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Genetic variation in *Goniolimon speciosum* (Plumbaginaceae) reveals a complex history of steppe vegetation

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We hypothesized that the spatial pattern of genetic variation in different ecological groups of steppe plant species is not similar. To test this hypothesis, we studied genetic variation of the typical steppe plant *Goniolimon speciosum* (Plumbaginaceae), which is spread across the whole of the Asian steppe, and compared it with the published data on genetic variation of several mountain-steppe species. To elucidate the phylogenetic position of and genetic structure in *G. speciosum*, we sequenced the nuclear ribosomal internal transcribed spacer (ITS) and plastid *trnH-psbA* and *trnQ-rps16* regions. *Goniolimon speciosum* was shown to have originated in Central Asia in the Pliocene. We revealed two genetic groups in *G. speciosum*: a south-eastern (Dahuria, north-eastern Mongolia, southern shore of the lake Baikal and eastern Tyva) group and a broadly distributed western group. This split of evolutionary lineages is estimated to have occurred in the mid-Pliocene. Some samples from the central part of the species area (riverheads of Yenisei and Ob) formed an intermediate genetic group, where most plants had western ITS ribotypes and south-eastern plastid haplotypes. This polyphyletic group could have originated due to multiple secondary contacts and subsequent hybridization events. The geographically structured genetic subdivision of the western lineage, based on the ITS data, indicates multiple northward colonizations of *G. speciosum* from Central Asia that occurred, according to our estimates, in the early Pleistocene. Thus, the history of steppe vegetation is more complicated than has been previously suggested, basing upon limited taxonomic sampling of steppe plants. Species with different ecological preferences have a different history.

ADDITIONAL KEYWORDS: divergence time estimates – Eurasian steppe formation – historical biogeography – phylogeny.

INTRODUCTION

Traditionally, the history of the Eurasian steppe belt has been deduced only by florogenetic methods (comparative analysis of modern distribution of different plant taxa: Popov, 1963). Another well-established method of reconstructing ancient vegetation, the analysis of palaeontological data, is an inadequate approach to apply to steppe biome as xerophytes usually leave no fossils (Peshkova, 2001). The Eurasian steppe belt began to form in Central Asia in the early Miocene (Velichko, 1999), and florogenetic reconstructions usually attribute the origin of steppe species to this region (reviewed by Peshkova, 2001). An alternative theory is that the Siberian steppe flora developed autochthonously, arising in the mountains of north-eastern Siberia and subsequently migrated to its current position (Peshkova, 2001). It is probable that these two florogenetic scenarios do not contradict each other, as steppe vegetation is composed of species with different ecological preferences, which probably have different histories. Peshkova (2001) distinguished typical steppe species (originating in the Pliocene in Central Asia), mountain-steppe species (appearing in north-eastern Eurasia in the Miocene–Pliocene), meadow-steppe species (evolving in Altai in the Miocene–Pliocene) and desert-steppe species (arising in the early Palaeogene before the separation of Africa, America and Australia).

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The advent of molecular methods facilitated further testing of all these florogenetic hypotheses by the use of the phylogeographic approach. For example, it has been shown recently that dated phylogenetic trees for two steppe genera of Brassicaceae mirror Eurasian steppe dynamics (Friesen *et al.*, 2016). Nowadays, as far as we know, the only steppe plant species with a wide Eurasian distribution for which genetic diversity has been investigated over its entire distribution is the mountain-steppe species *Clausia aprica* (Stephan) Korn.-Trotzky (Franzke *et al.*, 2004). This species appeared to consist of three main genetic groups (one distributed to the west of the river Ob, another distributed to the east; and genetically intermediate populations representing the third group which were situated between populations of the two first groups). This genetic split was explained by existence of unsuitable habitats for steppe plants in the Ob basin in the Pleistocene, mainly due to rivers dammed by glaciers (Franzke *et al.*, 2004). The same genetic split was demonstrated in *Capsella* Medik., which is predominately confined to steppe-like habitats (Hurka *et al.*, 2012), and between two close species of onions, assigned earlier to mountain-steppe *Allium globosum* M.Bieb. ex DC.: western *A. cretaceum* N.Friesen & Seregin and eastern *A. montanostepposum* N.Friesen & Seregin (Seregin, Anackov & Friesen, 2015).

