2008a).

Common morphological features for *Silene*, as circumscribed by Oxelman et al. (2001), are flowers with 10 stamens and three or five styles, five free petals, a synsepalous calyx, and a capsule that usually splits open into twice as many teeth as the number of styles. Two important characters in identification of *Silene* species are anthophore length and the coronal scales. The anthophore is a structure that separates the attachment of the calyx and corolla. The coronal scales are present as small appendages on the border between the petal limb and the petal claw (the part of the petal that is hidden in the calyx).

*Silene multinervia* S. Watson (Caryophyllaceae) is a Californian taxon (Hitchcock and Maguire 1947) that always has been placed into the otherwise Eurasian section *Conoimorpha* (e.g. Watson 1890; Hitchcock and Maguire 1947; Šourková 1971, as the separate genus *Pleconax* Raf.). *Silene multinervia* and section *Conoimorpha* share a distinctive morphological feature: all species have several (15–60) unbranched prominent parallel veins on the calyx (all other *Silene* species have 10 principal veins and/or branching nervature). *Silene* section *Conoimorpha* also has a base chromosome number of  $x = 10$  (Greuter 1995), whereas all other *Silene* have  $x = 12$  with one known exception (*S. fortunei* Vis.,  $2n = 30$ ; Bari 1973). Members of section *Conoimorpha* have elevated nucleotide substitution rates in chloroplast (Erixon and Oxelman 2008b) and mitochondrial DNA (Sloan et al. 2009), compared to other members of the genus. The circumscription of the group (e.g., Rohrbach 1868; Chowdhuri 1957) has been uncontroversial since its first appearance in the taxonomic literature (Otth 1824). The species currently recognized in the group (*Silene ammophila* Boiss. & Heldr., *S. conica* L., *S. coniflora* Nees ex Otth, *S. conoidea* L., *S. lydia* Boiss., *S. macrodonta* Boiss., *S. subconica* Friv., *S. grisebachii* (Davidov) B. Pirker & Greuter, and *S. sartorii* Boiss. & Heldr.; Pirker and Greuter 1997) have their native distribution in Europe and southwest to central Asia (Table 1), although *S. conica* and *S. conoidea* are introduced as weeds around the

TABLE 1. Native distribution and number of calyx veins of *Silene multinervia* and the members of *Silene* section *Conoimorpha*. *Silene grisebachii* and *S. sartorii* were not included in the molecular analyses.

Species	Native distribution	Number of calyx veins	References
<i>Silene ammophila</i> Boiss. & Heldr.	Greece (Crete and Karpathos)	15–20	Pirker and Greuter 1997
<i>Silene conica</i> L.	Europe to Central Asia	30	Schischkin 1970
<i>Silene coniflora</i> Nees ex Oth	Southwest to Central Asia	20	Schischkin 1970
<i>Silene conoidea</i> L.	Mediterranean to Southwest and Central Asia	30	Schischkin 1970
<i>Silene griesebachii</i> (Davidov) B. Pirker & Greuter	Greece	30	Pirker and Greuter 1997
<i>Silene lydia</i> Boiss.	Southeastern Balkans and Western Anatolia	30	Greuter 1995
<i>Silene macrodonta</i> Boiss.	Eastern Mediterranean	60	Greuter 1995
<i>Silene multinervia</i> S. Watson	California and Mexico	20	Watson 1890; Jepson 1914; Hartman and Rabeler 2008
<i>Silene sartorii</i> Boiss. & Heldr.	Greece	30	Pirker and Greuter 1997
<i>Silene subconica</i> Friv.	Mediterranean	30	Pirker and Greuter 1997

world (e.g. Rozefelds et al. 1999; Morton 2005; Global Compendium of Weeds 2007). *Silene multinervia* has recently been put into synonymy with the southwest/central Asian species *S. coniflora* (Morton 2005; followed by Hartman and Rabeler 2008). On the other hand, Popp and Oxelman (2007) and Rautenberg et al. (2010) showed, based on cpDNA and nrDNA ITS sequence data, that *S. multinervia* does not form a monophyletic group with Eurasian samples from the section *Conoimorpha*. However, the sampling in either of these two studies was not focused on *S. multinervia* or section *Conoimorpha*.

Using DNA sequences from samples of *S. multinervia*, *S. coniflora*, and six other taxa from *Silene* section *Conoimorpha*, a chromosome count of *S. multinervia*, and sequence data from several outgroup species with emphasis on potentially closely related species in *Silene* subgenus *Behenantha*, we address the following questions: Is there any morphological or molecular support for the synonymization of *S. multinervia* with *S. coniflora*? Is there any morphological or molecular support for the inclusion of *S. multinervia* in *Silene* section *Conoimorpha*? Does *S. multinervia* represent a recent introduction to the Californian flora? What is the phylogenetic position of section *Conoimorpha*?

## MATERIALS AND METHODS

**Study Species**—The present study includes *Silene multinervia* and seven of the nine species from *Silene* section *Conoimorpha* (Table 1), as well as a large outgroup sampling, with special emphasis on potentially closely related species in *Silene* subgenus *Behenantha*.

The members of section *Conoimorpha* are briefly characterized in Table 1, but a few of them deserve mention here. *Silene multinervia* grows in California and Mexico on burnt open ground, after forest fires, and is recognized by 20 calyx veins and no coronal scales (Watson 1890; Jepson 1914; Hartman and Rabeler 2008). *Silene coniflora* grows from southwest to central Asia and has 20 calyx veins and oblong coronal scales (Schischkin 1970). *Silene lydia* is a species sharing some of the features of section *Conoimorpha* (more than 10 unbranched parallel veins), but also having enough features to be placed in a section of its own (*S. section Lydiae* Greuter) by Greuter (1995). *Silene lydia* has a chromosome number of  $2n = 20$ , or possibly  $2n = 22$  (preliminary data by B. Pirker, discussed in Greuter 1995), long glandular hairs on the calyx, and no anthophore (Greuter 1995). It is distributed in the southeastern Balkans and western Anatolia (Greuter 1995). The Greek endemics *S. griesebachii* and *S. sartorii* were not included in the molecular analysis. They are similar to *S. subconica*, but differ in petal shape and venation, and the former has distinct seeds and longer anthophore (Pirker and Greuter 1997). Rautenberg et al. (2008) found some indications of a close relationship between *S. noctiflora* and section *Conoimorpha*. Previous molecular phylogenetic studies (e.g. Oxelman and Lidén 1995; Oxelman et al. 2001; Popp

and Oxelman 2001, 2004, 2007; Rautenberg et al. 2010) have revealed that section *Conoimorpha* is confidently embedded in subgenus *Behenantha*, which has poorly resolved basal relationships, possibly due to a rapid radiation some six to seven million years ago (Erixon and Oxelman 2008; Frajman et al. 2009). We therefore sampled outgroup taxa primarily to represent major lineages from subgenus *Behenantha*.

