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Genetic and morphological conservatism of *Calophaca wolgarica* (Fabaceae), a paleoendemic species of the Southern East European Plain

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Keywords: dispersal, ISSR, morphology, population polymorphism, targeted sequencing.

Summary. *Calophaca wolgarica* is a relict paleoendemic species of the Southern East European Plain. In this study, we assessed the degree of morphological and genetic variation in its 24 natural and 2 reintroduced populations. Nonmetric multidimensional scaling (nMDS) on 17 traits was used to evaluate morphological variability. To assess genetic variation, we sequenced 8 chloroplast DNA regions (*atp*B – *rbc*L, *mat*K, *trn*L – *trn*F, *trn*S – *trn*G, *rbc*L, *rps*16, *rpo*C1, *ycf*1) and ITS1-5.8S-ITS2 nuclear DNA region and performed inter simple sequence repeat (ISSR) analysis as well. We found that there was no interpopulation morphological variation. Sequencing of chloroplast and nuclear DNA regions also showed no differences between populations of the species. Based on the results of Bayesian analysis using ISSR markers in STRUCTURE and NEW HYBRIDS programs, we identified genetic groups, the spatial location of which indicates that during the last glaciations and regressions of Caspian Sea, one ancestral population was preserved in a refugium on the Stavropol Upland. The refugium of another ancestral population covered the south of Volga Upland up to the Ergeni Upland. We argued that the species dispersed from the Tien Shan Mountains to the Volga Upland, bypassing the Caspian Sea from the north, and then spread to the Stavropol Upland. We suggest that the endemism of *C. wolgarica* in the absence of geographical barriers linked with low ecological heterogeneity of Southern East European Plain could arise not only, or not so much from the gene flow limitation, but from morphological and genetic conservatism associated with species adaptation to a narrow ecological niche. Over millions of years of the species' existence, its range has probably experienced multiple constrictions and expansions depending on changing climatic conditions associated with glacial and interglacial periods and dynamics of Caspian Sea level.

Генетический и морфологический консерватизм *Calophaca wolgarica* (Fabaceae) – палеоэндемика юга Восточно-Европейской равнины

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Аннотация. *Calophaca wolgarica* – реликтовый палеоэндемик юга Восточно-Европейской равнины. В данной работе мы оценили уровень морфологической и генетической изменчивости в 24 естественных и 2 реинтродуцированных популяциях вида. Для оценки морфологической изменчивости использовалось неметрическое многомерное шкалирование (nMDS) по 17 признакам. Для оценки генетической изменчивости мы секвенировали 8 участков хлоропластной ДНК (*atp*B – *rbc*L, *mat*K, *trn*L – *trn*F, *trn*S – *trn*G, *rbc*L, *rps*16, *rpo*C1, *ycf*1) и участок ядерной ДНК ITS1-5.8S-ITS2, а также провели анализ межмикросателлитных последовательностей (ISSR). Мы обнаружили отсутствие межпопуляционной морфологической изменчивости. Секвенирование участков хлоропластной и ядерной ДНК также не выявило различий между популяциями вида. На основе результатов байесовского анализа с использованием ISSR-маркеров в программах STRUCTURE и NEW HYBRIDS нами были выявлены генетические группы, пространственное расположение которых свидетельствует о том, что во время последних оледенений и регрессий Каспия одна прародительская популяция сохранялась в рефугиуме на Ставропольской возвышенности. Рефугиум другой прародительской популяции охватывал юг Приволжской возвышенности вплоть до возвышенности Ергени. Мы утверждаем, что вид распространился от гор Тянь-Шаня до Приволжской возвышенности, обходя Каспийское море с севера, а затем распространился на Ставропольскую возвышенность. Мы предполагаем, что эндемизм *C. wolgarica* при отсутствии географических барьеров, связанных с низкой экологической гетерогенностью юга Восточно-Европейской равнины, мог возникнуть не только или не столько из-за ограничения потока генов, сколько из-за морфологического и генетического консерватизма, связанного с адаптацией вида к узкой экологической нише. За миллионы лет существования вида его ареал, вероятно, неоднократно сужался и расширялся в зависимости от меняющихся климатических условий, связанных с ледниковыми и межледниковыми периодами, а также динамикой уровня Каспийского моря.

Introduction

It is known that one of the main environmental problems of our time is biodiversity conservation. Considering the vulnerability of ecosystems and its individual components, the only way out is constant monitoring of biodiversity (genetic, species, biocenotic) for systematic and purposeful organisation of its exploitation, protection and renewal, optimisation of the structure of all its main levels, development of measures to increase the number of populations of endangered species.

The genus *Calophaca* Fisch. includes 8 shrub species distributed mainly in Central Asia (Zhang et al., 2015). According to modern molecular genetics data, it is considered as a monophyletic branch within *Caragana* Lam. genus (Duan et al., 2016).

Calophaca wolgarica (L. f.) Pall. ex Fisch. is a palaeoendemic species of Eastern Europe, a Miocene relict with a disjunctive range and the single species of the genus native to European Russia (Zhang et al., 2015). The species is included in the Red Data Book of Russian Federation (Shmaraeva, Baranova, 2024). The main range of *C. wolgarica* covers the Rostov and Volgograd Regions, as well as Stavropol Territory and the Republic of Kalmykia. Occurrences in Orenburg, Samara, Saratov and Ulyanovsk Regions, the Republic of Dagestan and Crimea have not been confirmed by modern field studies. Reports from North-Western Kazakhstan in areas bordering Volgograd and Orenburg Regions have also not been confirmed by field studies (Efimenko et al., 2024). This indicates the catastrophic rate of species decline and the need for urgent conservation measures.

Studies of *C. wolgarica* have been focused mainly on certain local natural populations (Kashin et al., 2015; Belous, 2016; Shishlova et al., 2019) or on introduced populations (Shmaraeva et al., 2023).

C. wolgarica has been included in two recent molecular genetic studies, in Zhang et al. (2015) and Duan et al. (2016). The first of these studies was focused on phylogenetic relationships and biogeographic distribution of *Calophaca* and included all 8 *Calophaca* species and representatives of *Caragana* Fabr., *Halimodendron* Fisch. ex DC., *Hedysarum* L. and *Astragalus* L. genera as outgroups. The study of Duan et al. (2016) covered the subtribes *Caraganinae* and *Chesneyinae* in the tribe *Caraganeae* and supported the monophyly of *Calophaca*. Both studies proposed the inclusion of *Calophaca* in the genus *Caragana* as an independent monophyletic section. Intraspecific genetic polymorphism of *C. wolgarica* as well as the origin and dispersal of haplo- and ribotypes within its range have never been investigated before.

Genetic diversity is known to be the most widely used indicator for assessing the evolutionary potential of a species. Species with a restricted distribution are thought to be more susceptible to diversity loss (Octavio-Aguilar et al., 2024).

Therefore, the aim of this study was to identify biogeographic and phylogeographic patterns in distribution of polymorphism of *C. wolgarica* populations throughout its range.

Material and methods

Sampling

The field studies were carried out in 24 natural populations of *C. wolgarica* located in several administrative subjects of Russia: Volgograd and Rostov Regions, Stavropol Territory, and the Republic of Kalmykia (Table 1). Additionally, we studied two populations (Sr-1 and Sr-2) in the Saratov Region that were reintroduced in 2015 by using donor material from populations of the Volgograd Region.

In 19 out of 24 natural populations of *C. wolgarica*, 17 morphological parameters were measured in 23–30 randomly selected generative plants: plant height; plant diameter; number of first-order shoots; number of second-order shoots; first-order shoot length; second-order shoot length; internode length; stem diameter; number of leaves; leaf length; leaf width; number of leaflets; leaflet length; leaflet width; number of third-order shoots; length of third-order shoot; number of beans. In population from the Stavropol Territory (St-1), the morphological data was obtained only for 7 plants, as the rest of individuals were in pre-generative state.

Per one leaf from ten plants was dried in silica gel for further molecular analyses. Herbarium specimens were stored in the herbarium of the Botanical Garden of Saratov State University, Saratov, Russia (SARBG).

Morphological analysis

To reveal the main patterns of morphological variation in *C. wolgarica* populations, a nonmetric multidimensional scaling (nMDS) was used (Oksanen et al., 2020). In order to identify the fluctuations of morphological variation between years, the analysis was carried out on datasets, collected in 2013, 2014 and 2024.

Population genetic analysis

DNA extraction and sequencing

DNA was extracted from the leaves dried in silica gel using the diaGene Kit for DNA extraction from plant tissue on spin columns (Dia-M, Russia) according to the manufacturer's protocol.