We hypothesized that the spatial pattern of genetic variation in other ecological groups of steppe plant species differs from previously investigated mountain-steppe plants, as the former could be characterized by a different history. To test this hypothesis we studied nuclear (ITS) and plastid (*trnH-psbA* and *trnQ-rps16*) genetic variation of a typical steppe plant (according to Peshkova, 2001) *Goniolimon speciosum* (L.) Boiss. (Plumbaginaceae), which is distributed across the whole Asian steppe (Fig. 1). The only divergence time estimation for the genus available is that of Lledó *et al.* (2005) which was based on a single calibration point (the age of La Gomera, Canary Islands) and included only two representatives of *Goniolimon*. The use of geographical events for calibrating molecular clocks,

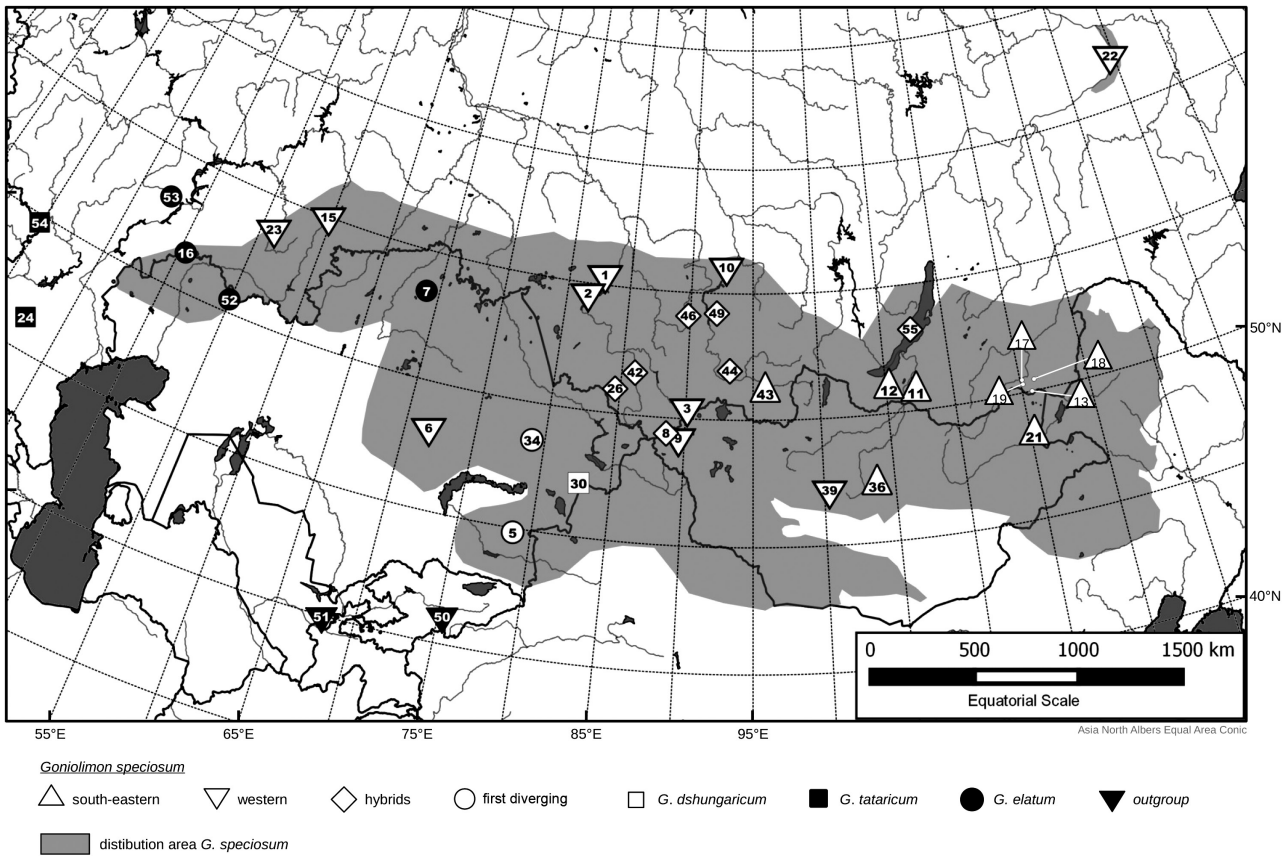

Figure 1. Distributional range of *Goniolimon speciosum* and sampling sites of *Goniolimon* spp. and outgroup *Acantholimon* spp. We included only accessions for which we managed to amplify all the three DNA regions. Symbols indicate species and phylogeographic subgroups (cf. Fig. 3). Overall distribution of *G. speciosum* is given according to Steinberg (1986), Gamayunova (1964), Gubanov (1996), Pen & Kamelin (1996) and Kovtonyuk (2006); some isolated north-eastern populations on the rivers Olenek and Yana are not shown.

such as the formation of an island, may lead to errors, for example the species could be older than the island they occupy (Renner, 2005). We conducted a divergence time estimation relying on published mean ITS substitution rates by Kay, Whittall & Hodges (2006) for the same reasons as Friesen *et al.* (2016).