**Chromosome Count**—A chromosome count was determined for *S. multinervia* based on a plant grown from seeds collected in Napa County, California (Appendix 1). Prior to fixation in Carnoy I solution (3 volumes absolute alcohol and 1 volume glacial acetic acid), growing roots were pretreated with equal parts 0.1% colchicine and 0.002M 8-hydroxyquinoline for 2 hrs. After fixation and hydrolysis in 1N HCl at 60°C for 2 mins, root-tip meristems were prepared. Flower buds were fixed and hydrolyzed in Carnoy I solution. All tissues were stained with aceto-orcein on clean slides and squashed under a coverslip.

**Morphology**—Herbarium specimens from CAS, G, GB, LE, MW, S, UPS, and WU (abbreviations according to Holmgren and Holmgren 1998), and Arne Strid's private herbarium (in Ørbaek, Denmark), of *S. multinervia*, *S. coniflora*, and other representatives of *Silene* section *Conoimorpha* were studied as physical specimens or as images deposited in the *Sileneae* database (<http://www.sileneae.info>). Specimens were compared to keys, descriptions, and illustrations in the literature (Oth 1824; Boissier 1867; Rohrbach 1868; Watson 1890; Williams 1896; Jepson 1914; Post 1932; Hitchcock and Maguire 1947; Blakelock 1957; Khoshoo and Bhatia 1963; Mouterde 1966; Zohary 1966; Bajtenov 1969; Schischkin 1970; Ghazanfar and Nasir 1986; Melzheimer 1988; El-Oqlah and Karim 1990; Hosny et al. 1992; Chater et al. 1993; Greuter et al. 1997; Boulos 1999; Morton 2005; Hartman and Rabeler 2008; Calflora 2009).

**DNA Extraction, Amplification, and Sequencing**—DNA was extracted from living or herbarium material using a modified Carlson/Yoon method (Oxelman and Lidén 1995). Voucher details and GenBank accession numbers are listed in Appendix 1. Three cpDNA regions (the *matK* gene, the *rps16* intron, and the *trnL* gene and *trnL-trnF* intergenic spacer), three mitochondrial DNA (mtDNA) regions [the protein-encoding ATP synthase subunit 1 (*atp1*), cytochrome *c* oxidase subunit 3 (*cox3*), and NADH dehydrogenase subunit 9 (*nad9*)], ITS from nuclear ribosomal DNA, and four low-copy nuclear regions (parts of the RNA polymerase genes *RPA2*, *RPB2*, *RPD2a*, and *RPD2b*) were amplified. The PCR products were either purified using Millipore multiscreen PCR plates in a vacuum manifold (Millipore, Billerica, Massachusetts) and sequenced by MacroGen Inc. in Seoul, South Korea or purified with Exonuclease I and shrimp alkaline phosphatase (USB Corporation, Cleveland, Ohio), cycle sequenced with BigDye v3.1 (Applied Biosystems, Foster City, California), and analyzed on an ABI 3130xl capillary sequencer. In addition to already published PCR and sequencing primers for *matK* (Fior et al. 2006; Mower et al. 2007; Sloan et al. 2009; Rautenberg et al. 2010), *rps16* (Oxelman et al. 1997), *trnLF* (Oxelman et al. 2005), *RPA2* (Popp and Oxelman 2004), *RPB2* (Popp and Oxelman 2001), *RPD2* (Popp and Oxelman 2004), *cox3* (Duminil et al. 2002), *nad9* (Duminil et al. 2002), and ITS (Popp and Oxelman 2001), the following primers were used for amplification and sequencing: *atp1\_Conoi\_F* (GCKGGAGAAATGGYKGAATTG), *atp1\_Conoi\_F2* (ATGCAAACYGCTTAAAGGC), *atp1\_Conoi\_F3* (ATTCTGTAGCAGC CACTGC), *atp1\_Conoi\_R* (CCWACATTAATAGCWGGTCTA) *atp1\_Conoi\_R2* (TCCAATCGCTACATAAACAC), *atp1\_Conoi\_R3* (CSGCTCTTCTAA GAGACG), *cox3\_Conoi\_F* (GAATAACCAAACACTACGTCCAC), *cox3\_Conoi\_R*

(GGBGGTGAATMCTGCTCAG), nad9\_Conoi\_F (ACCACNCGTTTTCTGGATC), nad9\_Conoi\_R (CAAGAARTGGGTCAAAAAGATG). Eighty sequences were new to this study, and additional sequences were obtained from GenBank Appendix 1).

**Sequence Alignment and Analysis**—Sequence reads were assembled into contigs and edited using the Staden package version 1.6.0 for Mac OS X (Staden 1996) with phred version 0.020425.c (Ewing and Green 1998; Ewing et al. 1998) and phrap version 0.990319 (<http://www.phrap.org>) or using Sequencher v4.5 (Gene Codes, Ann Arbor, Michigan). Base polymorphisms were coded using the NC-IUPAC ambiguity codes. Sequence alignment was performed manually in QuickAlign (Müller and Müller 2003), following the criteria of Popp and Oxelman (2004). The alignments of the three cpDNA regions were analyzed separately and checked for strongly supported conflicts (see definition below). As such conflicts were not found, the alignments were concatenated into a cpDNA data set. The mtDNA regions were analyzed both separately and concatenated into a single data set. The nuclear regions were analyzed separately. Simple indel coding (Simmons and Ochoterena 2000) was applied to the alignments using SeqState version 1.36, build 19.10.2007 (Müller 2005) for use in the PAUP\* and MrBayes analyses.

Maximum parsimony analyses and maximum parsimony bootstrap support measures were performed with PAUP\* v.4.0b10 (Swofford 2002). Maximum parsimony analyses were carried out using heuristic searches with TBR branch swapping, the multrees option on (but a limit of maxtrees set to 5,000), and 10 random addition sequences. For bootstrap support, 1,000 replicates were performed, with the multrees option off.

Bayesian phylogenetic analysis was performed using MrBayes 3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with nucleotide models as proposed by MrModeltest version 2.2 (Nylander 2004), using the Akaike information criterion. Four MCMC chains were run for five million generations with trees and parameter values saved every 1,000th generation, in two parallel runs. Convergence of MrBayes analyses was checked using the split frequency diagnostic (runs with average standard deviations of < 0.01 were considered as converged), Tracer v1.5 (Rambaut and Drummond 2007), and AWTY (Wilgenbusch et al. 2004; Nylander et al. 2008). The first 25% of the trees were discarded as burn-in.