To analyze the genetic variation, a total of 8 cpDNA markers (*atp*B – *rbc*L, *mat*K, *trn*L – *trn*F, *trn*S – *trn*G, *rbc*L, *rp*S16, *rpo*C1, *ycf*1) and ITS1-5.8S-ITS2 rDNA spacers were used. The choice of cpDNA markers was due to its previously detected high level of polymorphism in *Calophaca* species (Zhang et al., 2015; Duan et al., 2016) and the opportunity to compare the obtained *C. wolgarica* sequences with sequences of other *Calophaca* species from a part of its range inaccessible to us. Sequencing was performed on an ABI PRISM 3130 XL sequencer using the BIG DYE TERMINATOR Kit ver. 3.1 according to the manufacturer's protocol at the Sintol Research and Production Complex (Moscow, Russia). Forward and reverse sequences were assembled and aligned manually using BioEdit 7.0.5.3 (Hall, 1999).

Inter simple sequence repeats (ISSR)

Considering the high information content and efficiency for detecting genetic polymorphism of endemic species with low level of variation in nuclear and plastid sequences (El-Bakatoushi, Ahmed, 2018; Stathi et al., 2020), the ISSR method was also applied to study *C. wolgarica*. The DNA concentration was measured using a Qubit fluorimeter (Thermo Fisher Scientific, USA), after which all samples were diluted to a concentration of 10 ng/µl.

The Polymerase Chain Reaction (PCR) was performed in Mastercycler Gradient amplifier (Eppendorf, Germany) with pre-selected ISSR primers based on literature data and synthesized by Sintol Research and Production Complex (Moscow). A total of 20 µl of reaction mixture were prepared, including 4 µl of "5X MasDDTaqMIX -2025" (200 µM of each dNTP, 1.5 mM $MgCl_2$, 1.5 units of SmarTaq polymerase and buffer; Dialat LLC, Moscow), 15 µl of deionized water, 3.4 pmol of each primer and 1 µl of DNA. Amplification procedures included initial denaturation for 5 min at 95 °C, then 35 cycles of 30 s at 95 °C, 30 s at 44° C and 2 min at 72 °C, with a final elongation for 10 min at 72 °C. The DNA fragments were separated on a 2 % agarose gel after electrophoresis at 5 V/cm for 2 hours. Gels were photographed using a Doc-print VX2 gel-documenting system (Germany). To determine the length of the fragments, we used a DNA ladder with a step of 100 bp.

For each sample, two technical replicates were performed with each ISSR primer (gel reproducibility estimate ~95 %). The typing of ISSR fragments was presented as a presence-absence matrix. Only informative (polymorphic) markers were included in the subsequent analysis. A preliminary analysis was carried out in SplitsTree 6.1.10 using the NeighbourNet method (Huson, Bryant, 2006).

The informativeness of ISSR markers based on the Polymorphic Information Content (PIC),

Table 1. Location of studied *Calophaca wolgarica* populations

Population	Locality	Geographical coordinates (N/E)		Elevation (m a. s. l.)
		Latitude	Longitude	
Rs-2	Russia, Rostov Region, Martynovskiy District, Nesmeyanovka	47.306518	41.462232	36
St-1	Russia, Stavropol Territory, Grusheviy	45.014219	41.808267	569
Rs-3	Russia, Rostov Region, Proletarskiy District, Ganchukov	47.019167	41.627746	88
Rs-1	Russia, Rostov Region, Ust-Donetskiy District, Vinogradniy	47.66116	40.72048	89
Vl-2	Russia, Volgograd Region, Chernyshkovskiy District, Verkhnetsimlyanskiy	48.408607	42.606803	81
Rs-7	Russia, Rostov Region, Dubovskiy District, Aldabulskiy	47.666308	42.688688	86
Kl-2	Russia, Republic of Kalmykia, Sarpinskiy District, Godzhur	47.69674	44.52064	112
Vl-10	Russia, Volgograd Sity, Gorodischenskiy Area	48.88147	44.6186	108
Rs-6	Russia, Rostov Region, Tsimlyanskiy District, Khoroshevskaya	47.73257	42.22988	74
Kl-4	Russia, Republic of Kalmykia, Maloderbetovskiy District, Plodovitoe	48.122126	44.521845	83
Rs-5	Russia, Rostov Region, Konstantinovskiy District, Belyanskiy	47.790162	41.571264	75
Vl-7	Russia, Volgograd Region, Surovikinskiy District, Mayorovskiy	48.82411	43.18031	133
Rs-4	Russia, Rostov Region, Remontnenskiy District, Veseliy	46.480803	43.255555	66
Vl-4	Russia, Volgograd Region, Oktyabrskiy District, Abgonerovo	48.108906	44.165680	108
Kl-3	Russia, Republic of Kalmykia, Sarpinskiy District, Sadovoe	47.720606	44.530756	101
Kl-1	Russia, Republic of Kalmykia, Tselinniy District, Burgusta	46.30128	44.09273	160
Vl-9	Russia, Volgograd Region, Ilovlinskiy District, Trekhostrovskaya	49.084594	43.904202	80
Vl-12	Russia, Volgograd Region, Pallasovskiy, Bulukhta Lake	49.16867	46.23280	21
Vl-8	Russia, Volgograd Region, Kalacheevskiy District, Golubinskiy	49.123995	43.442834	105
Vl-3	Russia, Volgograd Region, Oktyabrskiy District, Oktyabrskiy	47.90095	43.5489	93
Vl-11	Russia, Volgograd Region, Olkhovskiy District, Oktyabrskiy	49.66394	44.56081	119
Vl-6	Russia, Volgograd Region, Svetloyarskiy District, Prudoviy	48.33815	44.341067	114
Vl-5	Russia, Volgograd Region, Svetloyarskiy District, Tinguta	48.28009	44.32539	106
Vl-1	Russia, Volgograd Region, Kotelnikovskiy District, Zaharov	47.700588	43.086919	56
Sr-1	Russia, Saratov Region, Fedorovskiy District, Dolina	51.000237	47.503193	75
Sr-2	Russia, Saratov Region, Pugachevskiy District, Maksutovo	51.848780	49.630739	74

Resolving Power (R), and Discriminating Power (D) was assessed using the iMEC online calculator: Online Marker Efficiency Calculator (Amiryousefi et al., 2018).

A number and percentage of polymorphic bands (P), observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), Nei's genetic diversity (h), total genetic differentiation (GST) and gene flow (NM) were calculated using POPGene ver. 1.32 (Yeh, 1997). Analysis of molecular variance (AMOVA) was performed in ARLEQUIN ver. 3.5 (Excoffier, Lischer, 2010). The dependence of gene flow on geographic distances between populations was estimated using the Mantel test.

The population structure analysis using Bayesian approach was performed in STRUCTURE 2.3 software (Pritchard et al., 2000; Evanno et al., 2005; Jakobsson, Rosenberg, 2007). The analysis was conducted twice using the admixture model. The preliminary selection of Markov chain starting point (burn-in) was carried out after 200 000 replications. Then, Markov chain was constructed with 500 000 iterations for each K (the hypothetical number of clusters) from 1 to 10 in three runs. The STRUCTURE output was interpreted using StructureSelector (Li, Liu, 2018).

Identification of gene flow limitation between local populations due to geographic barriers was performed in NEW HYBRIDS ver. 3.1.1 (Anderson et al., 2000). The data was analyzed in ten replications. Following the recommendations (Anderson et al., 2000; Anderson, Thompson, 2002) each replication included 500 000 iterations after a 200 000 iterations burn-in.

The maps were prepared in QGIS ver. 3.16.0 (2020).

Results

Morphological analysis

Analysis of the main patterns of morphological variation in *C. wolgarica* populations did not reveal a clear separation of any particular samples or populations in ordination space (Fig. 1).

All studied populations, except one (Vl-12), have an extremely wide range of morphological parameters' values. The population from the Stavropol Territory (St-1) with a small sample size (7 individuals) also demonstrates a high level of morphological variation. Samples from Vl-12 (Volgograd Region, Lake Bulukhta) population have a smaller range and are located more compactly in the lower right quadrant of ordination plot (Fig. 1).

