# Molecular taxonomic identification of a *Silene* plant regenerated from Late Pleistocene fruit material

Tatiana E. Kramina, Elizaveta P. Iakovenko, Larissa A. Koppel, Tahir H. Samigullin, Yulia A. Karpova, Edith N. Gakhova & Eugeny E. Fesenko (Jr.)

*Summary:* The article presents the results of taxonomic identification of two *Silene* L. somaclones regenerated *in vitro* from Late Pleistocene and the extant fruit material. Molecular phylogenetic analyses based on two DNA markers (nrDNA ITS region and cpDNA *rps*16 intron) have been conducted. The analyses were carried out separately for each marker and included sequences of 31 herbarium specimens of additional nine *Silene* species from Siberia, the Russian Far East and northeast of European Russia as well as those of a worldwide set of *Silene* species from GenBank. The obtained results clearly demonstrate that both clones (the ancient *Silene* SS1 and the recent *Silene* SS2) belong to *Silene linnaeana* Vorosch. group being part of the section *Physolychnis* (Benth.) Bocquet. The morphological and molecular analysis of both *Silene* clones yielded the adequate results of these two methods. These results confirm taxonomic identification of the ancient *Silene* clone by B. Oxelman et al. (in: Proc. Natl. Acad. Sci. U.S.A. 109(41): E2735, 2012. https://doi.org/10.1073/pnas.1207774109) on rejecting previous determination of the ancient *Silene* as *S. stenophylla* Ledeb.

*Keywords:* cpDNA *rps*16, *in vitro* culture, Kolyma Lowland, Late Pleistocene, nrDNA ITS, *Silene linnaeana*

The permafrost of the Russian Arctic zone is a natural cryobank providing the ideal conditions for conserving large amounts of various microorganisms, fungi, plant and animal remains, preserved in the past millennia. All of them may be considered as objects suitable for genetic studies. At constant sub-zero temperatures of the permafrost, microbial communities retain their viability much longer than in any other habitats (Gilichinsky et al. 1995; Rivkina et al. 2000). Recently discovered 100–200,000-year-old fungi of the genus *Penicillium*, extracted from the permafrost deposits of the Arctic and Antarctica and the frozen volcanic ash of Kamchatka Peninsula, were able to synthesize biologically active substances (Antipova et al. 2011). On the other hand, successful attempts to regenerate more highly organized ancient organisms are quite sparse. The first trial on growing plants from 10,000-year-old seeds of the arctic lupine (*Lupinus arcticus* S. Watson, Fabaceae) was reported by Porsild et al. (1967). The lupine seeds extracted from the ancient rodent burrow were believed to be the oldest ancient seeds that had ever been successfully germinated. However, an independent radiocarbon dating of the seeds found out that the ancient burrow was contaminated with modern lupine seeds (Zazula et al. 2009). La Farge et al. (2013) managed to obtain through *in vitro* a bryophyte meristem culture that had survived for 400 years being entombed in the ice. Recently, viable soil nematodes were found in the samples of the Pleistocene permafrost deposits of the Kolyma Lowland dated back to 40–30,000 years ago (Shatilovich et al. 2018). However, the first successful attempt for growing plants from the Pleistocene deposits was performed by Yashina et al. (2012a).

The Duvanny Yar bluff is one of the best studied exposures of Quaternary deposits in Western Beringia, the explorations of which provided the most extensive knowledge about Late Pleistocene

environments in the North-East Siberian Arctic (Zanina et al. 2011). It is located in the Kolyma Lowland, on the right bank of the Kolyma River, 35–43 km downstream the estuary of the Omolon River in Northeastern Yakutia (68°38'N; 159°03'E). The length of the outcrop along the river is over 8 km (Kaplina et al. 1978). This locality contains Late Pleistocene and Holocene deposits. The Duvanny Yar section is rich in nests of fossil rodents, especially the ground squirrels (*Urocitellus parryii*), which feeding chambers contain numerous and diverse paleobiological material. The fossil burrows were found at the depth of 20–40 m from the surface. The structure of ice complexes revealed that these burrows and their content have never been thawed after their initial burial. The presence of sublimated ice as well as the position and structure of the fossil nests rule out the possibility that the ancient material found in these nests could have been contaminated by modern admixtures. Therefore, the ancient age of the seeds is beyond doubt (Gubin & Khasanov 1996; Gubin et al. 2003). Fossilised seeds and plant remains of 40 taxa collected from the ancient rodent burrows provide a uniquely preserved genetic material for further morphogenetic and molecular genetic studies (Lopatina & Zanina 2006; Gubin et al. 2011).

The plant seeds and fruits were excavated from the fossil burrows of ancient ground squirrels. Using AMS radiocarbon dating yielded the age of the fossils being 31,800 ± 310 years old (Gubin et al. 2011). The seeds and fruits of *Silene* L., *Arctous* (A. Gray) Nied. and *Polygonum* L. found in fossil nests appeared to be viable and were cultivated by *in vitro* methods by Yashina et al. (2002).

The ancient *Silene* seeds were studied using SEM and LM methods and then compared to the seed samples of four extant species coming from the same geographic region (*S. latifolia* Poir. subsp. *alba* (Mill.) Greuter & Burdet, *S. chlorantha* Ehrh., *S. nutans* L. and *S. stenophylla* Ledeb.) (Başlı et al. 2009; Gyulai et al. 2011). Gyulai et al. (2011) considered the morphological features of the ancient *Silene* seeds to be very similar to those of contemporary *S. stenophylla* seeds, except for a smaller size of the former. On these grounds, the ancient *Silene* was identified as *S. stenophylla*. Meanwhile, Oxelman et al. (2012) noted that the seeds of *S. linnaeana* group are half the size of those of *S. stenophylla* and suggested that ancient seeds matched better the *S. linnaeana* group.

Successful regeneration of the whole *Silene* plants was carried out *in vitro* from the placentary tissue of three different immature fruits of the ancient campion. The organogenesis of the ancient campion was immediately followed by regeneration of the modern campion *in vitro* culture, also grown from a tissue of immature fruits collected in the Kolyma Lowland. The regenerated plants of the ancient and modern *Silene* clones were adapted to *ex vitro* conditions and reached the stages of flowering and mature fruiting (Yashina et al. 2012a).

All regenerated plants were tested morphologically and identified as *Silene stenophylla* (Yashina et al. 2012a). However, the photo of the ancient *Silene* plants published by Yashina et al. (2012a: Figs 2–3) made Oxelman et al. (2012) question the validity of identification of the species based on several morphological features. Particularly, the key morphological features, such as leaf morphology, inflorescence structure, floral morphology, indumentum and seed characteristics, indicated that the plants belonged to the *Silene linnaeana* Vorosch. (= *Lychnis sibirica* L.) group. In their reply to Oxelman et al. (2012), Yashina et al. (2012b) only partially agreed with the arguments. At the same time, they pointed at the following features: the calyx and inflorescence structure of the regenerated plants were similar to the *S. linnaeana* group, while their number of styles (preferentially three) and the number of teeth in the dehiscing capsule (twice as many as the number of styles) resembled *S. stenophylla*.

The genus *Silene* L. (Caryophyllaceae-Sileneae) includes approximately 870 species (Oxelman et al. 2013; Jafari et al. 2020). The recent classification of the tribe Sileneae as well as the corresponding database with the information on taxonomy, geography, herbarium vouchers and GenBank accessions are available online at http://www.sileneae.info (Oxelman et al. 2013).