The \*BEAST (starbeast) mode in BEAST v1.5.4 (Drummond and Rambaut 2007) infers gene trees and, at the same time, estimates a species tree that is compatible with the gene trees given a coalescent process. \*BEAST was used to infer a species tree for the genera *Lychnis* and *Silene* based on the combined information from the cpDNA, *RPA2*, *RPD2a*, and *RPD2b* regions. Because of the strong incongruence between the gene trees from *RPB2* and the other genes regarding the positions of *Lychnis* and the two subgenera in *Silene*, *RPB2* was excluded from the \*BEAST analysis. Input files for BEAST were created with BEAUti v1.5.4 (Drummond and Rambaut 2007) and with additional manual editing of the xml file, using a relaxed clock model (Drummond et al. 2006), with branch rates following a lognormal distribution, and the same substitution models as in the MrBayes analysis. We used a Yule prior for the species tree. Differences in effective population size will influence the coalescence times. The  $N_e$  of chloroplasts and mitochondria are generally considered to be  $\frac{1}{4}$  of the  $N_e$  of the nuclear genes in a dioecious plant,

assuming uniparental inheritance and an equal sex ratio [but see Lynch et al. (2006)]. In hermaphroditic plants however, the chloroplast  $N_e$  is  $\frac{1}{2}$  that of the nuclear genes. Therefore, the ploidy level of the cpDNA partition was adjusted manually in the xml file to accommodate this twofold difference. A prior on the age of the root of the species tree was set to 12.39 million years, with a normally distributed standard deviation of 2.1, based on the posterior age of the node containing *Silene* and *Lychnis* in a fossil calibrated *matK* tree of Caryophyllaceae (Frajman et al. 2009). One MCMC chain was run for 100 million generations with trees and parameter values saved every 1,000th generation. The tree files were summarized using TreeAnnotator v1.5.4 (Drummond and Rambaut 2007) into one maximum credibility tree with median node heights (discarding the first 10% of the trees as burn-in). To assess the effect of the priors on the posteriors, the run was compared to a run performed with the same settings but on an empty alignment.

The resulting trees were visualized using FigTree version 1.2 (Rambaut and Drummond 2008). Posterior probabilities (PP)  $\geq 0.95$ /bootstrap values (BS)  $\geq 85\%$  were considered as strong support, while values of 0.85–0.94 PP/75–84% BS were considered as moderate support, and values of 0.70–0.84 PP/50–74% BS as low support. We define incongruence as the presence of strongly supported conflicts between tree topologies. Data matrices and phylogenetic trees are available on TreeBASE (study number S11178).

## RESULTS

Statistics for the alignments and phylogenetic analyses, as well as the model of evolution proposed by MrModeltest for the DNA regions are presented in Table 2.

**Chloroplast Genes**—In the concatenated chloroplast data set, *Silene* section *Conoimorpha* is a well-supported monophyletic group containing all species reported to belong to the section except *S. multinervia* (Fig. 1a). All *S. multinervia* accessions form a monophyletic group placed in an unresolved position in subgenus *Behenantha*, outside the rest of section *Conoimorpha*. *Silene lydia* is placed as sister to the rest of section *Conoimorpha*. All species relationships within section *Conoimorpha* are strongly supported. The pattern is congruent between all included cpDNA regions (data not shown), and between phylogenetic methods (Fig. 1a).

**Mitochondrial DNA**—As in the cpDNA tree, the European and Asian members of section *Conoimorpha* form a strongly supported monophyletic group in the mtDNA tree (Fig. 1b). In the concatenated mtDNA data set, section *Conoimorpha* groups with *S. noctiflora* + *S. turkestanica* with strong support and *S. multinervia* is weakly to moderately supported as sister

TABLE 2. Statistics for the data sets used in the maximum parsimony (PAUP\*), MrBayes, and \*BEAST analyses. The \*BEAST data set included 38–54 sequences from four of the regions, representing 39 species from *Silene* and *Lychnis*, and had 22.9% missing data. The mitochondrial and chloroplast sequences were analyzed both separately and concatenated into one mtDNA data set (*atp1*, *cox3*, and *nad9*) and one cpDNA data set (*matK*, *rps16*, and *trnLF*). In order to make each species represented in each region in the \*BEAST analysis, empty sequences were added to some data sets.

Region	Number of terminals in PAUP* and MrBayes/ (sequences + empty in *BEAST)	Number of included characters (nucleotides/indels)	Number/% of parsimony informative characters	Percentage of missing data	CI (RI)	Substitution model	Average SD of split frequencies (MrBayes)
<i>RPA2</i>	58/(54 + 1)	2,560 (2,483/77)	246/9.6%	2.7%	0.804 (0.860)	GTR + $\Gamma$	0.003984
<i>RPB2</i>	50	1,100 (984/116)	295/26.8%	4.4%	0.759 (0.833)	GTR + $\Gamma$	0.003924
<i>RPD2a</i>	43/(38 + 10)	2,148 (2,013/135)	242/11.3%	14.9%	0.831 (0.869)	GTR + $\Gamma$	0.003065
<i>RPD2b</i>	45/(42 + 12)	838 (743/95)	216/25.8%	6.1%	0.815 (0.890)	GTR + $\Gamma$	0.003385
ITS	52	888 (853/35)	164/18.4%	9.3%	0.537 (0.729)	GTR + I + $\Gamma$	0.006320
mtDNA	43	2,099 (2,099/0)	434/20.7%	3.5%	0.693 (0.848)	GTR + $\Gamma$	0.009918
<i>atp1</i>	44	960 (960/0)	195/20.3%	0.5%	0.680 (0.823)	GTR + I + $\Gamma$	0.005406
<i>cox3</i>	43	674 (674/0)	127/18.8%	5.2%	0.735 (0.897)	GTR + I + $\Gamma$	0.006095
<i>nad9</i>	43	464 (464/0)	112/24.1%	5.0%	0.771 (0.894)	GTR + $\Gamma$	0.007122
cpDNA	55/(51 + 0)	4,182 (3,944/238)	513/12.2%	32.3%	0.782 (0.819)	GTR + I + $\Gamma$	0.005501
<i>matK</i>	45	1,722 (1,708/14)	157/9.1%	33.8%	0.820 (0.849)	GTR + $\Gamma$	0.003329
<i>rps16</i>	52	1,048 (966/82)	172/16.4%	4.7%	0.753 (0.848)	GTR + $\Gamma$	0.004645
<i>trnLF</i>	40	1,405 (1,272/133)	176/12.5%	8.1%	0.796 (0.784)	GTR + $\Gamma$	0.005577