Comparative analysis showed that in different years (2013, 2014 and 2024) there was a synchronized fluctuation of morphological variation in all populations as a clear separation of scattering clouds

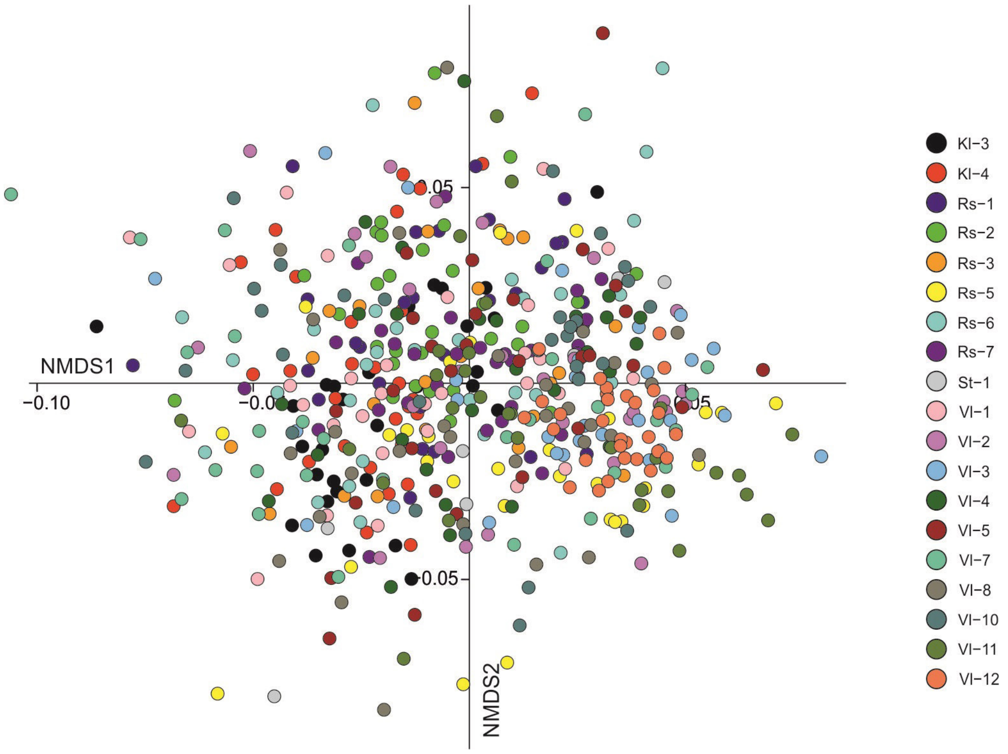

Fig. 1. Ordination of *Calophaca wolgarica* samples obtained by nMDS with 17 morphological parameters.

was observed (Fig. 2). Interpopulation differences within each year of observation were not revealed.

Consequently, the growth conditions in different years significantly affect the morphological variation in *C. wolgarica* populations. However, plants across its entire range react to conditions changes in a similar way. This observation indirectly indicates a genetic homogeneity of *C. wolgarica* in studying territory.

Population genetic analysis

DNA sequencing

The studied *C. wolgarica* populations showed complete genetic homogeneity both in chloroplast and nuclear DNA markers. Moreover, no substitutions in ITS1-5.8S-ITS2 region were found between *C. wolgarica* and two Tien Shan *Calophaca* species – *C. tianschanica* (B. Fedtsch.) Boriss. and *C. pskemica* Gorbunova; they differ only by one two-nucleotide indel. The obtained sequences were deposited in GenBank (accession numbers: PQ559904, PQ569627, PQ569628).

Inter simple sequence repeats (ISSR)

Using PCR with 8 ISSR primers (Table 2), 217 polymorphic bands were obtained. The number of bands per primer varied from 21 (UBC841, UBC845) to 36 (UBC810), with an average of 27 bands per primer. All primers had similar PIC and D values. According to these parameters, UBC843, UBC810 and UBC827 had the highest information content compared to the others, and UBC836 and UBC 841 had the lowest information content. Primers UBC810 and UBC827 were characterized by the highest resolving power and UBC845 by the lowest.

The average observed number of alleles per population (Na) varied from 1.263 (St-1, Stavropol Territory, Grusheviy) to 1.590 (Vl-2, Volgograd Region, Verkhnetsimlyanskiy) (Table 3). The highest levels of allelic diversity (Ne) were noted in populations: Vl-2 (Volgograd Region, Verkhnetsimlyanskiy), Rs-1 (Rostov Region, Vinogradniy) and Rs-7 (Rostov Region, Aldabulskiy). The percentage of polymorphic bands (P) in *C. wolgarica* populations ranged from 58.99 % (Vl-2) to 26.27 % (St-1). The index of total genetic differentiation of all populations (GST) was 0.346, corresponding to an average gene flow of (NM) = 0.947.

The NeighbourNet analysis yielded a dendrogram with a star-shaped structure, indicating the absence of any clear separation of population groups (Supplemental information).

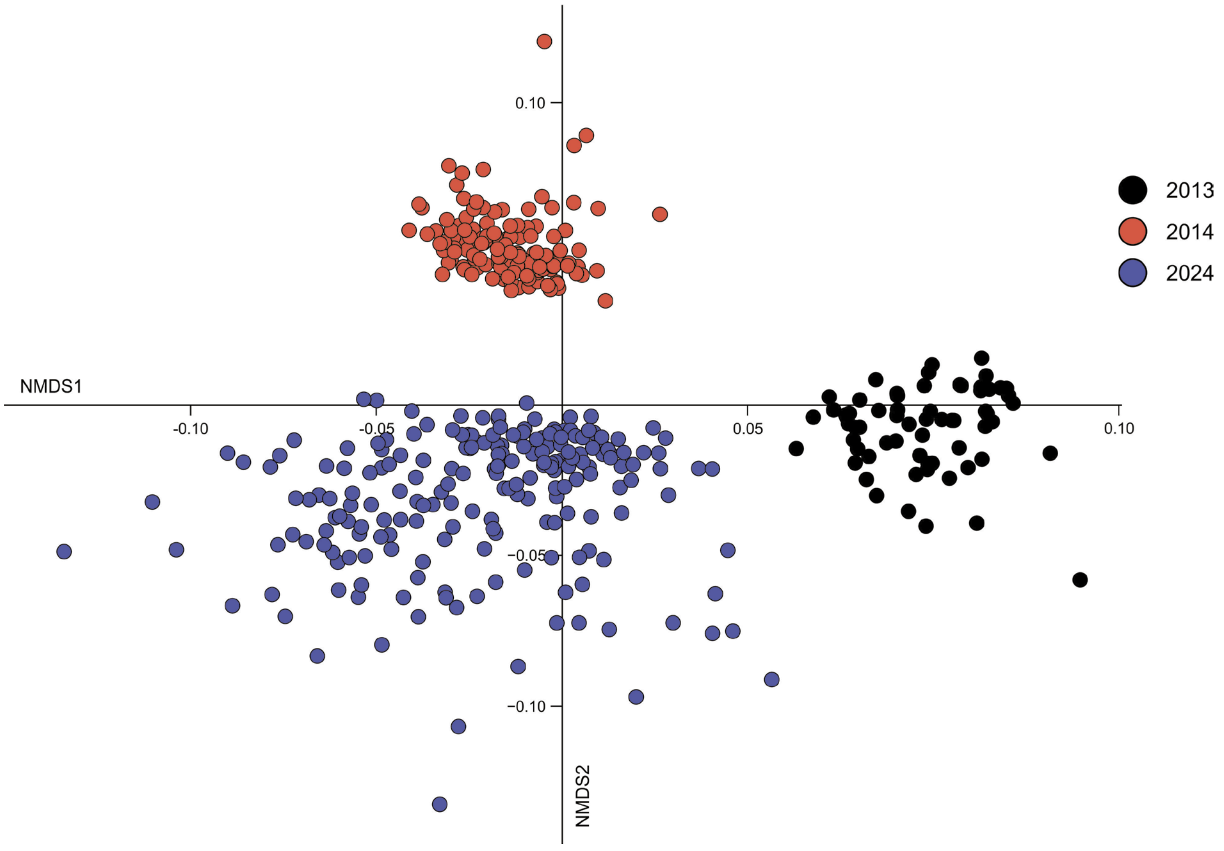

Fig. 2. Ordination of *Calophaca wolgarica* samples obtained by nMDS with 13 morphological parameters in three years of observation: 2013, 2014 and 2024.

Table 2. ISSR primers used for population genetic analysis of *Calophaca wolgarica* populations

Primer	Sequences 5'–3'	Number of bands	PIC	D	R
UBC810	(GA)8T	36	0.369	0.903	16.531
UBC816	(CA)8T	28	0.351	0.860	10.646
UBC827	(AC)8G	30	0.360	0.885	15.562
UBC836	(AG)8YT	25	0.339	0.803	10.354
UBC841	(GA)8YT	21	0.336	0.765	8.385
UBC843	(CT)8RA	31	0.392	0.939	11.846
UBC845	(CT)8RG	21	0.346	0.843	6.938
UBC851	(GT)8YG	25	0.355	0.871	8.523

Note: PIC – polymorphic information content; D – discriminating power; R – resolving power.