The genus *Silene* has been extensively studied through molecular phylogenetic methods. The most well-studied DNA markers are the nuclear ribosomal ITS region (Oxelman & Lidén 1995; Popp & Oxelman 2001, 2004, 2007; Popp et al. 2005; Eggens et al. 2007; Mikhaylova et al. 2014; Naciri et al. 2017; Jafari et al. 2020), the plastid *rps*16 intron (Oxelman et al. 1997; Popp & Oxelman 2001, 2004, 2007; Popp et al. 2005; Eggens et al. 2007; Petri & Oxelman 2011; Naciri et al. 2017; Jafari et al. 2020), and low copy nuclear RNA polymerase (RP) introns (Popp & Oxelman 2001, 2004, 2007; Popp et al. 2005; Eggens et al. 2007; Petri & Oxelman 2011; Naciri et al. 2017). Widely encompassing molecular phylogenetic studies have allowed Jafari et al. (2020) to lean towards a broad taxonomic concept of the genus *Silene* s. l., which includes many small-sized genera. As compared to Oxelman et al. (2001), these authors further expanded on the scope of the genus *Silene* including in it *Lychnis* L. and *Uebelinia* Hochst.; at the same time, they retain *Agrostemma* L., *Atocion* Adans., *Eudianthe* Rchb., *Heliosperma* Rchb., *Petrocoptis* A. Braun and *Viscaria* Bernh. as separate genera. Greuter (1995) considered the genus *Silene* even more broadly. On the contrary, some Russian researchers support the concept of small-sized genera within the tribe Sileneae (Mikhaylova et al. 2014).

Phylogenetic studies based on examining nrDNA ITS and cpDNA *rps*16 regions clearly demonstrated that the genus *Silene* was subdivided into two large clades (Oxelman et al. 2001), later interpreted as two subgenera, subgenus *Silene* and subgenus *Behenantha* (Otth) Torr. & A. Gray (= *Behen* (Dumort.) Rohrb.) (Popp & Oxelman 2004; Petri & Oxelman 2011). It seems to be consistent with the early concept by Rohrbach (1868). According to Jafari et al. (2020), genus *Silene* comprises three subgenera: subg. *Lychnis* (L.) Greuter (4 sections and some unplaced taxa), subg. *Behenantha* (18 sections and some unplaced taxa) and subg. *Silene* (11 sections and some unplaced taxa) as well as *incertae sedis* section *Atocion* Otth.

Popp & Oxelman (2007) partially clarified the taxonomy of *Silene* section *Physolychnis* (Benth.) Bocquet. Their studies were continued by Petri & Oxelman (2011). Using two low copy nuclear genes and three chloroplast markers, they proved that the section was divided into two clades: the Siberian *S. ajanensis* group and the Asian-American group. The Siberian group includes the Far Eastern species *S. ajanensis* (Regel & Tiling) Vorosch. as well as closely related species *S. linnaeana* s. str., *S. samojedorum* (Sambuk) B. Oxelman and *S. villosula* (Trautv.) V.V. Petrovsky & Elven, sometimes considered the group to be one species, *S. linnaeana* s. l. (Voroshilov 1985). Originally, *S. linnaeana* was described as *Lychnis sibirica* L. (Linnaeus 1753). Voroshilov transferred this species to the genus *Silene* under the name *Silene linnaeana* Vorosch. (Voroshilov 1985). Oxelman et al. (2001) and Popp et al. (2005) confirmed that the taxon belongs to the genus *Silene*. In the monograph "Arctic Flora of the USSR", Yurtsev (1971) recognised three subspecies within *Lychnis sibirica* (= *S. linnaeana*), which were accepted as separate species by Oxelman et al. (2001). Lazkov & Sennikov (2016) argued that *S. linnaeana* Vorosch. is a junior synonym to *S. orientalimongolica* Kozhevn. (Kozhevnikov 1984). Here, we adhere to the name *S. linnaeana*, since the final decision on the priority name of this taxon requires a comprehensive study of both type materials as well as of *S. linnaeana* group as a whole, including their molecular analyses.

*Silene stenophylla* belongs to the section *Graminiformes* Lazkov (Lazkov 1997). According to Naciri et al. (2017), this species is part of the section *Siphonomorpha* Otth (Otth 1824). Naciri et al. (2017) introduced a broader interpretation of the section *Siphonomorpha* using nuclear markers ITS, RPA2 and chloroplast markers *trn*H-*psb*A, *rps*16 and *trn*S-*trn*G. As a result, the section *Siphonomorpha* s. l. includes the species that were traditionally classified within the following sections: *Saxifragoides* Willk., *Coronatae* Chowdhuri, *Tataricae* Chowdhuri, *Chloranthae* Rohrb., *Barbeyanae* Greuter, *Nanosilene* Otth, *Otites* Adans., *Koreanae* Lazkov, *Brachypodae* Boiss., *Graminiformes*, *Dianthoides* (Chowdhuri) Lazkov, *Longitubulosae* C.L. Tang and *Holopetalae* Chowdhuri. From the authors' viewpoint, monophyletic groups 'Italicae', 'Paradoxae' and 'Siphonomorpha s. str.' may deserve taxonomic recognition as sections (Naciri et al. 2017). Jafari et al. (2020) shared the broad concept of the section *Siphonomorpha* s. l., but considered it even more broadly, including in it the three groups listed above.

Thus, despite of obvious controversies in the taxonomy of *Silene* and the tribe Sileneae, there are clear differences between two groups of taxa, proposed for taxonomic identification of *Silene* plants regenerated from fossil material, *Silene linnaeana* group and *S. stenophylla* group. These differences could be revealed through both morphological and molecular approaches.

In this study we aimed to determine the taxonomic affiliation of the clones regenerated *in vitro* from fossil fruits and fruits of extant *Silene*. For this goal, we carried out a molecular genetic analysis of the plantlets and a representative sample of the *Silene* species. Firstly, we performed a molecular taxonomic identification of the *Silene* regenerants obtained *in vitro* from both fossil and modern fruit material using the sequences of two DNA markers (nuclear ribosomal ITS region and cpDNA *rps*16 intron). Secondly, we compared the sequences of the ancient and recent *Silene* regenerants with those obtained from the recently collected herbarium specimens of several *Silene* species from the same geographic area. Finally, we carried out molecular phylogenetic analyses based on both markers and available data of *Silene* species.

## Materials and methods

**Plant material.** The Cryobiology Laboratory of the Institute of Cell Biophysics of the Russian Academy of Sciences provided us with regenerated lines of *Silene*, which were obtained from fossil and extant material by micropropagation methods in the early 2000s and are currently maintained *in vitro*. Their micropropagation is regularly carried out with a transfer to a fresh culture medium. Thus, all ancient and extant regenerants obtained *in vitro* are vegetative clones.

Two *Silene* somaclones were used as material for the current study: SS1 (the regenerants obtained from fossil material, i.e. immature fruits with fragments of placentary tissue, further referred to as 'ancient *Silene*') and SS2 (the regenerants obtained from the similar tissue of an extant plant, further referred to as 'recent *Silene*'). All 31 herbarium specimens from the Herbarium of Moscow State University [MW] originated from Siberia, the Russian Far East and northeast of European Russia were sampled for DNA for further comparisons. The specimens were identified as follows: *Silene ajanensis*, *S. involucrata* (Cham. & Schltdl.) Bocquet ssp. *tenella* (Tolm.) Bocquet, *S. jenisseensis* Willd.*, S. linnaeana*, *S. samojedorum*, *S. stenophylla*, *S. uralensis* (Rupr.) Bocquet subsp. *porsildii* Bocquet, *S. villosula* and *S. violascens* (Tolm.) V.V. Petrovsky & Elven. Voucher information is presented in Appendix 1.