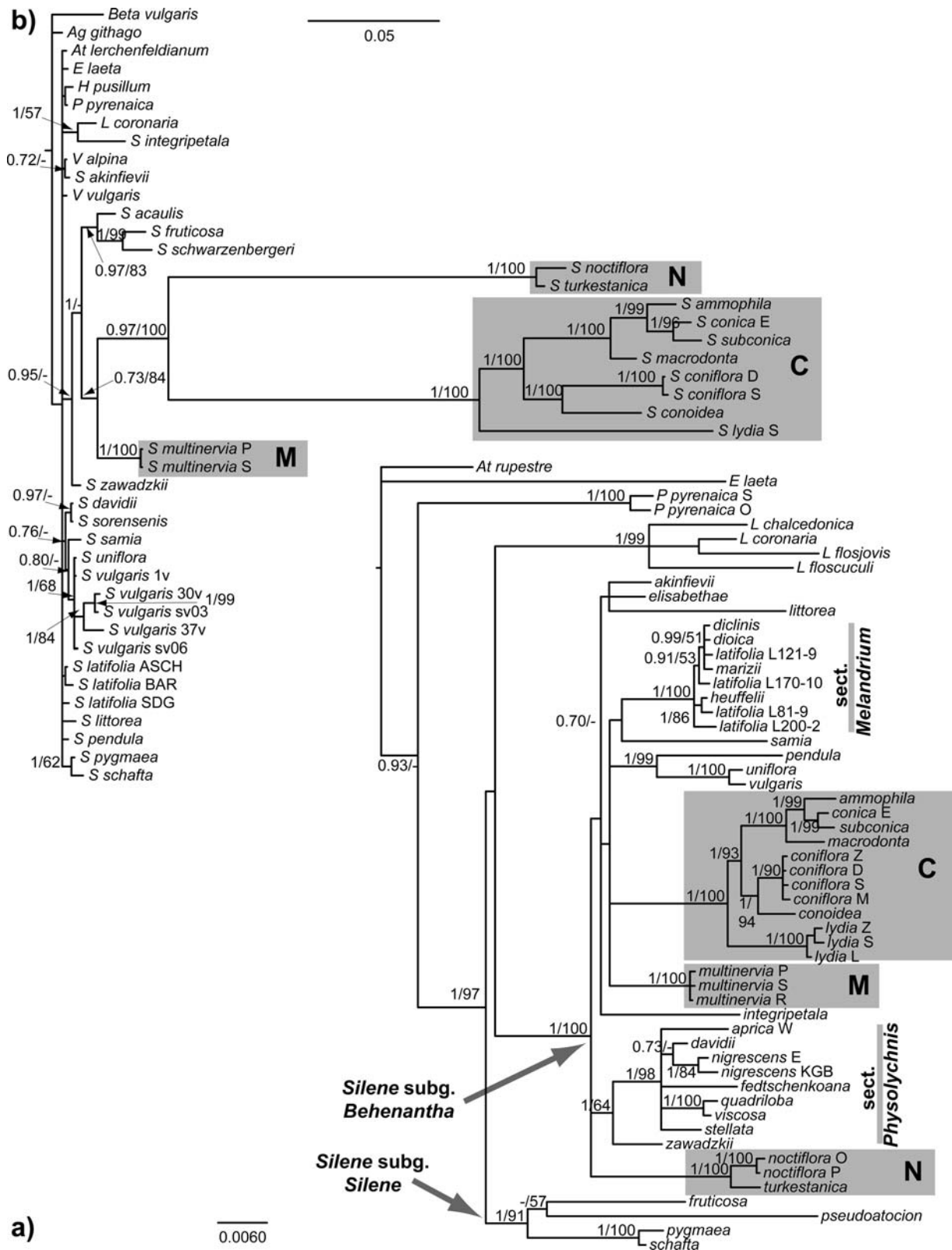


FIG. 1. Phylogram obtained from MrBayes for the concatenated chloroplast DNA (cpDNA) sequences (a), and for the concatenated mitochondrial DNA (mtDNA) sequences (b). Values associated with nodes are Bayesian posterior probabilities/parsimony bootstrap values. Posterior probabilities/ bootstrap values lower than 0.70/50 are not indicated. Branch lengths represent estimated number of changes per site. The gray boxes indicate the key groups C = section *Conoimorpha*, M = *S. multinervia*, and N = *S. noctiflora* + *S. turkestanica*. Numbers and letters after species names indicate different specimens (Appendix 1). Genera are represented as follows: *Ag* = *Agrostemma*, *At* = *Atocion*, *B* = *Beta*, *E* = *Eudianthe*, *H* = *Heliosperma*, *L* = *Lychnis*, *P* = *Petrocoptis*, *S* = *Silene*, and *V* = *Viscaria*.