Table 3. The main estimators of genetic diversity in studied *Calophaca wolgarica* populations. N = sample sizes, NP = number of polymorphic bands, P = percentage of polymorphic bands, Na = observed number of alleles, Ne = effective number of alleles, h = Nei's genetic diversity, I = Shannon's information index

Population	N	NP	P (%)	Na	Ne	h	I
Rs-2	10	109	50.23	1.502	1.258	0.155	0.237
St-1	10	57	26.27	1.263	1.178	0.104	0.153
Rs-3	10	123	56.68	1.567	1.311	0.186	0.283
Rs-1	10	120	55.3	1.553	1.323	0.188	0.282
Vl-2	10	128	58.99	1.590	1.344	0.201	0.302
Rs-7	10	122	56.22	1.562	1.315	0.186	0.282
Kl-2	10	113	52.07	1.521	1.304	0.178	0.268
Vl-10	10	103	47.47	1.475	1.279	0.164	0.246
Rs-6	10	109	50.23	1.502	1.305	0.177	0.264
Kl-4	10	105	48.39	1.484	1.273	0.161	0.243
Rs-5	10	100	46.08	1.461	1.266	0.155	0.233
Vl-7	10	103	47.47	1.475	1.269	0.158	0.238
Rs-4	10	124	57.14	1.571	1.293	0.178	0.274
Vl-4	10	106	48.85	1.489	1.273	0.162	0.245
Kl-3	10	106	48.85	1.489	1.284	0.169	0.255
Kl-1	10	109	50.23	1.502	1.295	0.172	0.258
Vl-9	10	97	44.7	1.447	1.269	0.157	0.235
Vl-12	10	87	40.09	1.401	1.237	1.139	0.208
Vl-8	10	105	48.39	1.484	1.267	0.157	0.237
Vl-3	10	112	51.61	1.516	1.312	0.181	0.271
Vl-11	10	90	41.47	1.415	1.229	0.137	0.207
Vl-6	10	98	45.16	1.452	1.261	0.155	0.234
Vl-5	10	111	51.15	1.512	1.279	0.164	0.249
Vl-1	10	114	52.53	1.525	1.285	0.170	0.258
Sr-1	10	100	46.08	1.461	1.249	0.149	0.226
Sr-2	10	121	55.76	1.558	1.309	0.184	0.278

Note: N – sample sizes, NP – number of polymorphic bands, P – percentage of polymorphic bands, Na – observed number of alleles, Ne – effective number of alleles, h – Nei's genetic diversity, I – Shannon's information index.

The optimal number of clusters (K) equal to 2 was obtained (Supplemental information) for dividing the set of all studied populations according to its genetic structure (Fig. 3).

The first genetic group included: all individuals from a single population of the Stavropol Territory (St-1); most individuals from 6 populations of the Rostov Region (Rs-1, Rs-2, Rs-3, Rs-5, Rs-6, Rs-7); most individuals from 3 populations of the Volgograd Region (Vl-2, Vl-7, Vl-10); individuals from 2 populations of the Republic of Kalmykia (Kl-2, Kl-4) (Fig. 3). The second group included most individuals from populations of the Volgograd Region (Vl-1, Vl-3, Vl-4, Vl-5, Vl-6, Vl-8, Vl-9, Vl-11, Vl-12); two populations of the Republic of Kalmykia (Kl 1, Kl-3).

Most individuals from one of the reintroduced populations were assigned to the second group (Sr-2), while individuals from another population (Sr-1) were more heterogeneous. One population from the Rostov Region (Rs-4) can be characterized as mixed in terms of the probability of assigning individuals to one group or another.

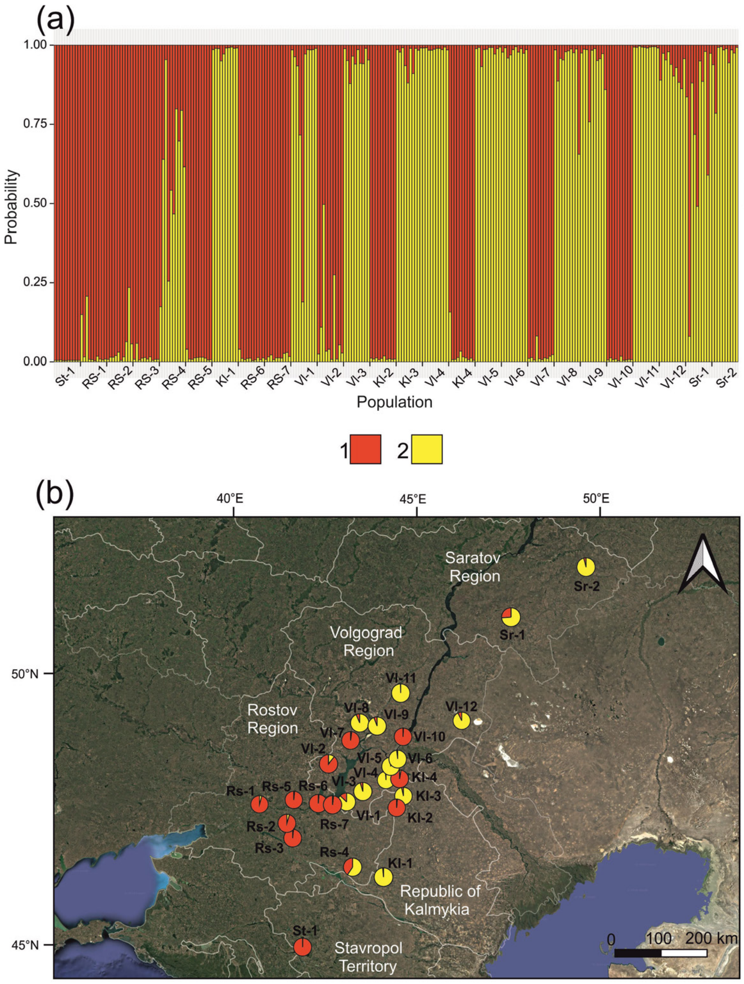

Fig. 3. Results of STRUCTURE analysis: a – The probability of each individual of *Calophaca wolgarica* being a member of a particular genetic group according to STRUCTURE clustering (at K=2); b – the geographical distribution of *C. wolgarica* populations with added diagrams showing the proportions of genetic groups in each local population.

Based on the results of Bayesian analysis in NEW HYBRIDS program, the studied populations can be conditionally divided into 3 groups: the first group included populations in which the majority of individuals were attributed to the first pure parent (pure_0) (Kl-1, Vl-1, Vl-3, Vl-4, Vl-5, Vl-6, Vl-8, Vl-9, Vl-11, Vl-12); the second group included one population St-1, with a large proportion of individuals attributed to the second pure parent (pure_1); the third group consisted of populations with the majority of individuals attributed to second-generation hybrids (F2) (Fig. 4).

The analysis of molecular variance was performed three times, according to the results obtained above: without dividing the populations into groups; with two groups, according to STRUCTURE clustering (two reintroduced and one mixed populations were removed from the analysis); with three groups, according to NEW HYBRIDS clustering (two reintroduced and one mixed populations were removed from the analysis). According to the results of AMOVA without dividing populations by groups (Table 4), the most of genetic variation (68.32 %) corresponds to intrapopulation polymorphism. The fixation index had a small value of 0.32, statistically not significant.

The division of populations according to STRUCTURE and NEW HYBRIDS clustering showed similar results: the proportion of variance explained by intrapopulation variation was higher than for intergroup and interpopulation differences, and amounted to 65.72 and 65.66 %, respectively. The fixation index values in both cases were statistically significant only at intrapopulation level and amounted to 0.34. The gene flow between populations (NM) had an average value of 0.947.

A Mantel test showed a weak, statistically significant correlation ($r = 0.42$, $p < 0.05$) between fixation index values and geographic distances (two reintroduced populations and mixed population Rs-4 removing from the analysis).

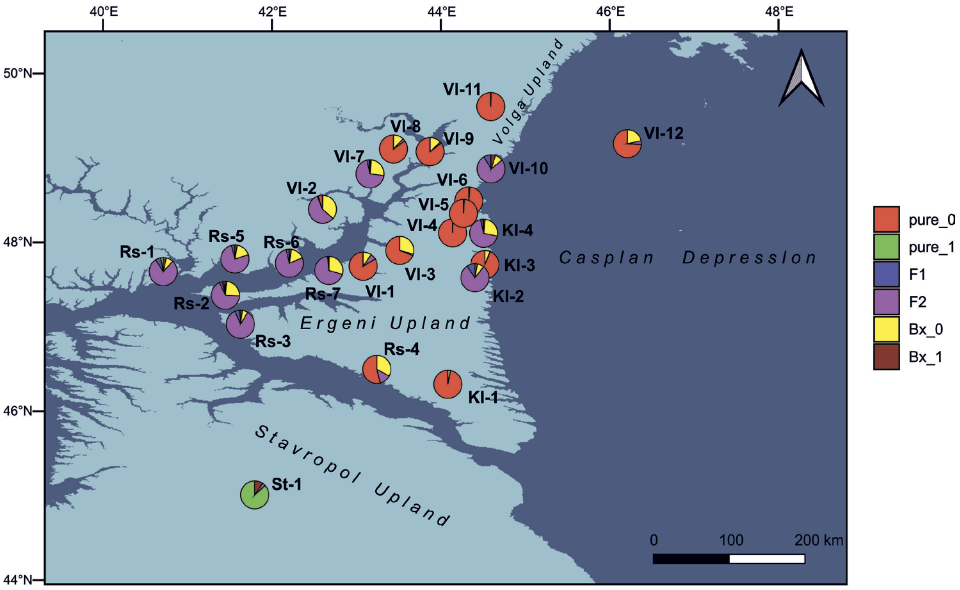

Fig. 4. The geographical distribution of *Calophaca wolgarica* populations with added diagrams showing the proportions of genetic groups in each local population according to NEW HYBRIDS clustering: pure_0 – the first pure parent; pure_1 – the second pure parent; F1 – first generation hybrids; F2 – second generation hybrids; Bx_0 – backcrosses to the first pure parent; Bx_1 – backcrosses to the second pure parent. The area of the Early Khvalynian transgression of Caspian Sea is shown in dark blue (up to 48 m a. s. l.).