We performed evaluation of morphological characters of the ancient and recent *Silene* regenerants cultivated *in vitro* and in soil, following Yurtsev (1971), Yurtsev et al. (1971), Zuev (1993), Kovtonyuk (1993) and Oxelman et al. (2012) and utilising herbarium specimens.

Morphological analysis of the regenerants of both *Silene* clones (i.e. the ancient somaclone SS1 and the recent somaclone SS2) was carried out using data from Yashina et al. (2012a, b), Oxelman et al. (2012) and photographs provided by Yashina et al. (2012a) and taken by Yu.A. Karpova, L.A. Koppel and S.R. Majorov in 2018–2019 in the Institute of Cell Biophysics of the Russian Academy of Sciences.

**DNA extraction, amplification and sequencing.** Total genomic DNA was extracted from stem and leaf tissues of ancient and recent *Silene* somaclones SS1 and SS2 using the CTAB method (Doyle & Doyle 1987). DNA of other samples was extracted from dry leaves of herbarium specimens (20 mg leaf tissue) using NucleoSpin Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions.

The sequences of the entire ITS1-5.8S-ITS2 region were amplified using primers NNC-18S10, C26A (Wen & Zimmer 1996), ITS2 and ITS3 (White et al. 1990). The sequences of *rps*16 intron were amplified using primers rpsF and rpsR2 (Oxelman et al. 1997).

Polymerase chain reactions (PCR) were conducted in 20 µl reaction volumes containing 4 µl of Ready-to-Use PCR Mas$^{DD}$TaqMIX (200 µM of each dNTP, 1.5 mM MgCl$_2$, 1.5 U SmarTaqDNA Polymerase and reaction buffer; Dialat Ltd., Moscow, Russia), 15 µl deionised water, 3.2 pmol of each primer and 1 µl of template DNA of unknown concentration. PCR amplification was performed with a T100™ Thermal Cycler Bio-Rad using the following thermocycling conditions: an initial denaturing step at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C and 1 min at 72°C and a final extention for 10 min at 72°C.

PCR products were checked on agarose gels and purified using the Cleanup Standard DNA cleaning kit (*Evrogen,* Moscow, Russia) following the manufacturer's instructions. Direct sequencing was performed on the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 for cycle sequencing reactions following the manufacturer's instructions. Forward and reverse strands of all samples were sequenced. Polymorphic sites in ITS sequences were IUPAC coded. The sequences newly obtained in the present study are deposited in GenBank (Appendix 1).

Additionally, 135 GenBank accessions were included in the phylogenetic analyses, i.e. 75 and 59 accessions for nrDNA ITS and cpDNA *rps*16 regions respectively (Appendix 2). The majority of sequences taken from GenBank (74 DNA sequences of ITS region and 58 DNA sequences of *rps*16) belong to various species of the genus *Silene* and cover the main intrageneric diversity of the genus. Sequences of *Agrostemma githago* L. were used as an outgroup. A taxonomic system of the genus *Silene* developed by Jafari et al. (2020) was used in this study. Two separate datasets were obtained, i.e. nrDNA ITS dataset with 107 sequences and plastid *rps*16 dataset with 92 sequences. Both datasets were then aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013) and corrected manually using the program BioEdit (Hall 1999). The aligned data matrices (cpDNA *rps*16 intron and nrDNA ITS sequences) are available on request from the corresponding author.

**Phylogenetic analyses.** The Maximum Likelihood (ML) analyses were performed using MEGA X (Kumar et al. 2018) with GTR+G model of nucleotide substitutions for *rps*16 intron matrix and GTR+G+I model for ITS1-5.8S-ITS2 matrix. The models were determined as the best choice for the corresponding datasets following the Model Selection option implemented in MEGA X based on the corrected Akaike information criterion (AICc). Bootstrap method with 500 bootstrap replications was used to assess the branch support. We use ML bootstrap consensus trees to compare them with the trees obtained by an alternative method, i.e. Bayesian Inference.

Phylogenetic relationships were also inferred with a Bayesian approach using MrBayes version 3.2.6 (Ronquist et al. 2012) with the GTR+G model of nucleotide substitutions for *rps*16 intron and SYM+G model for ITS1-5.8S-ITS2 sequences, the models were selected by AICc in PAUP version 4.0a (Swofford 2003). Bayesian inference was performed with two parallel runs with four Markov chains for each run. Trees were sampled every 1000th generations for a total of 20 million generations. The first 2000 (10%) sampled trees were discarded as burn-in, the number of discarded trees was determined by Höhna-Sahlin's ESS-based estimator in VMCMC (Ali et al. 2017). Then, a majority-rule consensus tree was constructed from the remaining trees. Trees were visualised using TreeView (Page 1996).

# Results

**Molecular phylogeny of *rps*16 sequences.** The cpDNA *rps*16 matrix included 1039 characters and 92 sequences and covered 19 *Silene* sections and *Agrostemma githago*. A total of 79 unique sequences were identified in the dataset.

As Bayesian inference and ML analyses produced the similar trees and did not contradict to each other, only Bayesian tree is presented (Fig. 1). Bayesian analysis of *rps*16 matrix revealed two large clades and two small separate branches. The first large clade, named subgenus *Behenantha* p. max. p., corresponds to *Silene* subgenus *Behenantha* (except for *S. cordifolia* All. of sect. *Cordifoliae* Chowdhuri and *S. cryptoneura* Stapf of sect. *Cryptoneurae* Aydin & Oxelman, which form separate branches) and is highly supported on the Bayesian tree (posterior probability PP = 0.99) (Fig. 1). The second large clade, containing species of *Silene* subgenus *Silene* and section *Atocion*, is low supported on the Bayesian tree (PP = 0.71). Noteworthy, the isolation of both large clades was not confirmed by ML analysis. However, each of the two large clades includes a main subclade, which is well supported by both Bayesian and ML analyses. For the clade *Behenantha* p. max. p., the main subclade is represented by the section *Physolychnis* clade (PP = 1, Bootstrap support in ML analysis (BS) = 97%). The main part of the *Physolychnis* clade is *S. linnaeana* group (PP = 1, BS = 96%).