MATERIAL AND METHODS

DNA extraction, amplification and sequencing

Total genomic DNA of *G. speciosum* was isolated from 37 herbarium and silica gel-dried specimens, representing the entire distribution (Fig. 1, Appendix 1), using the cetyl trimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987). We also included two closely related species [*G. tataricum* (L.) Boiss. (two accessions) and *G. elatum* (Fisch. ex Spreng.) Boiss. (four accessions)] and the taxonomically problematic *G. dshungaricum* (Regel) O.Fedtsch. & B.Fedtsch, which inhabits Central Asian mountain ridges on the border between Kazakhstan and China and which could be a race of *G. speciosum* (Steinberg, 1986). All voucher specimens are stored in MW or MHA (Moscow, Russia).

The complete ITS region (ITS1, 5.8S and ITS2) was amplified using the primers NNC-18S10 and C26A (Wen & Zimmer, 1996). We also amplified two plastid DNA intergenic spacers, *trnH-psbA* (Shaw *et al.*, 2005) and *trnQ-rps16* (Shaw *et al.*, 2007). Polymerase chain reactions (PCRs) were conducted in 20 μL reaction volumes containing 4 μL Ready-to-Use PCR MaGMix (200 μM each dNTP, 1.5 mM $MgCl_2$, 1.5 U SmarTaqDNA polymerase and reaction buffer; Dialat Ltd., Moscow, Russia), 15 μL deionized water, 3.4 pmol each primer and 1 μL template DNA of unknown concentration. PCR was performed in a MJ Research PTC-220 DNA Engine Dyad Thermal Cycler (BioRad Laboratories, USA) with the following parameters: initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C, ending with 10 min extension at 72 °C. Double-stranded PCR products were checked on agarose gels, purified using centrifugation with a solution of ammonium acetate in ethanol and sent to Syntol Company (Moscow, www.syntol.ru) for sequencing. Forward and reverse sequences from each sample were manually edited in SEQUENCHER 4.1.4 (Gene Codes Corporation) and combined in a single consensus sequence. DNA sequences were aligned using BioEdit 7.0.5.3 (Hall, 1999). The sequences of the two plastid intergenic spacers were combined into a single data set. The plastid DNA and ITS sequences were deposited to European Nucleotide Archive (Appendix 1).

Phylogenetic analyses

An heuristic search with the tree-bisection-reconnection algorithm was conducted for the ITS, *trnH-psbA* and *trnQ-rps16* sequences in PAUP*4.0b10 (Swofford, 2002). For Bayesian inferences, two runs with four Markov chains were run for two million generations in MRBAYES 3.2.1 (Ronquist & Huelsenbeck, 2003). We first selected the best-fitting sequence evolution models using the Akaike information criterion (AIC) in MODELTEST 3.7 (Posada & Crandall, 1998). Calculation of the bootstrap values was conducted with 100 replicates in PAUP* and the trees were visualized using FIGTREE 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). We calculated the user-specified starting tree for the divergence time estimation with 20 ITS sequences in the same manner. All the phylogenetic data were deposited to TreeBASE (study accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S19104).

Divergence time estimates based on ITS data

BEAST 1.8 was used to estimate the divergence times in *Goniolimon* (Drummond & Rambaut, 2007). The BEAUTI 1.8 interface was used to create input files for BEAST and, where necessary, the XML files were manually adjusted. For the estimation, a reduced subset of 11 taxa was selected from ITS sequences. We used the uncorrelated log-normal relaxed clock (ucld) and the Yule process was chosen as the speciation process. With the substitution models selected by the AIC we encountered problems with the convergence of the Markov chain, and we therefore reduced model parameters. The model with the best performance was HKY + G with six gamma rate categories. The ucld.mean for the ITS data was set to a normal distribution, with a mean of 4.13×10^{-9} substitutions per site per year and a standard deviation of 1.0×10^{-10} according to the works of Kay *et al.* (2006). The user-specified starting tree was inserted in the XML files in the newick format. Other parameters were set as the default. Several short BEAST runs were first performed to examine the performance of the Markov chain Monte Carlo (MCMC). The ucld.stdev did not abut zero and we reject a strict clock. Additional runs with empty alignments were carried out to ensure that the priors alone were not determining the results. Finally, three BEAST runs were performed for every substitution rate setting with the MCMC chain length of 10^8 generations and a sample frequency of every 100. The ESS was >200 with a 25% burn-in for all parameters as confirmed by analysing the output file with TRACER. The tree output files from the BEAST were summarized with LOGCOMBINER 1.8 and annotated with the

program TREEANNOTATOR 1.8 and the burn-in was set to 25% with the aid of TRACER. The mean node heights option was selected and the posterior probability set to 0.5. The trees were visualized using FIGTREE 1.4.0 with means and 95% highest posterior densities (HPDs) age estimates.