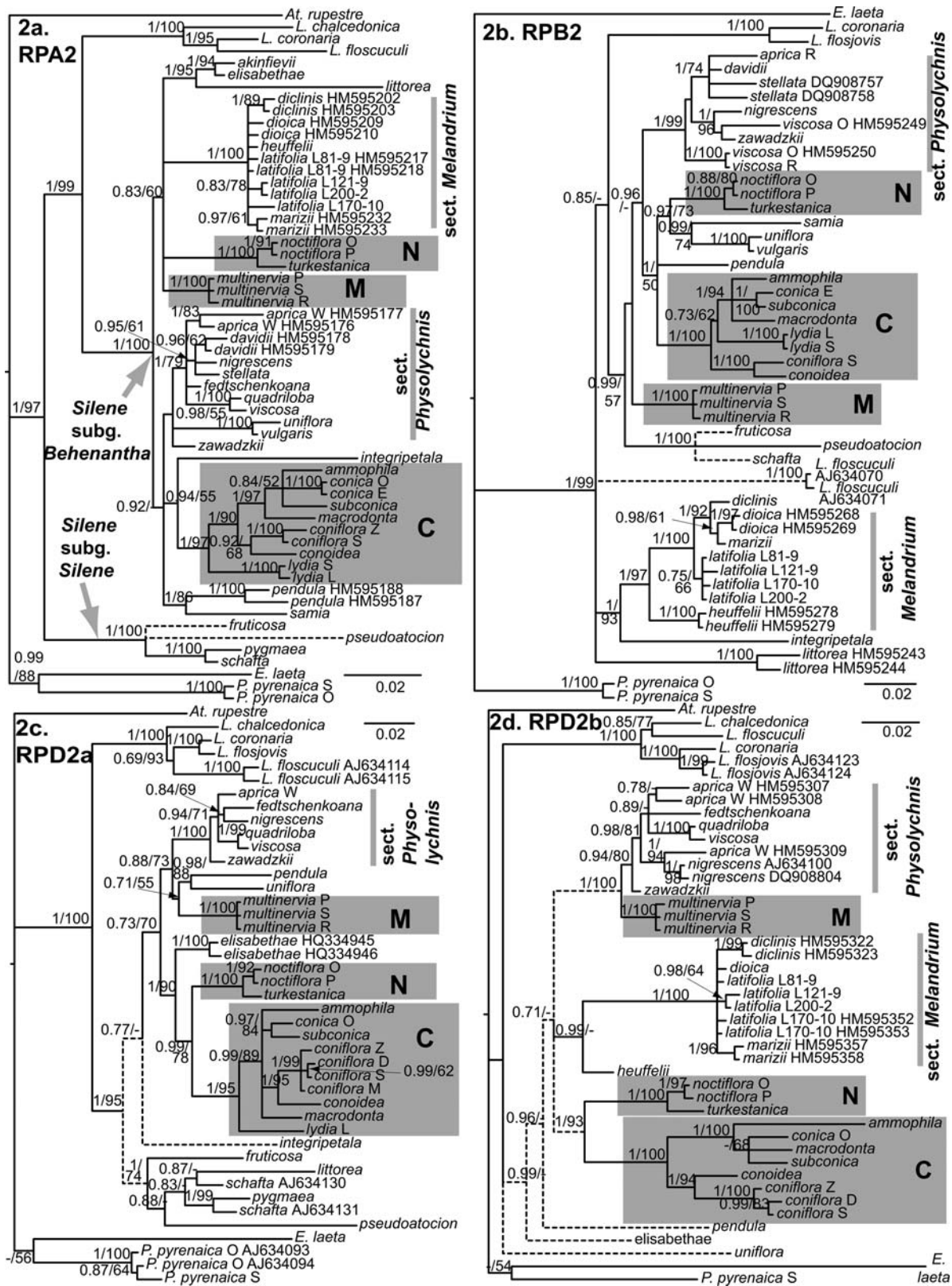


FIG. 2. Phylogram obtained from MrBayes for the low-copy nuclear RNA polymerase genes *RPA2* (a), *RPB2* (b), *RPD2a* (c), and *RPD2b* (d). Values associated with nodes are Bayesian posterior probabilities/parsimony bootstrap values. Posterior probabilities/bootstrap values lower than 0.70/50 are not indicated. Branch lengths represent estimated number of changes per site. Dashed lines represent parts of the tree where the maximum parsimony bootstrap consensus tree has a differing topology (bootstrap support lower than 60%). The gray boxes indicate the key groups C = section *Conoimorpha*, M = *S. multinervia*, and N = *S. noctiflora* + *S. turkestanica*. Numbers and letters after species names indicate different specimens (Appendix 1). GenBank accession numbers are used to identify different sequences from the same specimen. All species belong to *Silene* except *At.* = *Atocion*, *E.* = *Eudianthe*, *L.* = *Lychnis*, and *P.* = *Petrocoptis*.

to this clade (Fig. 1b). The branches are extremely long in section *Conoimorpha*, as well as in *S. noctiflora* + *S. turkestanica* (Fig. 1b). The different mtDNA gene trees show different patterns in terms of branch length variation (supplemental data S1). *Silene multinervia* occupies a branch that is somewhat longer than the majority of other *Silene* branches, but still much shorter than the extreme lineages (Fig. 1b). The position of *S. multinervia* is more or less ambiguously resolved in all three mtDNA gene trees (supplemental data S1). The internal relationships within Eurasian *Conoimorpha* are strongly supported and agree with the cpDNA tree (Fig. 1b).

**Nuclear Genes**—In all nuclear gene trees, the members of section *Conoimorpha*, with the exception of *S. multinervia*, form a strongly supported monophyletic group (Figs. 2–3). The relationships within *Conoimorpha* are generally well resolved, strongly supported, and congruent with other regions (Figs. 2–3). Generally, the topological relationships in *Behenantha* outside of section *Conoimorpha* are unresolved, or conflicting between different nuclear genes (Figs. 2–3). In the ITS tree the relationships between section *Conoimorpha*, *S. multinervia*, and *S. noctiflora* + *S. turkestanica* are unresolved (Fig. 3). In the *RPD2a* and *RPD2b* trees, *S. multinervia* is placed as a close relative of the *Physolychnis* group with moderate (*RPD2a*) or strong (*RPD2b*) support, while *S. noctiflora* + *S. turkestanica* form a moderately to strongly supported sister group to the members of section *Conoimorpha* (Fig. 2c–d). In *RPB2*, *RPD2a*, and *RPD2b*, *S. multinervia* and the rest of section *Conoimorpha* are separated by at least one moderately to strongly supported node (Fig. 2b–d).

**\*BEAST Analysis**—In the species tree obtained by the \*BEAST analysis based on cpDNA and data from the RNA polymerase genes *RPA2*, *RPD2a*, and *RPD2b*, the topological relationships between section *Conoimorpha*, *S. multinervia*, and *S. noctiflora* + *S. turkestanica* are poorly resolved (Fig. 4). There is no support for *S. multinervia* as the sister group to section *Conoimorpha*. In the *RPD2a* and *RPD2b* gene trees, *S. noctiflora* + *S. turkestanica* form a monophyletic group with section *Conoimorpha* (PP = 0.97 and 0.78, respectively; supplemental data Fig. S2), but in the species tree the PP for this grouping is 0.63 (Fig. 4).

**Dating**—The 95% HPD (highest posterior density) ages of the MRCA (most recent common ancestor) of the *S. multinervia* sequences vary in the different gene trees, between 0.0021 (*RPD2a*) and 0.64 million years (*RPA2*). In the combined species tree in the \*BEAST analysis, the 95% HPD ages of the MRCA of section *Conoimorpha* are 1.6–5.7 million years (Fig. 4). The age of the MRCA of *S. multinervia* and its closest sister group (section *Physolychnis*) has a 95% HPD interval of 1.9–7.1 million years in the combined species tree, although this node has a posterior probability of only 0.60 (Fig. 4).

**Chromosome Count**—Twenty-four chromosomes could readily be counted from several metaphase plates prepared from root-tips of *Silene multinervia*, and also from mitotic metaphase plates prepared from flower buds.