Both ML and Bayesian analyses of the *rps*16 data showed that the sequences of both experimental *Silene* somaclones (i.e. SS1 and SS2) belong to *S. linnaeana* group clade with a high degree of

**Figure 1.** Phylogenetic relationships in *Silene* inferred from Bayesian analysis of the cpDNA *rps*16 dataset. Branch length is proportional to the number of expected nucleotide substitutions, scale bar corresponds to 0.01 substitutions per site. The posterior probability of branches is indicated by the thickness and colour of the lines. For most discussed nodes, numbers above branches are posterior probabilities, numbers below branches are bootstrap values found in Maximum Likelihood analysis of the same dataset (values equal or more than 0.6/60% are shown). New sequences are highlighted in bold. Samples of *Silene* regenerants are highlighted in a box. For each sample the following information is given: Species name; GenBank number (for sequences taken from GenBank) or Sample code (for newly obtained sequences); Section (abbreviations according to Appendix 2) and Subgenus (B – *Behenantha*, L – *Lychnis*, S – *Silene*). Infrageneric names are given according to Jafari et al. (2020). See Appendix 1 for sample codes and voucher information.
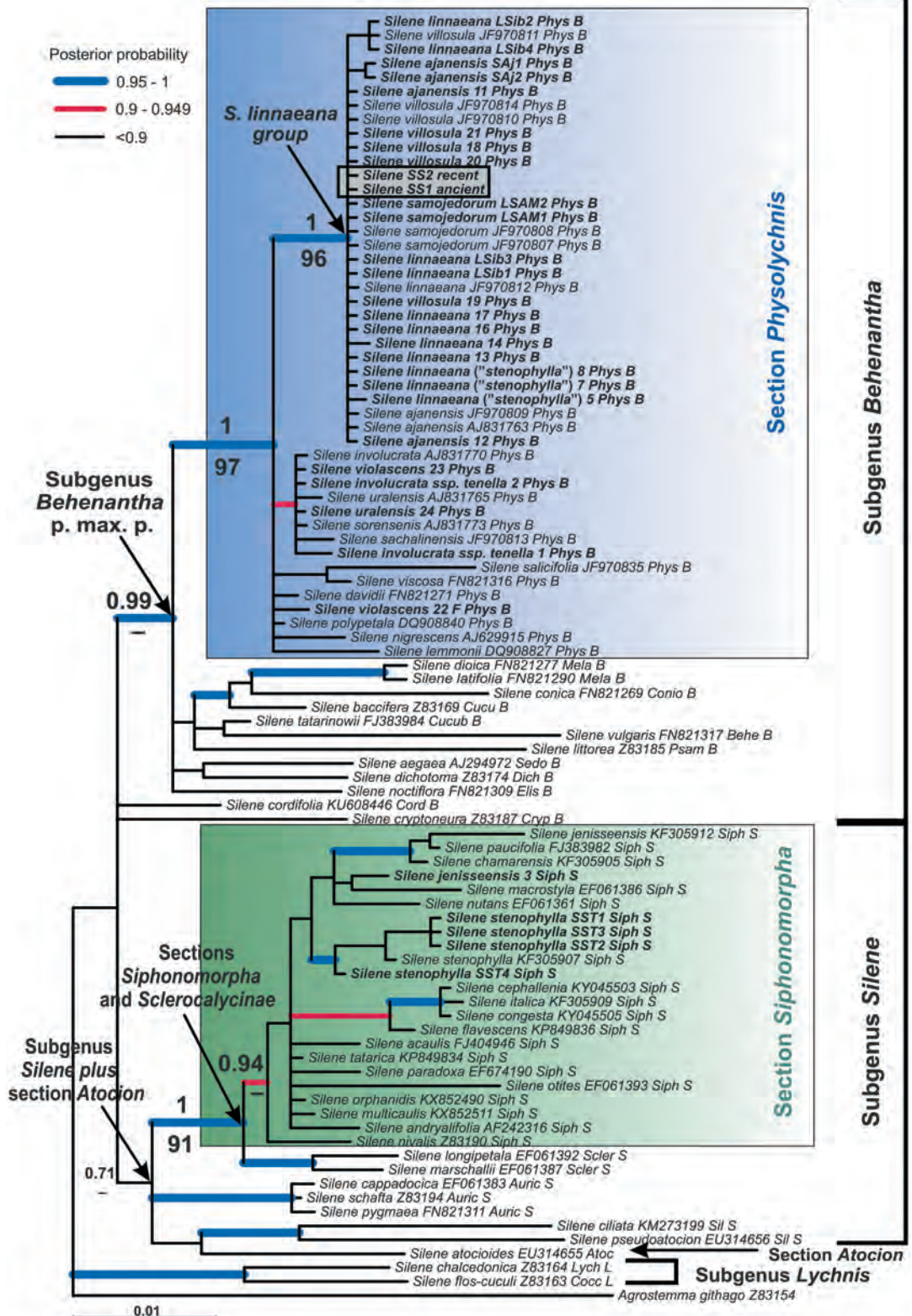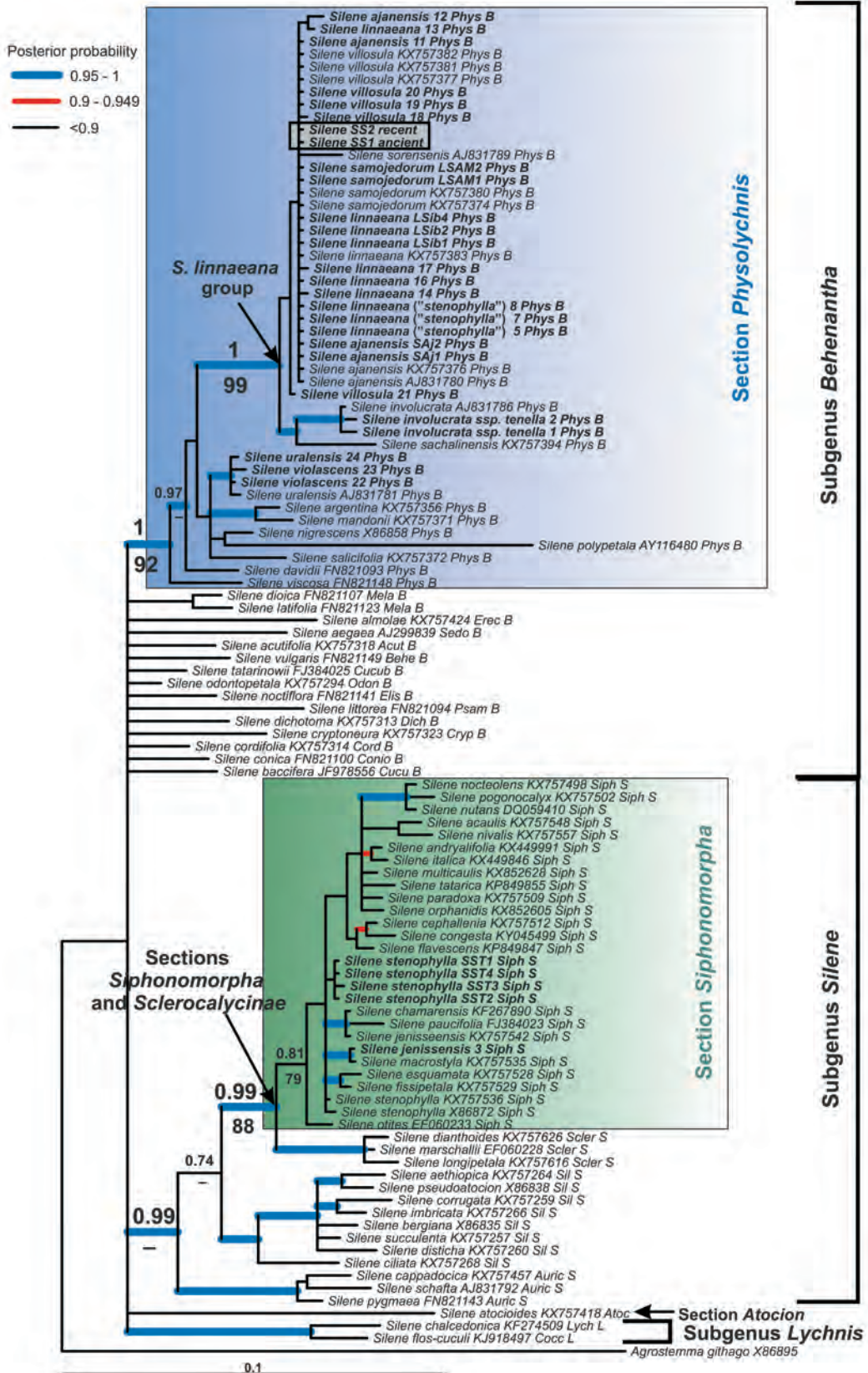
reliability (Fig. 1). This clade also includes the samples of *Silene linnaeana* and other species of *S. linnaeana* group, among which all newly sequenced samples of *S. ajanensis*, *S. samojedorum*, *S. villosula* and *S. linnaeana* s. str. Samples 5, 7 and 8 of *S. linnaeana*, previously identified by S.V. Maksimovich and S.P. Davydov as *S. stenophylla*, but later redetermined by S.R. Majorov, also belong to the *S. linnaeana* group. Samples *S. violascens* 23, *S. involucrata* ssp. *tenella* 1 and 2, and *S. uralensis* 24 are clustered in a separate subclade within the *Physolychnis* clade. The *rps*16 sequences of *Silene* regenerants SS1 and SS2 are 100% identical to each other and to the sequences of four other accessions, i.e. *Silene ajanensis* 11, *S. linnaeana* 8 and 13 and *S. villosula* 19.