DISTRIBUTION MAP

The distribution map was constructed in QGIS-2.8.1-Wien (QGIS Development Team, 2009) with an Asia North Albers equal area conical projection. We used free vector and raster map data from Natural Earth (www.naturalearthdata.com). The distribution area was compiled from the *Flora of U.S.S.R.* (Steinberg, 1986), the *Flora of Siberia* (Kovtonyuk, 2006), the *Flora of Kazakhstan* (Gamayunova, 1964), the *Conspectus of flora of outer Mongolia* (Gubanov, 1996), the *Flora of China* (Pen & Kamelin, 1996) and our own field observations.

RESULTS

Acantholimon tianschanicum Czerniak and *A. velutinum* Czerniak, as representatives of the sister group to the *Goniolimon* clade (according to Lledó *et al.*, 2005), were used as the outgroup. The summary of the statistics for the phylogenetic framework is presented in Table 1.

Phylogenetic trees based on ITS and plastid sequences demonstrated some incongruence (Figs 2, 3). Due to this incongruence, we included in the final analyses only those accessions for which we managed to amplify all the three DNA regions.

According to the ITS data, all three *Goniolimon* spp. [*G. speciosum s.l.* (including *G. dshungaricum*), *G. elatum*, *G. tataricum*] formed well-supported clades, of which *G. elatum* diverged first. In the resolved *G. speciosum s.l.* clade, *G. dshungaricum* diverged first. In the *G. speciosum s.s.* clade (excluding *G. dshungaricum*), the two samples from eastern Kazakhstan (5 and 34) were first diverging. The remaining accessions represented two genetic groups, one of which was restricted to the south-eastern part of the species area (Figs 1, 2A).

According to the plastid DNA data, all *Goniolimon* samples, after the branching of *G. tataricum*, formed two genetic groups, one of which consisted of the three subclades: eastern *G. elatum* samples, *G. dshungaricum* and south-eastern accessions of *G. speciosum*. This south-eastern group spread further to the west than that was observed in the ITS analyses. The second genetic group included the rest of the *G. speciosum* samples and the most western *G. elatum* specimen (Figs 1, 2B).

Divergence time estimates are shown in Fig. 4. Estimated mean ages and 95% HPDs are presented in Appendix 2. Main lineages in *G. speciosum* evolved in the mid-Pliocene. Further branching in these lineages occurred during the late Pliocene–Pleistocene.

DISCUSSION

Incongruence between phylogenetic tree topologies, based on maternally inherited plastid DNA and biparentally inherited nuclear sequences, can provide evidence for hybridization between different evolutionary lineages (Rieseberg & Soltis, 1991). We also should not exclude the possibility of incomplete lineage sorting (Schaal *et al.*, 1998), but hybridization is likely to play a significant role in our case, as it is usual when *G. speciosum* and *G. elatum* grow in sympatry

Table 1. Summary of phylogenetic analysis of *Goniolimon* (including outgroup *Acantholimon* species) from Modeltest and maximum parsimony analysis of separate and combined datasets

	ITS	*trnH-psbA*	*trnQ-rps16*	Combined *trnH-psbA* + *trnQ-rps16*
Number of included accessions	37	45	43	37
Number of included characters	620	242	344	583
Number of constant characters	520	210	304	514
Number of variable characters	200	32	40	69
Number of potentially parsimony-informative sites	88	28	32	55
Number of most parsimonious trees	9	10 000 (maxtree limit set)	9	507
Number of steps (tree length)	165	39	46	87
CI	0.66	0.90	0.96	0.87
RI	0.80	0.88	0.97	0.95
Model selected by AIC	GTR + I + G	K81uf + G	K81uf + G	TIM + I

AIC, Akaike information criterion; CI, consistency index; RI, retention index.