Table 4. The analysis of molecular variance (AMOVA) in *Calophaca wolgarica* populations

Interaction level	Degrees of freedom	Sum of squares	Variance	Explained variance (%)	Fixation index
Without clustering					
Between populations	25	3002.938	9.88124	31.68	0.316
Between individuals in population	234	4985.400	21.30513	68.32	
Total	259	7988.338	31.18637		
STRUCTURE clustering					
Between groups	1	268.848	1.31394	4.09	0.04092
Between populations in groups	21	2478.639	9.69270	30.19	0.31474
Between individuals in population	207	4368.400	21.10338	65.72	0.34278*
Total	229	7115.887	32.11003		
NEW HYBRIDS clustering					
Between groups	2	420.760	1.51259	4.71	0.04706
Between populations in groups	20	2326.727	9.52330	29.63	0.31095
Between individuals in population	207	4368.400	21.10338	65.66	0.34338*
Total	229	7115.887	32.13927		

Note: statistically significant values of Fixation index marked by asterisk.

Discussion

It is believed that the *Calophaca* genus distributed in several global biodiversity hotspots: the Pamir and western Tien Shan Mountains, the Caucasus, and East Asia (Zachos, Habel, 2011). Zhang et al. (2015) conducted a molecular genetics study of *Calophaca* and estimated the origin, relationships and diversification time of all 8 species, including *C. wolgarica*. The authors dated the origin of the genus to the global cooling and aridization process in the late Miocene 8–7 million years ago. The Pamir Mountains was considered to be the center of *Calophaca* origin. It is assumed that the dominant speciation type of *Calophaca* was dispersal. Four main dispersal routes were identified from the late Miocene to the Pleistocene: from the Pamir to western Tien Shan Mountains about 7 million years ago; to East Asia about 7 million years ago; north to the Tarbagatai Mountains about 2.2 million years ago; west from western Tien Shan Mountains to the Caucasus and European Russia 2.6 million years ago. The authors believe that natural barriers in the form of large arid and semi-arid zones associated with global cooling in the Middle Miocene were the reasons for species areas disjunction with the absence of dispersal traces. Consequently, based on the results of previous phylogeographic studies, the origin of *C. wolgarica* is assumed to be in Pamir-Western Tien Shan and its closest relatives are *C. pskemica* Gorbunova and *C. tianschanica* (B. Fedtsch.) Boriss. The closest relationship between *C. wolgarica*, *C. pskemica*, and *C. tianschanica* is also confirmed by present study, since no significant differences were found between the sequences of these species. Therefore, the origin of *C. wolgarica* in Pamir-Western Tien Shan is also beyond doubt.

However, the assumption that *C. wolgarica* spread from the Western Tien Shan to Eastern Europe via the Caucasus Mountains "bypassing" the Caspian Sea from the southern side (Zhang et al., 2015) raises some doubts. This way is questionable, since the Iranian Plateau and the Main Caucasus Range have the high altitudes and formed long ago (28–23 million years ago) before the supposed dispersal of *C. wolgarica* to Eastern Europe. In addition, the authors point out that increased aridization in the inner regions of Asia associated with global cooling occurred in the Middle Miocene 8–7 million years ago, but *C. wolgarica* spread to the Caucasus and European Russia only 2.6 million years ago, i. e. much later than the appearance of the impassable arid zone.

Taking into account the occurrence records of *C. wolgarica* from different sources (Ryabinina, Knyazev, 2009; Ismailov, 2018; Efimenko et al., 2024; GBIF, 2024), the following way of dispersal to Eastern Europe seems more logical: through the Kazakh Upland to the territory of modern North-West Kazakhstan or the Southern Urals, where species was noted until recently, and perhaps still grows fragmentarily (Ryabinina, Knyazev, 2009), then to the Volga and Ergeni Uplands, and further to the Stavropol Upland. The conducted analysis of genetic structure using ISSR markers confirms this assumption.

According to NEW HYBRIDS clustering, the majority of samples from populations occupying the northeastern and central parts of the studied territory, i. e. the south of the Volga and Ergeni Uplands, were assigned to the first ancestral population. The southernmost and territorially isolated population St-1, growing on the Stavropol Upland, was identified as the second ancestral population. The geographic distances to the closest Rs-4 and Kl 1 populations, growing on the southern slope of the Ergeni Upland and separated from St-1 by the Kuma-Manych Depression, was about 200 and 230 km, respectively. In the remaining populations, located mainly in the west and, partially, in the east of the studied territory, the overwhelming majority of individuals were assigned to second generation hybrids. In terms of representation the third group was backcross to the first pure parent; it was found with a noticeable contribution both in the first ancestral population and in populations with second generation hybrids (Fig. 4).

Phylogeographic analysis of *C. wolgarica* cannot be carried out without taking into account global climate change, glacial-interglacial rhythms of East European Plain and the evolution of Ponto-Caspian region, in particular the Caspian Sea. Global changes in climatic conditions in the Pleistocene had a decisive influence on both the transgressive-regressive state of Pontus (Black Sea) and Caspian basins and on glaciers dynamics in East European Plain. Whereas, the glaciers also had a regional impact on the development of these basins (Yanina, 2014; Hurka et al., 2019).

The modern area of *C. wolgarica* covers the Lower Volga, Middle and Lower Don basins, and the Stavropol Upland. Previously, the species was also noted in the Middle Volga, in the southern part of the Cis-Urals and in Crimea, i. e. at the end of the 20th century it covered a significant part of the south of East European Plain. According to Zhang et al. (2015), its dispersal occurred from the western Tien Shan Mountains westward to European Russia about 2.6 million years ago, i. e. at the boundary of Pliocene and Pleistocene. At this stage of Earth's evolution, glaciation of Greenland, Scandinavia and North America began in the northern hemisphere. After long warm period, subtropical vegetation was replaced by boreal in Central Europe, and continentality and aridity of Eastern Europe were increased. The climate at this stage resembled the modern climate, to which *C. wolgarica* is well adapted. Subsequently, during the early Pleistocene (2.6 million – 850 thousand years ago), a general cooling occurred consistently with an increase in the long-term volume of global ice and the spread of permafrost in northern Europe. The seasonality increased, and in the lower early Pleistocene (1.8 million – 850 thousand years ago) periglacial components began to dominate in European floras. In the middle and late Pleistocene (850–10 thousand years ago), the periods of deep cooling and warming replaced each other. At this time, large continental glaciations occurred, and open-arid landscapes such as tundra-steppe, semi-deserts, and deserts began to predominate in Eastern Europe. The climate on the East European Plain during this period was extremely unfavorable for *C. wolgarica*, and the species had to repeatedly survive for long time intervals in refugia, which, as follows from the results of our study, were in Stavropol and Volga Uplands. After the end of the last Late Valdai glaciation (25–10 thousand years ago) and the retreat of the glacier, the species may have completely restored its range within its maximum boundaries. However, the Early Khvalynian transgression of Caspian Sea (20–11 thousand years ago) did not allow doing this in the flood zone, when its absolute marks reached 48–50 m a. s. l. (Dolukhanov et al., 2009; Tudryn et al., 2013; Yanina, 2014). This territory began to be populated by the species at later times.