Within the large clade [Subgenus *Silene* plus section *Atocion*], the main subclade combines the sections *Siphonomorpha* and *Sclerocalycinae*. This subclade is fairy well-confirmed by both methods of phylogenetic reconstruction (PP = 1, BS = 91%). The support of the section *Siphonomorpha* appears to be weaker. All studied *rps*16 sequences of *Silene stenophylla* and *S. jenisseensis* from the section *Siphonomorpha* are confirmed to belong to the clade [Sections *Siphonomorpha* and *Sclerocalycinae*].

**Molecular phylogeny of nrDNA ITS sequences.** The nrDNA ITS matrix included 776 characters and 107 sequences and covered 22 sections of the genus *Silene* and *Agrostemma githago*. In ITS sequences of the cultivated *Silene* somaclones SS1 and SS2, the same polymorphic sites were observed: 10 sites in ITS1 and 6 sites in ITS2. The regenerant SS2 also included two additional polymorphic sites, one in ITS1 and another in ITS2. In the sequences obtained from herbarium specimens of *S. linnaeana* group, the number of polymorphisms was a bit lower: up to nine in ITS1 and up to four in ITS2. The identity of ITS sequences of the ancient and recent *Silene* somaclones was 0.972.

As compared to Bayesian *rps*16 tree, in which the separation of two large clades at about subgeneric level was observed, in the Bayesian ITS tree only the subgenus *Silene* clade was distinguished (Fig. 2). The subgenus *Behenantha* does not form a common clade in ITS tree, but is represented by the clade *Physolychnis* (PP = 1, BS = 92%) and a number of separate small branches. The *Physolychnis* clade is consistent with the corresponding clade on the plastid *rps*16 tree. *S. linnaeana* group, which constitutes the main part of the clade *Physolychnis*, is well supported (PP = 1, BS = 99%) and in whole is similar to the corresponding clade on the *rps*16 tree in its sample composition, but has slight differences in position of several samples. Both Bayesian and ML phylogenetic analyses of ITS sequences fairly well confirm the inclusion of SS1 and SS2 *Silene* somaclones in *S. linnaeana* group along with other samples of the group, including samples *S. linnaeana* ('*stenophylla*') 5, 7 and 8 redetermined by S.R. Majorov.

The large clade of subg. *Silene* in the Bayesian ITS tree differs from the corresponding clade in *rps*16 tree, since the latter formed a weakly supported clade with the sections *Auriculateae* (Boiss.) Schischk. and *Silene*. This clade is supported by Bayesian analysis only, but its major part –

**Figure 2.** Phylogenetic relationships in *Silene* inferred from Bayesian analysis of the nrDNA ITS dataset. Branch length is proportional to the number of expected nucleotide substitutions, scale bar corresponds to 0.1 substitutions per site. The posterior probability of branches is indicated by the thickness and colour of the lines. For most discussed nodes, numbers above branches are posterior probabilities, numbers below branches are bootstrap values found in Maximum Likelihood analysis of the same dataset (values equal or more than 0.6/60% are shown). New sequences are highlighted in bold. Samples of *Silene* regenerants are highlighted in a box. The information for each sample is given as in Fig. 1. ◄ See Appendix 1 for sample codes and voucher information.

**Figure 3.** Flowering *Silene* plant regenerated from tissue of fossil fruit material (the ancient *Silene* somaclone SS1), cultivated in soil. Image: S.R. Majorov 2018.

[Sections *Siphonomorpha* and *Sclerocalycinae*] clade – tends to be well confirmed by both methods (PP = 0.99, BS = 88%). This clade includes all ITS sequences of *S. stenophylla* and *S. jenisseensis*.

**Taxonomic identification of *Silene* herbarium specimens and regenerated plants of ancient and recent *Silene* based on morphology.** The list of morphological characteristics allowing to distinguish *S. stenophylla* of the section *Siphonomorpha* from *S. linnaeana* s.l. of the section *Physolychnis* was compiled following Yurtsev (1971), Yurtsev et al. (1971) and Oxelman et al. (2012).

In *S. stenophylla* vegetative rosette shoots are well-developed, the plant is glabrous; the leaves are linear, sometimes almost filiform, reaching a considerable length (12–75 mm); the leaves are fused at 2–3 mm from the base; the inflorescence is a thyrsus of dichasia; the calyx is ellipsoidal with a maximum width in the middle, its size is 10–15 mm long, being larger than that of *S. linnaeana* s.l.; veins on the calyx have purple colour.

In the plants of *S. linnaeana* s.l., the vegetative rosette shoots are expressed to a much lesser degree; the plant is pubescent, but the pubescence density varies; the leaves are linear-lanceolate with shorter length (12–40 mm); the leaves are fused at 1 mm from the base, the inflorescence is a compound dichasium; the calyx is campanulate and narrowed to the base; the calyx is about ⅓ shorter than in *S. stenophylla*, 5.5–8 mm, its maximum width is above the middle; calyx veins are usually green, sometimes coloured purple.

The specimens labelled 5, 7, 8, 13, 14, 16, 17 and LSib1–LSib4 were identified as *S. linnaeana* s.l. according to their morphological characters, among them the specimens 5, 7 and 8 that were previously erroneously identified as *S. stenophylla*. Morphological characters of the specimens SST1–SST4 correspond to the diagnosis of *S. stenophylla*. The specimen 1, earlier misidentified as *Lychnis sibirica* (accepted name *S. linnaeana*), was reidentified as *S. involucrata* ssp. *tenella*.