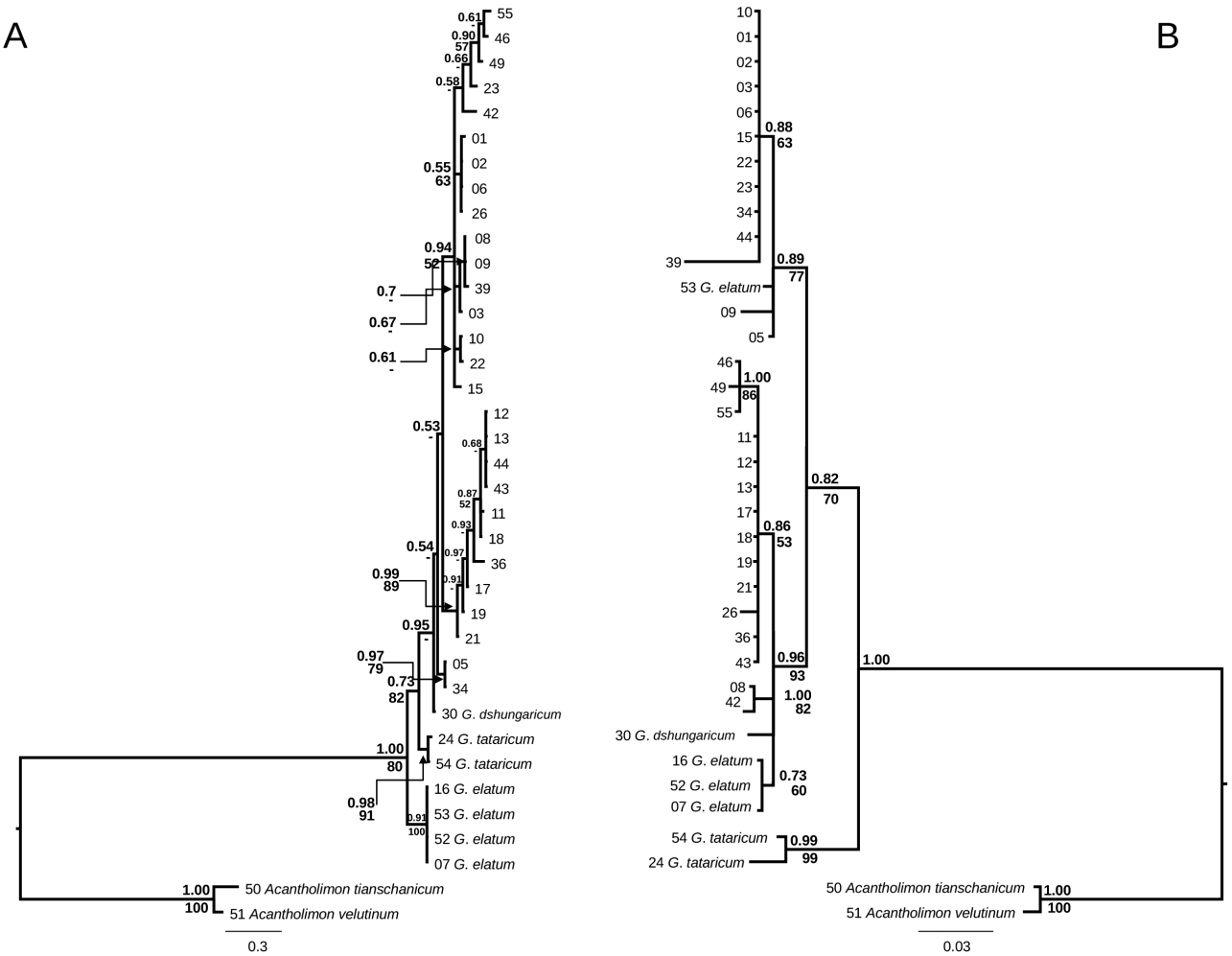

Figure 2. Phylogenetic trees resulting from Bayesian analyses of the (A) ITS sequences and (B) combined plastid DNA sequences of *Goniolimon*. Species from the related genus *Acantholimon* were used as outgroup taxa. Numbers by nodes represent Bayesian posterior probabilities above branches (>0.5) and bootstrap support below branches (>50%).

(Steinberg, 1986). It could have resulted in a polyphyly of *G. elatum* in the plastid DNA tree, where accessions of this species growing in sympatry and in allopatry with *G. speciosum* formed different genetic groups (Figs 1, 3). The same introgression between different lineages of *G. speciosum* could also take place.

The phylogenetic tree based on the ITS data reveals monophyly of the studied *Goniolimon* spp. and, thus, does not appear to be influenced by introgression. The first diverging monophyletic position of *G. dshungaricum* in the *G. speciosum s.l.* clade, according to the ITS data, suggests that this taxon could be treated as a subspecies of *G. speciosum*, or even as a separate ancestral species. However, more samples of *G. speciosum* from Central Asia and other accessions of *G. dshungaricum* should be analysed to enable a more definite decision. The early divergence of the two samples from eastern Kazakhstan in the *G. speciosum s.s.* clade serves as evidence for Central Asian origin of the species. This fits well into the history of subfamily Staticoideae, which has a Mediterranean and Irano-Turanian origin (Lledó *et al.*, 2001).

Our data clearly reveal two genetic groups in *G. speciosum*: a south-eastern group (Dahuria, north-eastern Mongolia, southern shore of the lake Baikal and eastern Tyva) and another broadly distributed western group (Figs 1, 3). This split of evolutionary lineages is estimated to have occurred in the mid-Pliocene *c.* 3.74 Mya (95% HPD: 1.07–8.36) (Fig. 4: node 6), when formation of the steppe zone had just finished (Velichko, 1999). We estimated the first branch in *Goniolimon* at an age of 6.69 Mya (95% HPD: 2.31–12.94) (Fig. 4: node 3), which matches the estimation of the group age, according to the calibrated plastid phylogenetic tree of Plumbaginaceae of Lledó *et al.* (2005), in which *Goniolimon* diversified *c.* 7 Mya. However, this genus might be older because none of the estimations has been based on complete taxon sampling of *Goniolimon*.