Probably, the most favorable conditions for the existence of *C. wolgarica* were during its dispersal from the Western Tien Shan to Eastern Europe, i. e. at the boundary of the Pliocene and Pleistocene (about 2.6 million years ago). The pattern of genetic polymorphism in populations (Fig. 4) indicates that in later times the species was preserved in refugia and only relatively recently (on evolutionarily significant time scale) finally dispersed within the boundaries of its modern range. The dispersal to the Caspian Lowland and Kuma-Manych Depression was significantly affected by the Early Khvalynian transgression of Caspian Sea. Population St-1 defined as the ancestral is located on the Stavropol Upland. The habitat of this population is characterized by the highest elevation among all studied populations – about 570 m. a. s. l. Populations attributed to the second ancestral group are also located on uplands – Ergeni and the southern spurs of the Volga Upland. It should be noted that, in general, the habitats of the ancestral populations have a higher elevation than the habitats of most hybrid populations (Table 1, Fig. 4). This confirms the role of the Stavropol Upland, the south of the Volga Upland and Ergeni as refugia for *C. wolgarica*. In this case, hybrid populations are located mostly to the west, and partly to the east (along the western border of the flood zone of the most extensive Early Khvalynian transgression of Caspian Sea; Kl-2, Kl-4, Vl-10) from the ancestral populations. This confirms that it formed later as a result of secondary settlement of the territory along the geographic latitude in both directions from the ancestral populations.

The results of STRUCTURE clustering potentially reflect the *C. wolgarica* distribution in the Late Pleistocene and Holocene. Two groups of populations were identified. Group 1 united populations located mainly in the west and partially in the east of the studied territory (Fig. 3). Some populations from this group were characterized by maximum intrapopulation diversity (Table 3). The most genetically diverse populations were Vl-2 (Volgograd Region, Verkhnetsimlyanskiy), Rs-1 (Rostov Region, Vinogradniy), Rs-3 (Rostov Region, Ganchukov) and Rs-7 (Rostov Region, Aldabulskiy). Populations from group 2 are concentrated in the north-eastern and central parts of the studied area. Population Rs-4 had a mixed genetic nature. The analysis also included samples from two reintroduced populations that have existed in the Saratov Region since 2015. Most of the samples were attributed to group 2, since the seeds for sowing were selected from populations of the Volgograd region (Vl-4, Vl-12). However, some seeds for the Sr-1 population were also taken from the Vl-10 population, whose individuals were most likely assigned to group 1. As a result, we observe a fairly large proportion of individuals (about 25 %) assigned to group 1. According to the results of long-term monitoring, Sr-2 population distinguished by better characteristics of age and vitality structure than Sr-1 population (Efimenko et al., 2024). Probably, the genotypes of Sr-2 population assigned to group 2 have greater adaptive capabilities. In Sr-1 population, the genotype of samples taken from the natural Vl-10 population, whose individuals are most likely assigned to group 1, brings wide range of vitality index, i.e. it has a lower adaptive capacity.

According to the results of AMOVA, *C. wolgarica* has a high level of intrapopulation genetic variation, exceeding the level of variation both between individual populations and between groups of populations identified based on STRUCTURE and NEW HYBRIDS clustering (Table 4). The fixation index values were not statistically significant when compared between groups and populations within groups. It was shown that along with the high diversity within populations, there is also a gene flow (NM = 0.947) of medium intensity between populations, sufficient to prevent their genetic divergence. A visual assessment of spatial distribution of genetic groups identified as a result of STRUCTURE and NEW HYBRIDS suggests that populations located closer to each other have a more similar genetic structure. This assumption was confirmed by Mantel test, which showed the presence of a weak but statistically significant correlation between genetic differentiation and geographic distances. The observed patterns of spatial distribution of genetic diversity can be partly explained by isolation by distance, which results in limited gene flow between the most distant habitats.

Probably, the gene flow from the first ancestral population St-1 was less intense than from the second ancestral population. The refugium of the second ancestral population was either more extensive, covering the entire south of the Volga Upland up to the Ergeni Upland, or the gene flow from the second ancestral population was much more intense. This is confirmed by the fact that backcrosses to its genotypes are much more widespread than backcrosses to the genotypes of the first ancestral population.

C. wolgarica has a very low rate of dispersal. Over the past 13–15 thousand years, species has not occupied the territory of the main water area of the Early Khvalynian transgression of Caspian Sea after its regression with the exception of Bulukhta Lake surroundings, situated about 70 km from the western boundary of the flood zone. Consequently, the territory to the west of the Early Khvalynian transgression boundary, covered by backcrosses to the second ancestral population and second-generation hybrids, is most likely the result of an earlier dispersal.

Morphological variability also indicates a low rate of species dispersal. This is clearly seen in the example of population from the Bulukhta Lake (Vl-12) surroundings, which is characterized by lower morphological diversity and being the only one population located within the flood zone of the Early Khvalynian transgression of Caspian Sea (Fig. 4), i. e. over the past 10–15 thousand years from the supposed dispersal time it has remained morphologically conservative.

Similar genetic and morphological conservatism was found in another endemic relict species of Eastern Europe with a disjunctive range, *Globularia bisnagarica* L. and its relative *G. trichosantha* Fisch. et C. A. Mey. (Kondratieva et al., 2024). Endemic relicts *G. bisnagarica* and *C. wolgarica* are distributed across the flat southern part of European Russia characterized by low elevations (21–160 (569) m a. s. l. for *C. wolgarica*), a high degree of ecological homogeneity and the absence of geographic barriers. Only the range of *G. trichosantha* is divided by mountain systems and sea waters. But the polymorphism in its populations is higher than in the two previous ones (Kondratieva et al., 2024). Given the ecological homogeneity of the range and the absence of geographic barriers to gene flow, relict endemic species from the southern part of Eastern Europe has remained genetically and morphologically homogeneous (conservative) throughout the long history of their existence, even despite the disjunctive nature of range.

It has been previously hypothesized that the most likely cause of high plant species diversity is extreme geographic heterogeneity, which, combined with climate and sea level change, provides ample opportunities for evolutionary radiation through allopatric speciation (Qian, Ricklefs, 2000). It is assumed that relict endemic species exhibit evolutionary stagnation with long-term preservation of the climatic niche and species morphology (Ricklefs, Latham, 1992) and low genetic diversity of populations (Cabral et al., 2021). It has been shown that some species from eastern North America retain low polymorphism and weak interpopulation differentiation under conditions of low ecological heterogeneity and absence of geographic barriers within their range (Godt et al., 1996). Similar patterns are also found in the other ecologically homogeneous regions. For example, the endemic species *Cicer graecum*, growing at low altitudes in the northern Peloponnese Peninsula (Greece), was found to have a low level of interpopulation polymorphism (Stathi et al., 2020). The narrowly distributed endemic of Mexico *Yucca capensis* L. W. Lenz (Asparagaceae) was found to have high heterozygosity, high polymorphism, but low genetic differentiation between populations (Luna-Ortiz et al., 2022). We suggest a similar explanation for genetic homogeneity of *C. wolgarica*.

On the contrary, it has been shown that ecological heterogeneity of habitats and the presence of geographic barriers in East Asia cause high genetic differentiation between populations of relict endemics (Chen et al., 2009; Hu et al., 2010; Meng et al., 2015; Xu, Zhang, 2015; Ma et al., 2019; Zhao et al., 2019; Takahashi et al., 2022; Zhang et al., 2022; Takahashi, Suyama, 2023; De Luca et al., 2024). Exceptions to this pattern include the behavior of *Paeonia decomposita* Handel-Mazzetti, an endemic of remote mountainous regions of northwestern Sichuan Province (China), which has a disjunctive (sporadic) range (Wang, 2020). Despite this, its genetic differentiation was mainly observed at the intrapopulation level. Another example of such "atypical" behavior is *Rhododendron protistum* var. *giganteum* (Forrest) D. F. Chamb. (Wu et al., 2015) with only two known endemic populations in China. Amplified fragment length polymorphism (AFLP) analysis revealed high genetic diversity at species level and low genetic differentiation between two populations. The authors attributed this to life cycle features, pollen dispersal, and high gene flow, since the distance between the habitats was only about 20 km.

The above-mentioned behavior of plant species with a high degree of interpopulation genetic differentiation is also found in other ecologically heterogeneous regions. For the endemic of northern Andes, *Lupinus alopecuroides* Desr. (Fabaceae), it was shown that populations located on different mountain peaks have very low intrapopulation polymorphism with very high genetic differentiation between them (Vásquez et al., 2016). In the endemic of karst forests of Mexico *Pilea pteridophylla* (Urticaceae), only two populations do not have common haplotypes and have high morphological differentiation, as a consequence of long-term isolation (Lagos Baez et al., 2024). In the microendemic species of the mountainous part of Tamaulipas (Mexico) with a high degree of ecological heterogeneity, high genetic variability and strong differentiation were revealed even in the geographic proximity of the five studied populations. The authors attribute the high overall diversity to the bottleneck effect, followed by adaptive selection to local conditions (Octavio-Aguilar et al., 2024). In the highland Alpine endemic *Salix serpillifolia* Scop., the strong genetic pattern of differentiation along the Alps attributed to the local survival of small populations during the Pleistocene glacial periods (Kosiński et al., 2019). The Western European highland endemic, the Tertiary relict species *Meconopsis cambrica* L. (Papaveraceae) growing in the mountains of Iberian Peninsula, Pyrenees, Central Massif and in some western parts of the British Isles, also demonstrates high interpopulation variability along the latitudinal gradient (Valtuena et al., 2012). Relict populations of *Rhododendron ferrugineum* L. (Ericaceae) from the Apennine and Alpine Mountains of Western Europe also demonstrate a high degree of separation (Bruni et al., 2012). A low level of intrapopulation genetic diversity and a high level of population isolation were also found in the Tertiary relict of the mountainous regions of southwestern Eurasia *Platanus orientalis* L. (Platanaceae) with strong fragmentation of the range and the presence of geographic barriers (Rinaldi et al., 2019).