In the regenerated somaclones SS1 and SS2, there are fewer vegetative shoots (Yashina et al. 2012a: Photos 2–3) than it is recognised for *S. stenophylla*; the pubescence of the shoot is very dense (Figs 3 and 4); inflorescence is a compound dichasium (Yashina et al. 2012a: Photos 2–3);

**Figure 4.** Flowering *Silene* plant regenerated from tissue of fossil fruit material (the ancient *Silene* somaclone SS1), cultivated in vitro. Image: M. Laimer 2020.

the leaves are mainly linear-lanceolate (Figs 3 and 5), but the leaves of the plants in *in vitro* culture are usually linear (Yashina et al. 2012a: Photo 5B). The calyx shape of both *Silene* somaclones is campanulate, tapering to the base, with green veins (Fig. 3). The number of styles varies in different samples; there are usually 3 and less often 4–5 styles (Yashina et al. 2012b). The degrees of fusion of the leaves in experimental *Silene* regenerants were not studied. The analysis of the morphological features of the ancient and recent *Silene* somaclones indicates that both of them can be identified as *S. linnaeana* s. l.

Many herbarium individuals of *S. linnaeana* s. l. and *S. stenophylla* and especially *Silene* plants regenerated *in vitro* (both ancient and recent) are characterised by reduced and impoverished inflorescences, which complicate the use of this trait for taxonomic identification.

## Discussion

The results obtained in the present study are consistent with previously published data (Jafari et al. 2020) and indicate that *S. stenophylla* belongs to subg. *Silene* and *S.* sect. *Siphonomorpha* s. l., while *S. linnaeana* should be assigned to subg. *Behenantha* and *S.* sect. *Physolychnis* s. l. These taxa are distant from each other on the phylogenetic trees.

**Figure 5.** Microclonal propagation of ancient *Silene* regenerants. Image: E.E. Fesenko 2019.

The results of molecular phylogenetic analyses of nrDNA ITS and cpDNA *rps*16 sequences clearly demonstrate that the plants of the ancient and recent *Silene* somaclones SS1 and SS2 belong to *Silene linnaeana* group. Thus, the present study confirms the point of view by Oxelman et al. (2012) on the taxonomic identification of the ancient *Silene* regenerants from fossil material as *S. linnaeana* s. l.

Taxonomic identification of the ancient and recent *Silene* regenerants obtained by molecular methods was confirmed by morphological analysis of the plants. Obviously, determining the *in vitro* cultivated plants solely based on their morphology may be complicated by unnatural morphogenesis resulting in development of atypical morphologies of such plants (Ziv 1991). Even when transplanted to soil, these plants can develop somehow atypically. Cultivated in the soil, *Silene* regenerants from somaclones SS1 and SS2 were depressed in growth: they regularly failed to form high shoots and bloom annually. When blooming, they usually formed a very reduced inflorescence, which was often composed of one terminal flower. In this case the difference between thyrsoid inflorescence and a compound dichasium is impossible to detect. The atypical growth pattern of *Silene* plants *in vitro* may be one of the reasons, why they were confused with *S. stenophylla* and misidentified. Another reason for the erroneous identification is a very wide morphological variability of *S. linnaeana* group growing in natural conditions, inflicted by very wide distribution range of this species. The distribution area of *S. linnaeana* group covers Siberia, the Far East and Mongolia (Yurtsev 1971; Zuev 1993), and the morphological features of individuals occurring in the Arctic zone may significantly differ from those growing in the south. The misidentification of the ancient seeds of *Silene* by Başlı et al. (2009) and Gyulai et al. (2011) is probably related to a very narrow set of species incorporated for comparisons, which did not include *S. linnaeana*.

*Silene linnaeana* s. str. as well as other related species (*S. ajanensis*, *S. samojedorum*, *S. villosula* and a more distantly related *S. uralensis*) are usually diploids (2n = 2x). Using low copy nuclear genes

RPD2a and RPD2b along with the plastid data, Petri & Oxelman (2011) demonstrated that some of them take part in the formation of polyploid *Silene* species. The extent of polyploidy in the genus *Silene* and, in particular, in the section *Physolychnis*, is still poorly understood.

The results of the molecular phylogenetic analyses obtained in the present study convincingly show that individual species within *S. linnaeana* group can hardly be distinguished from each other using the nuclear and plastid markers, nrDNA ITS and cpDNA *rps*16 sequences, which is consistent with previously obtained data (Popp et al. 2005; Popp & Oxelman 2007; Petri & Oxelman 2011). Low sequence variability, reticulate evolution and polyploidy may be possible reasons for the observed phylogenetic pattern. Though these criteria do not allow us to attribute the experimental somaclones of *Silene* to a species within the group, their identification as belonging to the *S. linnaeana* group seems to be highly reliable. Moreover, the data of the DNA markers speaks in favour of this hypothesis. In addition, studying low copy genes, such as the second largest subunits of RNA polymerase gene family (Popp & Oxelman 2004), tends to be promising for enhancing identification of the ancient *Silene* material.

## Conclusion

In this work we determined the taxonomic affiliation of the clones regenerated *in vitro* from the fossil fruits and fruits of extant *Silene* through molecular phylogenetic analyses. The obtained results clearly demonstrate that the ancient *Silene* plants belong to *Silene linnaeana* group from the section *Physolychnis*. This study corroborates the point of view of Oxelman et al. (2012) on the taxonomic identification of the ancient *Silene* clone.

Our results imply a huge role of permafrost as a natural depository of the ancient gene pool. The recovery of ancient plants from permafrost can provide us with exceptional material to compare the ancient and modern species. Data from fossil plants in the Arctic make it possible to better understand the evolutionary processes taking place in the recent past at both molecular and morphological levels.

## Acknowledgements

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Addresses of the authors:

Tatiana E. Kramina (corresponding author) * https://orcid.org/0000-0002-6417-3678
Larissa A. Koppel  https://orcid.org/0000-0002-8579-6436
Biological Faculty, Lomonosov Moscow State University
Leniskie Gory 1, building 12
119234 Moscow, Russia
E-mail: tkramina@yandex.ru *

Elizaveta P. Iakovenko
Timiryazev Institute of Plant Physiology, Russian Academy of Sciences
Botanicheskaya 35
127276 Moscow, Russia

Tahir H. Samigullin
A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University
GSP-1, Leninskie Gory
119991 Moscow, Russia

Yulia A. Karpova
Edith N. Gakhova
Eugeny E. Fesenko (Jr.)
Institute of Cell Biophysics
Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences" (PSCBR RAS)
Institutskaya 3
142290 Pushchino, Moscow Region, Russia

**Appendix 1.** Taxa, sample codes, localities, voucher information and GenBank accession numbers of newly sequenced samples.