**Morphology**—There are several phenotypic differences between the allegedly synonymous *S. multinervia* and *S. coniflora*: *S. multinervia* lacks coronal scales and has basal leaves that are oblanceolate and cauline leaves that are lanceolate-linear (Fig. 5A). *Silene coniflora* has coronal scales and grass-like linear leaves (Fig. 5B). The number of calyx veins is 20 in both *S. multinervia* and *S. coniflora*. Although the protologue by Otth, citing the original author Nees, states the number of calyx veins to be 30 (Otth 1824), the examined *S. coniflora*

### 3. ITS

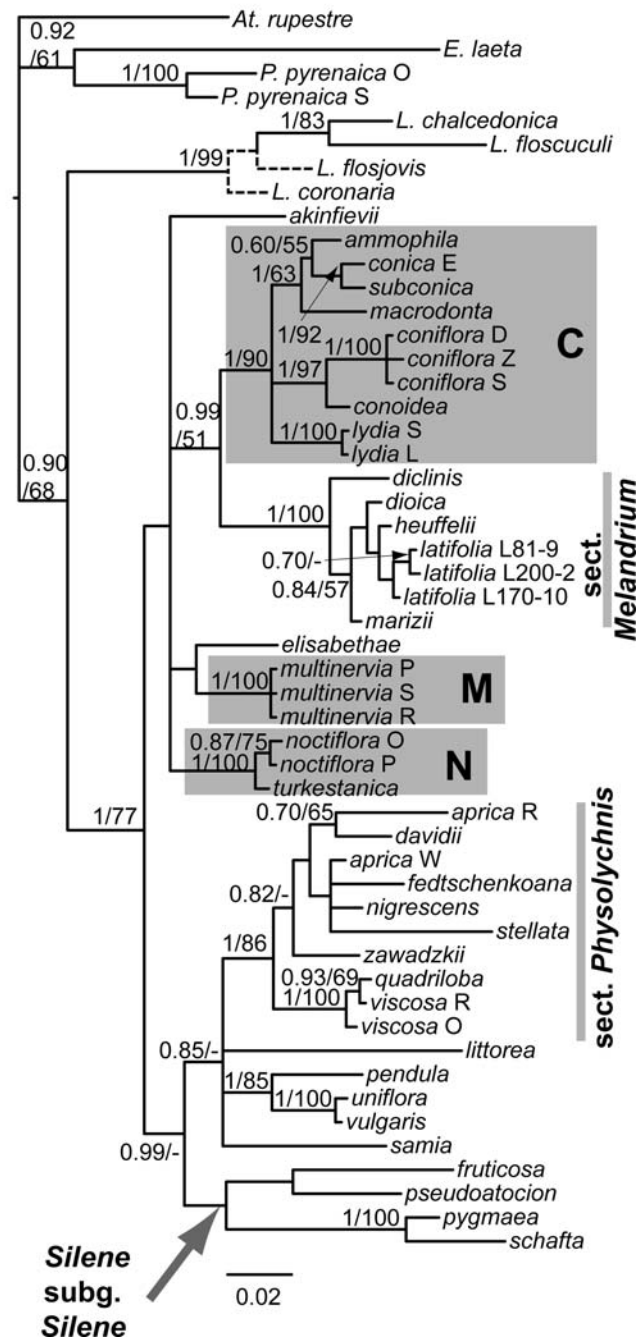


FIG. 3. Phylogram obtained from MrBayes for the ITS sequences of the nuclear ribosomal DNA. Values associated with nodes are Bayesian posterior probabilities/parsimony bootstrap values. Posterior probabilities/ bootstrap values lower than 0.70/50 are not indicated. Branch lengths represent estimated number of changes per site. Dashed lines represent parts of the tree where the maximum parsimony bootstrap consensus tree has a differing topology (bootstrap support lower than 60%). The gray boxes indicate the key groups C = section *Conoimorpha*, M = *S. multinervia*, and N = *S. noctiflora* + *S. turkestanica*. Numbers and letters after species names indicate different specimens (Appendix 1). All species belong to *Silene* except At = *Atocion*, E = *Eudianthe*, L = *Lychmis*, and P = *Petrocoptis*.

specimens have 20 calyx veins, a number that is also supported by previously published reports (Boissier 1867; Rohrbach 1868; Williams 1896; Post 1932; Blakelock 1957; Zohary 1966; Bajtenov 1969; Schischkin 1970; Hosny et al. 1992; Boulos