Conclusion

The relict species *C. wolgarica* demonstrates genetic homogeneity of nucleotide sequences in fragments of both nuclear and plastid DNA within the entire endemic range. Moreover, no significant differences were found between the sequences of *C. wolgarica* and the sequences of its closest relatives, *C. pskemica* and *C. tianschanica*. Detection of anonymous genetic polymorphism using ISSR markers also showed the absence of clear differentiation between *C. wolgarica* populations. Most of genetic variations were observed at intrapopulation level. Morphological variability of *C. wolgarica* at the interpopulation level is almost absent, but it is quite significant within populations. At the same time, growing conditions in each year significantly affect morphological variability, but plants across the entire range respond to the uniqueness of these conditions in a similar way.

All of the above justifies an assumption that the endemism of relict species in the south of East European Plain, in the absence of geographical barriers and low ecological heterogeneity of this territory, may be a consequence not only, or not so much, of gene flow, as a consequence of their morphological and genetic conservatism. They retain territories with stable "habitual" climatic conditions to which they are adapted, but do not go beyond its boundaries due to the weak adaptive capacity. At the same time, over millions of years of existence, the gene pool of these species has not actually changed, and the range in the past probably experienced multiple narrowing and expansions depending on changing climatic conditions associated with the change of glacial and interglacial periods and with the transgressive-regressive dynamics of Caspian Sea level.

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REFERENCES / ЛИТЕРАТУРА

Amiryousefi A., Hyvönen J., Poczai P. 2018. iMEC: Online Marker Efficiency Calculator. *Appl. Plant Sci.* 6(6): e1159. https://doi.org/10.1002/aps3.1159

Anderson E. C., Thompson E. A. 2002. A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* 160(3): 1217–1229. https://doi.org/10.1093/genetics/160.3.1217

Anderson E. C., Williamson E. G., Thompson E. A. 2000. Monte Carlo evaluation of the likelihood for Ne from temporally-spaced samples. *Genetics* 156(4): 2109–2118. https://doi.org/10.1093/genetics/156.4.2109

Belous V. N. 2016. The synecological features of *Calophaca wolgarica* (the Stavropol region's steppe landscapes as an example). *Problems of Botany of South Siberia and Mongolia* 15: 230–235. [In Russian] (***Белоус В. Н.*** Синэкологические особенности *Calophaca wolgarica* (L. fil.) DC. на примере степных ландшафтов Ставрополья // Проблемы ботаники Южной Сибири и Монголии, 2016. № 15. С. 230–235).

Bruni I., De Mattia F., Labra M., Grassi F., Fluch S., Berenyi M., Ferrari C. 2012. Genetic variability of relict *Rhododendron ferrugineum* L. populations in the Northern Apennines with some inferences for a conservation strategy. *Plant Biosyst.* 146(1): 24–32. https://doi.org/10.1080/11263504.2011.557093

Cabral A., Luebert F., Mello-Silva R. 2021. Evidence for Middle Miocene origin and morphological evolutionary stasis in a *Barbacenia Inselberg* clade (Velloziaceae). *Mol. Phylogenet. Evol.* 161: 107163. https://doi.org/10.1016/j.ympev.2021.107163

Chen F., Wang A., Chen K., Wan D., Liu J. 2009. Genetic diversity and population structure of the endangered and medically important *Rheum tanguticum* (Polygonaceae) revealed by SSR Markers. *Biochem. Syst. Ecol.* 37(5): 613–621. https://doi.org/10.1016/j.bse.2009.08.004

De Luca D., Del Guacchio E., Cennamo P., Minutillo F., Bernardo L., Caputo P. 2024. Genetics and distribution of the italian endemic *Campanula fragilis* Cirillo (Campanulaceae). *Plants* 13(22): 3169. https://doi.org/10.3390/plants13223169

Dolukhanov P. M., Chepalyga A. L., Shkatova V. K., Lavrentiev N. V. 2009. Late Quaternary Caspian: sea-levels, environments and human settlement. *Open Geogr. J.* 2(1): 1–15. https://doi.org/10.2174/1874923200902010001

Duan L., Yang X., Liu P. L., Johnson G., Wen J., Chang Z. Y. 2016. A molecular phylogeny of *Caraganeae* (Leguminosae, Papilionoideae) reveals insights into new generic and infrageneric delimitations. *PhytoKeys* 70: 111–137. https://doi.org/10.3897/phytokeys.70.9641

Efimenko S. F., Parkhomenko A. S., Shilova I. V., Grebenuk L. V., Kuliseva Yu. I., Kashin A. S. 2024. Results of monitoring of reintroduced populations of *Calophaca wolgarica* in the Saratov Region. *Izv. Sar. Univ. Ser. Chem. Biol. Ecol.* 24, 4: 315–320. [In Russian] (***Ефименко С. Ф., Пархоменко А. С., Шилова И. В., Гребенюк Л. В., Кулисёва Ю. И., Кашин А. С.*** Результаты мониторинга реинтродукционных популяций *Calophaca wolgarica* на территории Саратовской области // Изв. Сар. Унив. Сер. Хим. Биол. Экол., 2024. Т. 24, № 4. С. 315–320). https://doi.org/10.18500/1816-9775-2024-24-4-415-429

El-Bakatoushi R., Ahmed D. G. A. 2018. Evaluation of genetic diversity in wild populations of *Peganum harmala* L., a medicinal plant. *J. Genet. Eng. Biotechnol.* 16(1): 143–151. https://doi.org/10.1016/j.jgeb.2017.11.007

Evanno G., Reqnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* 14(8): 2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Excoffier L., Lischer H. E. L. 2010. Arlequin suite ver. 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* 10(3): 564–567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

GBIF [2024]. *The Global Biodiversity Information Facility*. URL: https://www.gbif.org/ (Accessed 21 Desember 2024).

Godt M.J. W., Johnson B. R., Hamrick J. L. 1996. Genetic diversity and population size in four rare Southern Appalachian plant species. *Conserv. Biol.* 10(3): 796–805.

Hall T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95–98.

Hu Y., Wang L., Xie X., Yang J., Li Y., Zhang H. 2010. Genetic diversity of wild populations of *Rheum tanguticum* endemic to China as revealed by ISSR analysis. *Biochem. Syst. Ecol.* 38(3): 264–274. https://doi.org/10.1016/j.bse.2010.01.006

Hurka H., Friesen N., Bernhardt K.-G., Neuffer B., Smirnov S. V., Shmakov A. I., Blattner F. R. 2019. The Eurasian steppe belt: status quo, origin and evolutionary history. *Turczaninowia* 22, 3: 5–71. https://doi.org/10.14258/turczaninowia.22.3.1

Huson D. H., Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23(2): 254–267. https://doi.org/10.1093/molbev/msj030

Ismailov A. B. 2018. Distribution and protection of *Calophaca wolgarica* (Fabaceae) in Russia. *Botanical Journal of the North Caucasus* 2: 13–20. [In Russian] (**Исмаилов А. Б.** Распространение и охрана *Calophaca wolgarica* (Fabaceae) в России // Ботанический вестник Северного Кавказа, 2018. № 2. С. 13–20).

Jakobsson M., Rosenberg N. A. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23(14): 1801–1806. https://doi.org/10.1093/bioinformatics/btm233

Kashin A. S., Petrova N. A., Shilova I. V., Grebennikov K. A., Kulikova L. V. 2015. Status of the *Calophaca wolgarica* (L.) Fisch. ex DC. (Fabaceae, Angiospermae) cenopopulations in the Volgograd Region. *Pov. J. Ecol.* 3: 253–267. [In Russian] (**Кашин А. С., Петрова Н. А., Шилова И. В., Гребенников К. А., Куликова Л. В.** Состояние ценопопуляций *Calophaca wolgarica* (L.) Fisch. ex DC. (Fabaceae, Angiospermae) в Волгоградской области // Поволжский экологический журнал, 2015. № 3. С. 253–267).