| Taxon | Code | Geographic location | Voucher | ITS1-2 | rps16 intron |
|---|---|---|---|---|---|
| ancient *Silene* | SS1 | Yakutia, lower Kolyma river, Duvanny Yar | *In vitro* culture grown from tissue of fossil fruit (ICB RAS) | MT733348 | MT742215 |
| recent *Silene* | SS2 | Yakutia, lower Kolyma river, Duvanny Yar | *In vitro* culture grown from a seed of an extant plant (ICB RAS) | MT733349 | MT742216 |
| *S. ajanensis* (Regel & Tiling) Vorosch. | SAJ1 | Khabarov Krai, Ayan | S. Kharkevich and T. Bush s.n., 08.08.1977 [MW0065811] | MT733126 | MT742186 |
| | SAJ2 | Magadan Oblast, Koni peninsula, Cape Taran | N.S. Pavlova s.n., 13.07.1989 [MW0065808] | MT733127 | MT742187 |
| | 11 | Magadan Oblast, Omsukchansky distr., S slope of Mount Ircha, left bank of Left Mongke stream | V. Kuvaev 157-1, 27.07.1980 [MW0065807] | MT733124 | MT742184 |
| | 12 | Magadan Oblast, Olsky distr., S coast of the Pyagin Peninsula | Antropova and Tuzov 2491, 17.08.1978 [MW0065802] | MT733125 | MT742185 |
| *S. involucrata* (Cham. & Schltdl.) Bocquet subsp. *tenella* (Tolm.) Bocquet | 1 | Yakutia, Nizhnekolymsky distr., near Chersky | S.V. Maksimovich 173, 11.08.2002 [MW] | MT733132 | MT742189 |
| | 2 | Komi, Vorkuta | S.V. Maksimovich 209, 10.07.2004 [MW0947980] | MT733149 | MT742210 |
| *S. jeniseensis* Willd. | 3 | Amurskaya Oblast, northern outskirts of Skovorodino | S.V. Maksimovich 240, 18.08.2006 [MW] | MT733128 | MT742188 |
| | LSIB1 | Yakutia, Oimyakon upland, Tomtor village | V. Ivanova s.n., 20.08.1958 [MW0065727] | MT733137 | MT742197 |
| | LSIB2 | Buryatia, Khamar-Daban, left bank of Temnik river | N. Gamova s.n., 30.06.2013 [MW0156383] | MT733138 | MT742198 |
| | LSIB3 | Yakutskaya ASSR, Zhigansky distr., Serki river | Fokina s.n., 21.07.1962 [MW0065626] | — | MT742199 |
| | LSIB4 | Amurskaya Oblast, Zeya distr., Sosnovy Bor village | M.S. Ignatov s.n., 02.06.1978 [MW0065618] | MT733139 | MT742200 |
| | 5 | Irkutsk Oblast, N of Ust'-Kut | S.V. Maksimovich 274, 26.06.2006 [MW0947957] | MT733129 | MT742194 |
| | 7 | Yakutia, Nizhnekolymsky distr., near Chersky, by Panteleikhiskaya stream | S.V. Maksimovich 27, 11.08.1989 [MW0947958-2] | MT733130 | MT742195 |
| *S. linnaeana* Vorosch. | 8 | Yakutia, Nizhnekolymsky distr., Chersky, The Station of The Pacific Geographical Institute of the Far Eastern branch of RAS | S.P. Davydov s.n., 23.06.2012 [MW0954587] | MT733131 | MT742196 |
| | 13 | Buryatia, Ivolginsky distr., 2 km E of Orongoy | S.G. Kazanovsky 301, 28.08.2003 [MW0160707] | MT733133 | MT742190 |
| | 14 | Krasnoyarsk Krai, Baykitsky (Evenkiysky) distr. | S.S. Shcherbina 92-27, 23.06.1991 [MW0065694] | MT733134 | MT742191 |
| | 16 | Magadan Oblast, Srednekansky distr., Zamkovoe | O. Khokhryakova, G. Antropova s.n., 22.06.1980 [MW0065613] | MT733135 | MT742192 |
| | 17 | Krasnoyarsk Krai, NW outskirts of Krasnoyarsk, right bank of Yenisei River | M.E. Ploshchinskaya s.n., 17.08.1992 [MW0065693] | MT733136 | MT742193 |

Appendix 1. Cont.

| Taxon | Code | Geographic location | Voucher | ITS1–2 | *rps16* intron |
|---|---|---|---|---|---|
| *S. samojedorum* (Sambuk) B. Oxelman | LSAM1 | Taimyr National Okrug, Agana river | E. Pavlova s.n., 12.07.1967 [MW0065702] | MT733140 | MT742201 |
| | LSAM2 | East Taimyr, Bolshaya Baty-Sala river | E. Pospelova s.n., 01.07.2012 [MW0157382] | MT733141 | MT742202 |
| | SST1 | Khabarov Krai, Verkhnebureinsky distr., mnt. Dusse-Alin' | V.A. Shtrik, K.V. Bakumenko 92–27, 28.07.1992 [MW0065923] | MT733142 | MT742203 |
| *S. stenophylla* Ledeb. | SST2 | Magadan Prov., Olsky distr., Atargan village | Ikonnikova, Tuzov s.n., 28.07.1978 [MW0065896] | MT733143 | MT742204 |
| | SST3 | Chukotka, Meinypilgyno village | S.A. Balandin s.n., 20.07.1985 [MW0162265] | MT733144 | MT742205 |
| | SST4 | Magadan Prov., Upper Olu river | I.M. Krasnoborov s.n., 23.07.1988 [MW0065889] | MT733145 | MT742206 |
| *S. uralensis* (Rupr.) Bocquet subsp. *porsildii* Bocquet | 24 | Chukotka, the upper river Palyavaam | S.A. Balandin et al. s.n., 14.07.1973 [MW0065244] | MT733146 | MT742207 |
| | 18 | Chukotka Autonomous Okrug, W Chukotka, Chaun distr., Ayon village | V.R. Filin s.n., 21.06.1958 [MW0065601] | MT733147 | MT742208 |
| | 19 | Krasnoyarsk Krai, Diksonsky (Taimyrsky) distr., Central Taimyr, the valley of Verkhnyaya Taimyra river | I.N.Pospelov 95–745, 14.07.1995 [MW0065609] | MT733148 | MT742209 |
| *S. villosula* (Trautv.) V.V. Petrovsky & Elven | 20 | Krasnoyarsk Krai, Taimyr Autonomous Okrug, Dudinsky district, the middle course of the Pyasina river | E. Pospelova s.n., 08.08.1970 [MW0065608] | MT733150 | MT742211 |
| | 21 | Wrangel Island, the floodplain of the Gusinaya River, 14km from the estuary | V.A. Shtrik s.n., 16.07.1991 [MW0065602] | MT733151 | MT742212 |
| | 22 | Krasnoyarsk Krai, SE Taimyr, the outskirts of the Anabar Plateau, the confluence region of the rr. Eriechka and Nyamakit-Daldyn | I.N. Pospelov 13-0621, 15.07.2013 [MW0158420] | MT733152 | MT742213 |
| *S. violascens* (Tolm.) V.V. Petrovsky & Elven | 23 | Krasnoyarsk Krai, Putorana Plateau, east tip of the lake Lama | E.B. Pospelova 16-0415, 02.08.2016 [MW0162268] | MT733153 | MT742214 |

**Appendix 2.** Infrageneric classification of *Silene* (according to Jafari et al. 2020), taxonomic position of studied species and accession numbers of ITS and *rps*16 sequences of *Silene* and *Agrostemma* taken from GenBank. The abbreviations of infrageneric taxa names used in the trees are given in square brackets.