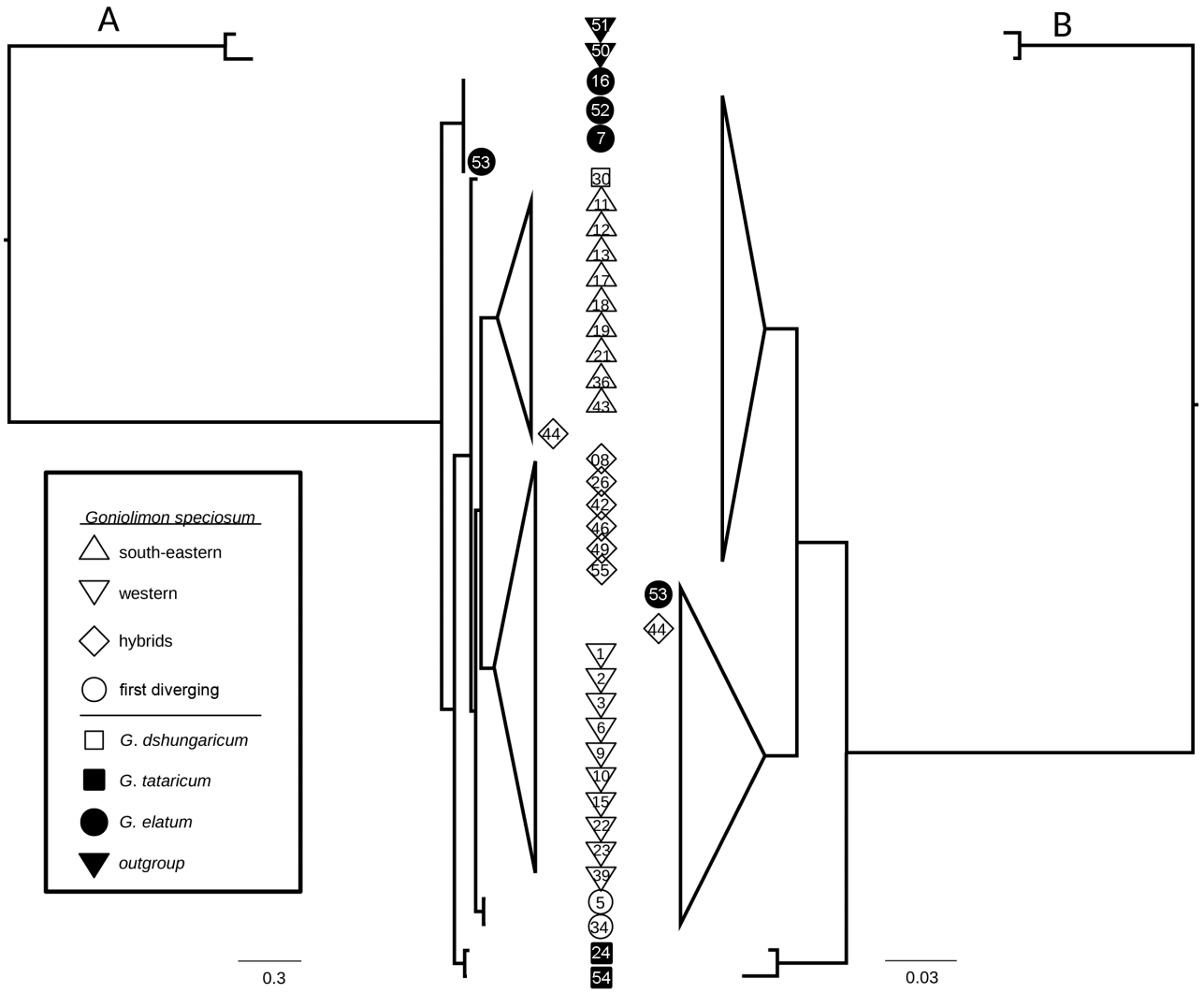

Figure 3. Collapsed phylogenetic trees resulting from a Bayesian analysis of the (A) ITS sequences and (B) combined plastid DNA sequences of the genus *Goniolimon*. Species from the related genus *Acantholimon* were used as outgroup taxa. Symbols indicate species and phylogeographic subgroups (cf. Fig. 1).

An inferred Central Asian origin of *G. speciosum* in the Pliocene fully supports the view on the history of typical steppe species that Peshkova (2001) formed, based on florogenetic data. Some samples from the central part of the species area (riverheads of Yenisei and Ob) formed an intermediate genetic group, in which most plants had western ITS ribotypes and south-eastern plastid haplotypes [the opposite situation was revealed for only one sample (44), Figs 1, 2]. During the Quarternary glaciations, the riverheads of Yenisei and Ob were periodically dammed with glaciers, subsequently turning the area into a system of lakes and swampy areas surrounded with taiga which was not suitable for the development of steppe vegetation (Arkhipov *et al.*, 1995). When the glaciers retreated and the steppe vegetation recovered, western and south-eastern lineages of *G. speciosum* came into secondary contact with each other and hybridized. Polyphyly of this intermediate group suggests multiple separation periods, followed by secondary contacts, which can be explained by repeated glaciation cycles and has been shown for *Clausia aprica* (Franzke *et al.*, 2004; Friesen *et al.*, 2016).