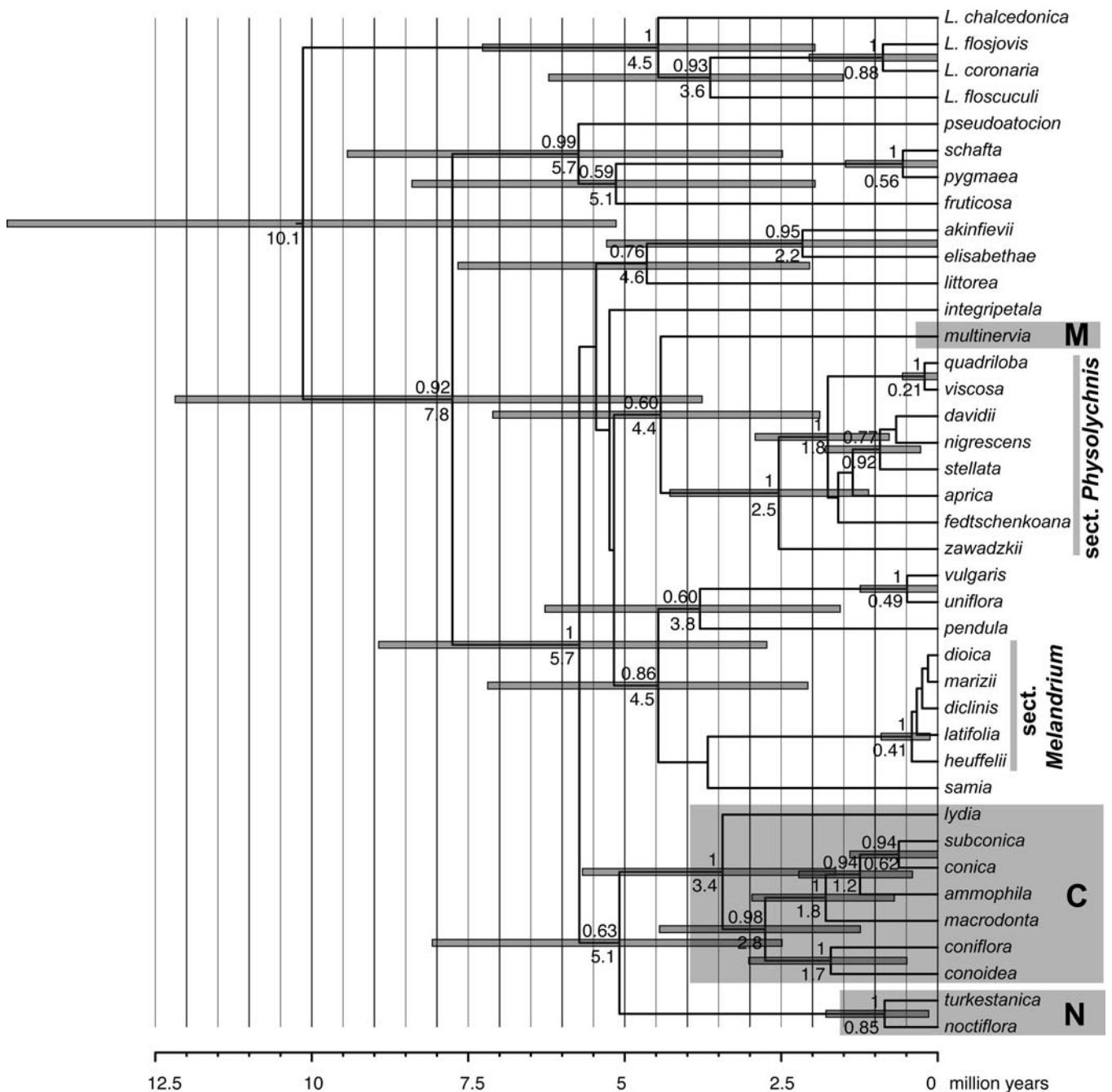


FIG. 4. Species tree obtained from \*BEAST for *Silene* and *Lychnis*, based on cpDNA, *RPA2*, *RPD2a*, and *RPD2b* sequences. Values associated with nodes are Bayesian posterior probabilities (above branches) and median node ages in million years (under branches). The horizontal bars represent 95% HPD (highest posterior density) intervals of node ages. The gray boxes indicate the key groups C = section *Conoimorpha*, M = *S. multinervia*, and N = *S. noctiflora* + *S. turkestanica*. All species belong to *Silene* except *L. Lychnis*.

1999). Ghazanfar and Nasir (1986) and Melzheimer (1988) give a number of 15–20 calyx veins for *S. coniflora*.

#### DISCUSSION

*Is There any Morphological or Molecular Support for the Synonymization of S. multinervia to S. coniflora?*—*Silene coniflora* is the representative of section *Conoimorpha* that most resembles the superficial appearance of *S. multinervia*, with the similarity mainly based on the number of calyx veins. Careful study of plant material, however, reveals that

the North American and southwest/central Asian species are two distinct entities that easily can be distinguished morphologically based on leaf morphology and presence/absence of coronal scales. None of the gene phylogenies show any support for the synonymy of *S. multinervia* and *S. coniflora*.

*Is There any Morphological Support for the Inclusion of S. multinervia in Silene Section Conoimorpha?*—The common characteristic nervature of *Silene* section *Conoimorpha* and *S. multinervia*, with 15 or more densely packed, prominent parallel calyx veins, is not present in any other members of the genus. Other *Silene* species have calyces with 10 veins,



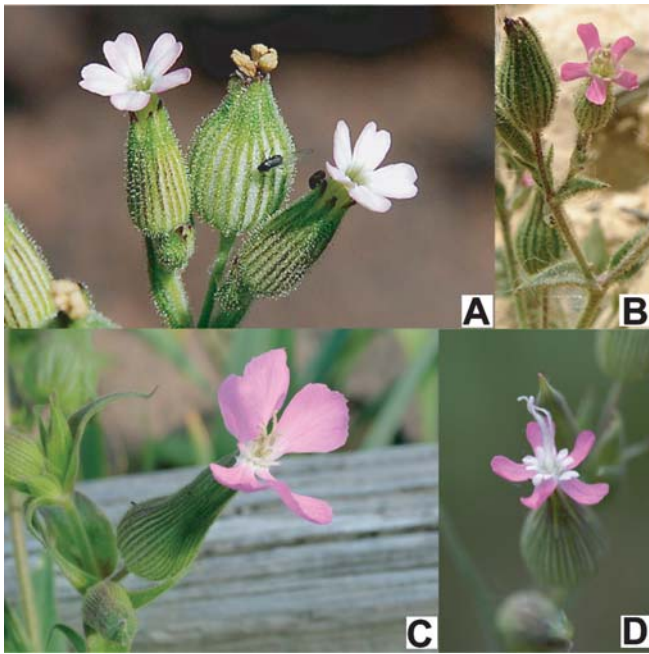


FIG. 5. Flowers of A. *Silene multinervia* (California, U. S. A., photo: Barry Breckling). B. *S. coniflora* (Israel, photo: Sara Gold, used with kind permission from <http://www.wildflowers.co.il>). C. *S. conoidea* (cultivated, Sweden, photo: Anja Rautenberg). D. *S. conica* (photo: Mikael Tholleson).

or with a different distribution of the veins. Among the close relatives in *Silene* subgenus *Behenantha*, the dioecious members of section *Melandrium* have female flowers with 20 branching veins and male flowers with 10 veins, *S. vulgaris* and its close relatives have an anastomosing pattern on the calyx, whereas the members of section *Physolychnis* have 10 veins. We have not found any synapomorphies for *S. multinervia* and section *Conoimorpha* other than the nervature. Our chromosome count of *S. multinervia* ( $2n = 24$ ) is the same as for most other diploid *Silene* species, but differs from what Morton (2005) reports for Asian *S. coniflora* material ( $2n = 20$ ). We have, however, not been able to find any original chromosome counts of *S. coniflora* in Bari (1973), the IPCN database (Goldblatt and Johnson 1979), the *S. coniflora* literature listed in Material and Methods, or other literature on chromosome counts in *Silene*. Several reports show that other members of section *Conoimorpha* have  $2n = 20$  (e.g. Khoshoo 1960; Khoshoo and Bhatia 1963; Greuter 1995), or possibly  $2n = 22$  in *S. lydia* (Greuter 1995). Thus, cytological evidence do not support the inclusion of *S. multinervia* in section *Conoimorpha*, whereas the presence of many densely packed calyx veins is a potential synapomorphy.

**Is There any Molecular Support for the Inclusion of *S. multinervia* in *Silene* Section *Conoimorpha*?**—The present study, based on a more thorough sampling of specimens and taxa, supports previous studies indicating that *S. multinervia* does not form a monophyletic group with the Eurasian species of section *Conoimorpha* (Popp and Oxelman 2007; Rautenberg et al. 2010). The relationships between the different groups from *Silene* subgenus *Behenantha* are largely unresolved, and hence it is difficult to pinpoint the phylogenetic position of *S. multinervia*. Although the \*BEAST species tree and the gene phylogenies of *RPA2*, *RPB2*, *RPD2a*, and *RPD2b* are somewhat incongruent regarding the rela-

tionships within subgenus *Behenantha*, they all indicate that *S. multinervia* is not the closest relative of section *Conoimorpha*. Other molecular studies also have had problems resolving the positions of several groups in subgenus *Behenantha* (e.g. Popp and Oxelman 2007; Rautenberg et al. 2010), and Erixon and Oxelman (2008a) suggested that an ancient radiation is responsible for the pattern seen in the cpDNA data.