| Subgenus | Section | Species | ITS1-2 | *rps*16 |
|---|---|---|---|---|
| | | **Genus *Silene*** | | |
| *Behenantha* (**Orth**) Torr. & A. Gray [B] | *Coniomorpha* Orth [Conio] | *S. conica* L. | FN821100 | FN821269 |
| | *Elisanthe* (Fenzl ex Endl.)Ledeb. [Elis] | *S. noctiflora* L. | FN821141 | FN821309 |
| | *Behenantha* Orth [Behe] | *S. vulgaris* (Moench) Garcke ssp. *angustifolia* Hayek | FN821149 | FN821317 |
| | *Sedoides* Oxelman & Greuter [Sedo] | *S. aegaea* Oxelman | AJ299839 | AJ294972 |
| | *Physolychnis* (Benth.) Bocquet [Phys] | *S. argentina* (Pax) Bocquet | KX757356 | — |
| | | *S. mandonii* (Rohrb.) Bocquet | KX757371 | — |
| | | *S. lemmonii* S. Watson | — | DQ908827 |
| | | *S. polypetala* (Walter) Fernald & B.G.Schub. | AY116480 | DQ908840 |
| | | *S. involucrata* (Cham. & Schltdl.) Bocquet | AJ831786 | AJ831770 |
| | | *S. uralensis* (Rupr.) Bocquet ssp. *arctica* (Th. Fr.) Bocquet | AJ831781 | AJ831765 |
| | | *S. sorensensis* (B. Boivin) Bocquet | AJ831789 | AJ831773 |
| | | *S. linnaeana* Vorosch. | KX757383 | JF970812 |
| | | *S. samojedora* (Sambuk) B. Oxelman | KX757380 | JF970808 |
| | | *S. samojedorum* (Sambuk) B. Oxelman | KX757374 | JF970807 |
| | | *S. ajanensis* (Regel & Tiling) Vorosch. | KX757376 | JF970809 |
| | | *S. ajanensis* (Regel & Tiling) Vorosch. | AJ831780 | AJ831763 |
| | | *S. villosula* (Trautv.) V.V. Petrovsky & Elven | KX757382 | JF970810 |
| | | *S. villosula* (Trautv.) V.V. Petrovsky & Elven | KX757381 | JF970811 |
| | | *S. villosula* (Trautv.) V.V. Petrovsky & Elven | KX757377 | JF970814 |
| | | *S. davidii* (Franch.) Oxelman & Lidén | FN821093 | FN821271 |
| | | *S. nigrescens* (Edgew.) Majumdar | X86858 | AJ629915 |
| | | *S. sachalinensis* F.Schmidt | KX757394 | JF970813 |
| | | *S. salicifolia* C.L. Tang | KX757372 | JF970835 |
| | | *S. viscosa* (L.) Pers. | FN821148 | FN821316 |
| | *Cucubaloides* Edgeworth & Hook.f. [Cucub] | *S. tatarinowii* Regel | FJ384025 | FJ383984 |
| | *Odontopetalae* Chowdhuri [Odon] | *S. odontopetala* Fenzl | KX757294 | — |

Appendix 2. Cont.

| Subgenus | Section | Species | ITS1-2 | rps16 |
|---|---|---|---|---|
| *Behenantha* (cont.) | *Cordifoliae* Chowdhuri [Cord] | *S. cordifolia* All. | KX757314 | KU608446 |
| | *Dichotomae* (Rohrb.) Chowdhuri[ Dich] | *S. dichotoma* Ehrh. | KX757313 | Z83174 |
| | *Melandrium* (Röhl.) Rabeler [Mela] | *S. dioica* (L.) Clairv. | FN821107 | FN821277 |
| | | *S. latifolia* Poir. | FN821123 | FN821290 |
| | *Erectorefractae* Chowdhuri [Erec] | *S. almolae* J. Gay ex Coss. | KX757424 | — |
| | *Psammophilae* (Talavera) Greuter [Psam] | *S. littorea* Brot. | FN821094 | Z83185 |
| | *Acutifoliae* Oxelman & F. Jafari [Acut] | *S. acutifolia* Link ex Rohrb. | KX757318 | — |
| | *Cryptoneurae* Aydin & Oxelman [Cryp] | *S. cryptoneura* Stapf | KX757323 | Z83187 |
| | *Cucubalus* (L.) Greuter [Cucu] | *S. baccifera* (L.) Roth | JF978556 | Z83169 |
| *Silene* [S] | *Auriculatae* (Boiss.) Schischk. [Auric] | *S. cappadocica* Boiss. & Heldr. | KX757457 | EF061383 |
| | | *S. pygmaea* Adams | FN821143 | FN821311 |
| | | *S. schafta* Hohen. | AJ831792 | Z83194 |
| | *Silene* [Sil] | *S. aethiopica* Burm.f. | KX757264 | — |
| | | *S. ciliata* Pourr. | KX757268 | KM273199 |
| | | *S. disticha* Willd. | KX757260 | — |
| | | *S. bergiana* Lindm. | X86835 | — |
| | | *S. succulenta* Forssk. | KX757257 | — |
| | | *S. pseudoatocion* Desf. | X86838 | EU314656 |
| | | *S. corrugata* Ball | KX757259 | — |
| | | *S. imbricata* Desf. | KX757266 | — |
| | *Sclerocalycinae* (Boiss.) Schischk. [Scler] | *S. dianthoides* Pers. | KX757626 | — |
| | | *S. marschallii* C.A. Mey. | EF060228 | EF061387 |
| | | *S. longipetala* Vent. | KX757616 | EF061392 |
| | *Siphonomorpha* Otth [Siph] | *S. acaulis* (L.) L. | KX757548 | FJ404946 |
| | | *S. nivalis* (Kit.) Rohrb. | KX757557 | Z83190 |
| | | *S. otites* (L.) Sm. | EF060233 | EF061393 |
| | | *S. multicaulis* Guss. | KX852628 | KX852511 |
| | | *S. orphanidis* Boiss. | KX852605 | KX852490 |

Appendix 2. Cont.

| Subgenus | Section | Species | ITS1-2 | rps16 |
|---|---|---|---|---|
| *Silene* (cont.) | *Siphonomorpha* (cont.) | *S. italica* (L.) Pers. | KX449846 | KF305909 |
| | | *S. andryalifolia* Pomel | KX449991 | AF242316 |
| | | *S. paradoxa* L. | KX757509 | EF674190 |
| | | *S. cephallenia* Heldr. | KX757512 | KY045503 |
| | | *S. congesta* Sm. | KY045499 | KY045505 |
| | | *S. esquamata* W.W. Sm. | KX757528 | — |
| | | *S. fissipetala* Turcz. | KX757529 | — |
| | | *S. flavescens* Waldst. & Kit. | KP849847 | KP849836 |
| | | *S. macrostyla* Maxim. | KX757535 | EF061386 |
| | | *S. tatarica* (L.) Pers. | KP849855 | KP849834 |
| | | *S. nutans* L. | DQ059410 | EF061361 |
| | | *S. pogonocalyx* (Svent.) Bramwell | KX757502 | — |
| | | *S. nocteolens* Webb & Berthel. | KX757498 | — |
| | | *S. chamarensis* Turcz. | KF267890 | KF305905 |
| | | *S. jenisseensis* Willd. | KX757542 | KF305912 |
| | | *S. pauciflora* Ledeb. | FJ384023 | FJ383982 |
| | | *S. stenophylla* Ledeb. | KX757536 | KF305907 |
| | | *S. stenophylla* Ledeb. | X86872 | — |
| *Lychnis* (L.) Greuter [L] | *Lychnis* [Lych] | *S. chalcedonica* (L.) E.H.L. Krause | KF274509 | Z83164 |
| | *Coccyganthe* (Rchb.) Greuter [Cocc] | *S. flos-cuculi* (L.) Clairv. | KJ918497 | Z83163 |
| *Incertae sedis* | *Atocion* Orth [Atoc] | *S. atocioides* Boiss. | KX757418 | EU314655 |
| | | **Genus *Agrostemma* L.** | | |
| | | *A. githago* L. | X86895 | Z83154 |