The steppe flora of Dahuro-Manchuria developed independently from the steppe flora of Buryatia since the Pliocene, when high mountain ridges (Yablonovy and adjacent ridges) rose and separated these steppe areas (Peshkova, 2010). Thus, the south-eastern lineage of *G. speciosum* could have spread from Central Asia to Buryatia and Dahuria in the first half of the Pliocene, before the steppe became separated by the ridges. A similar scenario based on florogenetic data was proposed for *Convolvulus chinensis* Ker Gawl. and *C. dahuricus* Herb. (Peshkova, 2010). Further northward

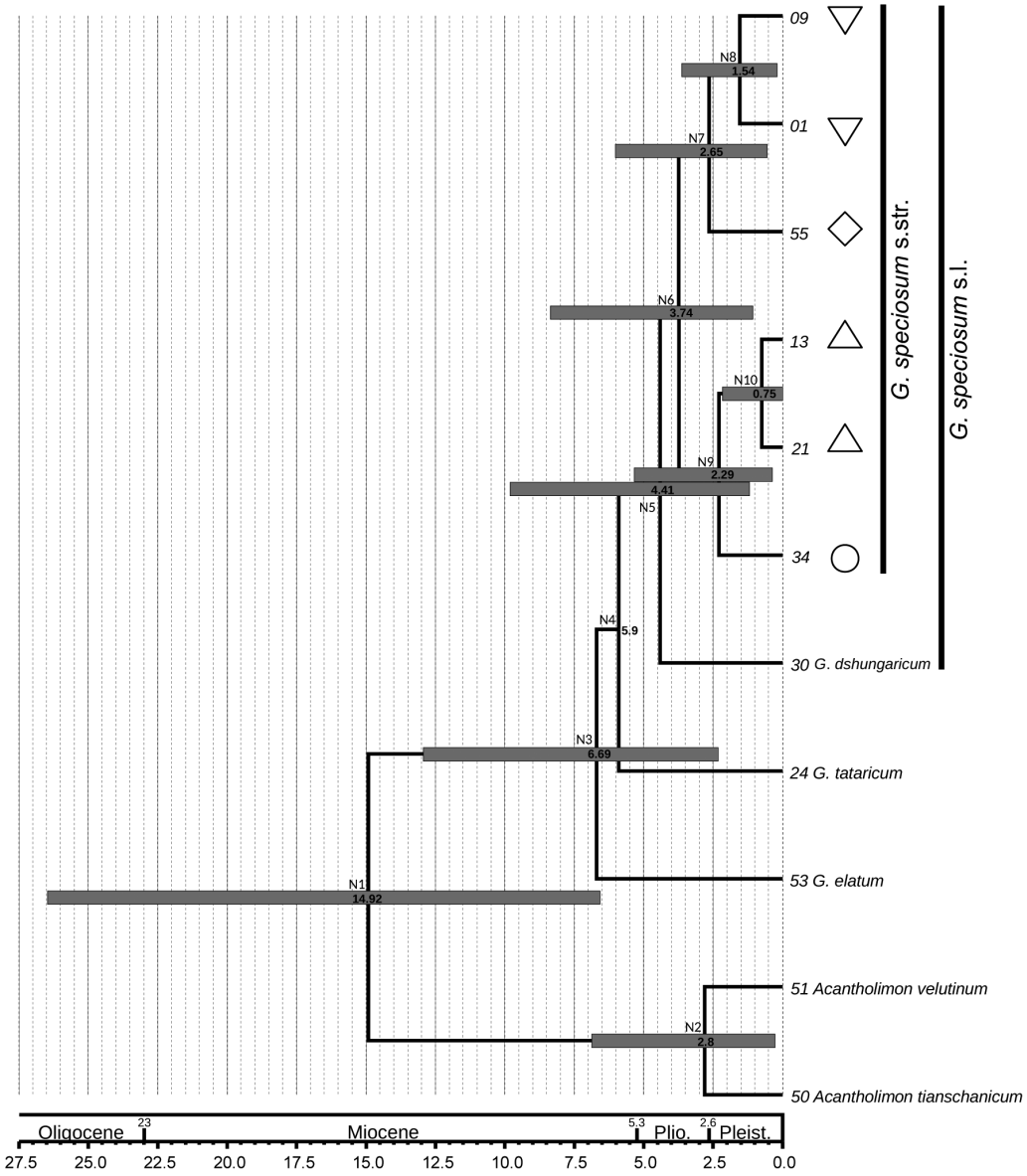

Figure 4. Divergence time estimates of the genus *Goniolimon* based on ITS sequences of 11 accessions. Maximum clade credibility tree for divergence times with a mean substitution rate according to Kay *et al.* (2006) is represented. The 95% highest posterior density (HPD > 50%) estimates for each node are represented by bars. Mean divergence time estimates are given along the bars. Node numbers (cf. Appendix 2) are given above the branches. Geological epochs according to the International Commission on Stratigraphy (ICS). Symbols indicate phylogeographic subgroups of *G. speciosum s.s.* (cf. Fig. 1).