If *S. multinervia* and *S.* section *Conoimorpha* are sister lineages, the non-monophyly of the groups could potentially be explained by incomplete lineage sorting effects, which would be reasonable if the branching events leading to the radiation of subgenus *Behenantha* were separated by short time spans and/or large effective population sizes. If *S. multinervia* and section *Conoimorpha* are not each other's closest relatives, the apparent morphological synapomorphy (many densely packed unbranched calyx veins) could be caused by convergent evolution or by a deep coalescent event of the gene(s) responsible for this feature. The chronograms indicate that the split between *S. multinervia* and section *Conoimorpha* lineages must be several million years old, so even if *S. multinervia* and section *Conoimorpha* are sister groups, the hypothesis of human-mediated dispersal of *S. multinervia* from Eurasia to America can be safely rejected.

The species from section *Conoimorpha* included in our species tree analyses (*S. ammophila*, *S. conica*, *S. coniflora*, *S. conoidea*, *S. lydia*, *S. macrodonta*, and *S. subconica*) form a strongly supported monophyletic group. *Silene grisebachii* and *S. sartorii* could unfortunately not be sampled for the present study, but given their great morphological, ecological, and geographical resemblance (Pirker and Greuter 1997) to the rest of the species, it is sound to hypothesize that they also belong to the section *Conoimorpha* clade.

***Silene* Section *Conoimorpha* and *S. noctiflora***—In accordance with Sloan et al. (2009), the members of *Silene* section *Conoimorpha* and the monophyletic group *S. noctiflora* + *S. turkestanica* both have extremely high substitution rates in the mitochondrial genes *atp1*, *cox3*, and *nad9*. *Silene multinervia* has slightly elevated rates, as compared to the rest of the genus. Due to the extreme variations in substitution rates between the sampled taxa, it is difficult to use the mtDNA phylogeny to draw conclusions on the relationships between different lineages. In the *RPD2a* and *RPD2b* phylogenies, *S. noctiflora* + *S. turkestanica* form a monophyletic group with section *Conoimorpha*. This topology is partly supported by a recent study, where the 3' part of the *SIX1/SIY1* gene indicates monophyly of *S. noctiflora* and section *Conoimorpha*, although with low support (Rautenberg et al. 2008). If this sister group relationship reflects the species phylogeny, it would support a single origin of the elevated substitution rates in section *Conoimorpha* and *S. noctiflora* + *S. turkestanica*. However, the incongruence of the tree topologies inferred from other nuclear and chloroplast genes (Figs. 1a, 2a–b, 3–4; and the 5' part of *SIX1/SIY1* gene in Rautenberg et al. 2008) makes this relationship remain ambiguous.

**Congruence Between Organellar Phylogenies**—Recent studies in *Silene vulgaris* have found evidence of paternal transmission and recombination in organelle genomes, resulting in incongruence between cpDNA and mtDNA gene trees within the species (McCauley et al. 2005; Houlston and Olson 2006; McCauley et al. 2007; McCauley and Ellis 2008). These results raise the possibility of phylogenetic conflicts between cpDNA and mtDNA at the interspecific level. Plant mtDNA sequences are often uninformative at local phylogenetic scales, because

substitution rates in plant mtDNA are generally low compared to those in plant chloroplast and nuclear genomes and compared to mtDNA of other organisms (Wolfe et al. 1987; Palmer and Herbon 1988). In our dataset, the extreme differences in branch lengths in the *Silene* mtDNA phylogenies preclude using mtDNA to infer relationships among the major lineages. On the other hand, the rate acceleration provides the rare opportunity to use plant mtDNA to resolve the relationships at a local phylogenetic scale within section *Conoimorpha*. Within section *Conoimorpha*, we found that the different mitochondrial regions are congruent with each other, with the cpDNA regions, and with the nuclear regions, except for a few weakly supported deviations. Therefore, if paternal leakage and recombination have occurred within section *Conoimorpha*, they do not appear to have generated significant phylogenetic conflicts between chloroplast and mitochondrial genomes.

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APPENDIX 1. Voucher details (collector, number and herbarium), origin (for section *Conoimorpha* specimens), and GenBank accession numbers for the specimens analyzed in the present study. Sequences HQ334894–HQ334976 were produced for this study. Herbarium abbreviations are according to Holmgren and Holmgren (1998), except Strid = Arne Strid's private herbarium, Ørbaek, Denmark. Superscripts are used as



*S. turkestanica* Regel—Kiseleva 20.VI.1970 (MW): FJ589303 (*atp1*), FJ589425 (*cox3*), FJ589494 (*nad9*), FN821195 (*matK*), FN821315 (*rps16*), FN821371 (*trnLF*), FN821147 (ITS), HM595192 (RPA2), HM595248 (RPB2), HQ334957 (RPD2a), HM595313 (RPD2b). *S. uniflora* Roth—Erixon 73 (UPS)<sup>E</sup>: FJ589304 (*atp1*), FJ589426 (*cox3*), FJ589495 (*nad9*), FJ589565 (*matK*), EU221620 (*trnLF*); Oxelman 2197 (GB)<sup>O</sup>: Z83173 (*rps16*), X86849 (ITS), DQ908710 (RPA2), DQ908759 (RPB2), DQ908807 (RPD2a), DQ908780 (RPD2b). *S. viscosa* (L.) Pers.—Rautenberg 104 (UPS)<sup>R</sup>: FN821200 (*matK*), FN821316 (*rps16*), FN821372 (*trnLF*), FN821148 (ITS), HM595194 (RPA2), HM595251 (RPB2), HQ334958 (RPD2a), HM595316 (RPD2b); Oxelman 2288 (GB)<sup>O</sup>: X86831 (ITS), HM595249, HM595250 (RPB2). *S. vulgaris* (Moench) Garcke—EF1394601, EF139465, EF139471, EF139480, EF139482 (*atp1*), EF139560, EF139565, EF139571, EF139580, EF139582 (*cox3*), EF139610, EF139615, EF139621, EF139630, EF139632 (*nad9*), *S. vulgaris* subsp. *angustifolia* (DC.) Hayek—Thulin 5717 (UPS): FJ376828 (*matK*), FN821317 (*rps16*), FN821374 (*trnLF*), FN821149 (ITS), HM595195 (RPA2), HM595252 (RPB2). *S. zawadzkkii* Herbich—Oxelman 2241 (GB): FJ589307 (*atp1*), FJ589429 (*cox3*), FJ589498 (*nad9*), FN821201 (*matK*), Z83177 (*rps16*), EU221621 (*trnLF*), X86893 (ITS), AJ629306 (RPA2), FJ376921 (RPB2), AJ634108 (RPD2a), AJ634109 (RPD2b). *Viscaria alpina* (L.) G. Don—Frajman & Schönswetter 11415 (LJU): FJ589308 (*atp1*), FJ589430 (*cox3*), FJ589499 (*nad9*), *V. vulgaris* Bernh.—Schönswetter & Frajman 11097 (LJU): FJ589309 (*atp1*), FJ589431 (*cox3*), FJ589500 (*nad9*).