spreading of the south-eastern lineage of *G. speciosum* was probably blocked by the Stanovoe upland, which served as an important border during the development of steppe vegetation (Peshkova, 2010).

Our data suggest close Quaternary connections of the steppe islands of central Yakutia with the zonal steppes of western Siberia and not with the steppes of Mongolia and Dahuria. Instead of their supposed origin due to northward migrations along the river Lena valley (Yurtsev, 1981), steppe plants in central Yakutia could be relicts of broadly distributed late Pleistocene steppe and tundra-steppe (Willerslev *et al.*, 2014; Friesen *et al.*, 2016 and references therein). *Goniolimon speciosum* from central Yakutia (accession 22) is genetically identical to plants from the Krasnoyarsk region (10), which supports the supposition of a young age (early Holocene) of disjunct distribution of steppe plants in northern Asia, as has previously been shown for *C. aprica* (Friesen *et al.*, 2016).

Geographically structured genetic subdivision of the western lineage, based on the ITS data, indicates multiple northward colonizations of *G. speciosum* from Central

Asia that occurred, according to our estimates, in the early Pleistocene (Fig. 4). One migration event gave rise to the plants from the above-mentioned Krasnoyarsk and Central Yakutia steppe islands. *Goniolimon speciosum* in the Khakassian steppe island (accessions 1 and 2) could have migrated there independently from Kazakhstan not long ago, as no genetic differences between plants from Khakassia and Kazakhstan (accession 6) have accumulated and the Ob basin was ice-dammed during the late Saalian glaciation (*c.* 150 ka: Svendsen *et al.*, 2004). One more sublineage of *G. speciosum* could have persisted in western Mongolia (accessions 3, 9 and 39) during the whole Pleistocene (Fig. 4). Plants from the southern Urals (15 and 23) probably evolved as a result of at least two independent migrations, one of which occurred in the early Pleistocene (accession 15). This can be explained by the long uninterrupted history of steppe vegetation in the southern Urals, which has persisted there since the end of the Pliocene (Kamelin, 1998; Velichko, 1999). The existence of such a long-term, relatively stable steppic area has resulted in a number of relicts of the Pleistocene periglacial steppe-like vegetation, many of them are endemic to the southern Urals (Gorchakovsky, 1969). The history of these species was revealed in detail by the phylogeographical approach by example of *Clausia agideliensis* Knjasev (Friesen *et al.*, 2016).

The geographical structure of genetic diversity of the only other steppe plant which has been investigated throughout its whole area (*C. aprica*; Franzke *et al.*, 2004; Friesen *et al.*, 2016) suggests another history of origin and migration. However, the authors described only the genetic subdivision of *C. aprica* along the river Ob (Franzke *et al.*, 2004) and deduced a Central Asian origin of the genus *Clausia* Trotzky (Friesen *et al.*, 2016), but refrained from reconstructing the history of *C. aprica*. The observed genetic diversity of the species does not contradict the scenario suggested by Kamelin (1973, cited by Peshkova, 2001) for another mountain-steppe species, *Amygdalus pedunculata* Pall. and other species from its section. They originated in the Miocene–Pliocene in north-eastern Eurasia and, when the climate cooled down, migrated southwards to their present area. This migration occurred via two routes (western and eastern), which were separated by the Western Siberian Sea (ice-dammed Ob and to a lesser extent the Yenisei rivers).

In conclusion, the history of steppe vegetation is more complicated than has been suggested (Friesen *et al.*, 2016), basing on limited taxonomic sampling of steppe plants. A different history of steppe species with varying ecological preferences was already suggested by Krasheninnikov (1937, cited by Kamelin, 1998), based upon florogenetic data. This view was further developed by Peshkova (2001). Phylogeographic studies of more steppe plants with different ecological preferences would help to verify florogenetic hypotheses and form a more complete knowledge of steppe history.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix 1. Geographical origin and European Nucleotide Archive accession numbers of the investigated samples of *Goniolimon* spp. and the outgroup *Acantholimon* spp.
Appendix 2. Estimated ages (million years) of the marked nodes in Figure 4. Posterior probabilities were calculated in Beast.