Kondratieva A. O., Parkhomenko A. S., Kritskaya T. A., Shilova I. V., Kasatkin M. Ju., Efimenko S. F., Kashin A. S. 2024. Variation patterns in populations of Eastern European *Globularia* (Plantaginaceae, Magnoliopsida) species due to habitat conditions. *Biol. Bull. Russ. Acad. Sci.* 51: 1–16. https://doi.org/10.1134/S1062359024980061

Kosiński P., Sękiewicz K., Walas Ł., Boratyński A., Dering M. 2019. Spatial genetic structure of the endemic alpine plant *Salix serpillifolia*: genetic swamping on nunataks due to secondary colonization? *Alp. Bot.* 129: 107–121. https://doi.org/10.1007/s00035-019-00224-4

Lagos Baez K., Licona Vera Yu., Gomez Dominguez H., Ornelas Ju. F., Vasquez-Aguilar A. A., Hurtado Reveles L., Ortiz Rodriguez A. E. 2024. Genetic and morphological differentiation among populations of the narrowly endemic and karst forest adapted *Pilea pteridophylla* (Urticaceae). *Org. Divers. Evol.* 24: 149–162. https://doi.org/10.1007/s13127-024-00638-x

Li Y. L., Liu J. X. 2018. Structure Selector: a web based software to select and visualize the optimal number of clusters using multiple methods. *Mol. Ecol. Res.* 18(1): 176–177. https://doi.org/10.1111/1755-0998.12719

Luna-Ortiz A., Arteaga M. C., Bello-Bedoy R., Gasca-Pineda J., Leon de la Luz J. L., Domınguez-Cadena R., Medel-Narvaez A. 2022. High genetic diversity and low structure in an endemic long-lived tree, *Yucca capensis* (Asparagaceae). *Plant Biol.* 24(1): 185–191. https://doi.org/10.1111/plb.13346

Ma S. M., Nie Y. B., Jiang X. L., Xu Z., Ji W. Q. 2019. Genetic structure of the endangered, relict shrub *Amygdalus mongolica* (Rosaceae) in arid northwest China. *Aust. J. Bot.* 67(2): 128–139. https://doi.org/10.1071/BT18188

Meng L., Chen G., Li Z., Yang Y., Wang Z., Wang L. 2015. Refugial isolation and range expansions drive the genetic structure of *Oxyria sinensis* (Polygonaceae) in the Himalaya-Hengduan Mountains. *Sci. Rep.* 5: 10396. https://doi.org/10.1038/srep10396

Octavio-Aguilar P., Rubio-Tobón C. A., Montelongo-Ruiz G., Galván-Hernández D. M. 2024. Evaluation of the diversity and genetic structure of *Ceratozamia kuesteriana* Regel (Zamiaceae) in Tamaulipas, Mexico. *Plant Spec. Biol.* 1–13. https://doi.org/10.1111/1442-1984.12455

Oksanen J., Blanchet F. G., Friendly M., Kindt R., Legendre P., McGlinn D., Minchin P. R., O'Hara R. B., Simpson G. L., Solymos P., Stevens M.H. H., Szoecs E., Wagner H. 2020. vegan: Community Ecology Package. R package version 2.5-7. URL: https://CRAN.R-project.org/package=vegan (Accessed 14 February 2025).

Pritchard J. K., Stephens M., Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155(2): 945–959. https://doi.org/10.1093/genetics/155.2.945

QGIS [2020]. *Geographic Information System version 3.16.0. QGIS Association.* URL: https://download.qgis.org/downloads/ (Accessed 02 February 2025).

Qian H., Ricklefs R. E. 2000. Large-scale processes and the Asian bias in species diversity of temperate plants. *Nature* 407: 180–182. https://doi.org/10.1038/35025052

Ricklefs R. E., Latham R. E. 1992. Intercontinental correlation of geographical ranges suggests stasis in ecological traits of relict genera of temperate perennial herbs. *Am. Nat.* 139(6): 1305–1321. https://doi.org/10.1086/285388

Rinaldi R., Cafasso D., Strumia S., Cristaudo A., Sebastiani F., Fineschi S. 2019. The influence of a relict distribution on genetic structure and variation in the Mediterranean tree, *Platanus orientalis*. *AoB PLANTS* 11(1): plz002. https://doi.org/10.1093/aobpla/plz002

Ryabinina Z. N., Knyazev M. S. 2009. *Opredelitel sosudistykh rasteniy Orenburgskoy oblasti* [*Determination key of vascular plants of the Orenburg Region*]. Moscow: KMK Scientific Press. 758 pp. [In Russian] (**Рябинина З. Н., Князев М. С.** Определитель сосудистых растений Оренбургской области. М.: Тов-во науч. изд. КМК, 2009. 758 с.).

Shishlova Zh. N., Shmaraeva A. N., Ermolaeva O. Yu. 2019. State of the coenopopulation of *Calophaca wolgarica* (L. fil.) DC. in the Ataman forestry (Rostov Region). In: K. Sh. Kazeev (ed.). *Aktualnyye problemy ecologii i prirodopolzovaniya* [*Current issues of ecology and nature management*]. Rostov-on-Don; Taganrog: Publ. house of the Southern Federal University. Pp. 237–241. [In Russian] (**Шишлова Ж. Н., Шмараева А. Н., Ермолаева О. Ю.** Состояние ценопопуляции *Calophaca wolgarica* (L. fil.) DC. в Атаманском лесничестве (Ростовская область) //

Актуальные проблемы экологии и природопользования. Под ред. К. Ш. Казеева. Ростов н/Д.; Таганрог: Изд-во Южн. федер. ун-та, 2019. С. 237–241).

Shmaraeva A. N., Baranova O. G. 2024. *Calophaca wolgarica* (L. f.) Pall. ex Fisch. In: *Krasnaya kniga Rossiyskoy Federatsii. Rasteniya i griby* [*Red Data Book of Russian Federation. Plants and fungi*]. Moscow: VNII "Ecologiya". Pp. 268–269. [In Russian] (***Шмараева А. Н., Баранова О. Г.*** Майкараган волжский – *Calophaca wolgarica* (L. f.) Pall. ex Fisch. // Красная книга Российской Федерации. Растения и грибы. М.: ВНИИ «Экология», 2024. С. 268–269).

Shmaraeva A. N., Kuzmenko I. P., Makarova L. I., Shishlova Zh. N., Firsova A. V. 2023. Results of introduction of rare and endangered plants of Rostov Region. In: *Botanicheskiy sad Yuzhnogo federal'nogo universiteta – Tsentr sokhraneniya biologicheskogo raznoobraziya rasteniy stepnoy zony* [*Botanical Garden of Southern Federal University – center of conservation of biological diversity of steppe zone plants*]. Rostov-on-Don; Taganrog: Publ. house of the Southern Federal University. Pp. 24–42. [In Russian] (***Шмараева А. Н., Кузьменко И. П., Макарова Л. И., Шишлова Ж. Н., Фирсова А. В.*** Результаты интродукции редких и исчезающих растений Ростовской области // Ботанический сад Южного федерального университета – центр сохранения биологического разнообразия растений степной зоны. Ростов н/Д.; Таганрог: Изд-во Южн. федер. ун-та, 2023. С. 24–42).

Stathi E., Kougioumoutzis K., Abraham E. M., Trigas P., Ganopoulos I., Avramidou E. V., Tani E. 2020. Population genetic variability and distribution of the endangered Greek endemic *Cicer graecum* under climate change scenarios. *AoB PLANTS* 12(2): plaa007. https://doi.org/10.1093/aobpla/plaa007

Takahashi D., Isagi Y., Li P., Qiu Y.-X., Setoguchi H., Suyama Y., Matsuo A., Tsunamoto Y., Sakaguchi S. 2022. Stable persistence of relict populations involved evolutionary shifts of reproductive characters in the genus *Tanakaea* (Saxifragaceae). *J. Syst. Evol.* 60(6): 1405–1416. https://doi.org/10.1111/jse.12849

Takahashi D., Suyama Y. 2023. Demographic history and conservation aspects of two relict shrubs (*Daphnimorpha* spp., Thymelaeaceae) showing disjunct and restricted distribution in the southern part of Japan. *Tree Genet. Genomes* 19: 43. https://doi.org/10.1007/s11295-023-01618-6

Tudryn A., Chalié F., Lavrushin Yu. A., Antipov M. P., Spiridonova E. A., Lavrushin V., Tucholka P., Leroy S. A. G. 2013. Late Quaternary Caspian Sea environment: Late Khazarian and Early Khvalynian transgressions from the lower reaches of the Volga River. *Quat. Int.* 292: 193–204. https://doi.org/10.1016/j.quaint.2012.10.032

Valtuena F. J., Preston C. D., Kadereit J. W. 2012. Phylogeography of a Tertiary relict plant, *Meconopsis cambrica* (Papaveraceae), implies the existence of northern refugia for a temperate herb. *Mol. Ecol.* 21(6): 1423–1437. https://doi.org/10.1111/j.1365-294X.2012.05473.x

Vásquez D. L. A., Balslev H., Møller Hansen M., Sklenář P., Romoleroux K. 2016. Low genetic variation and high differentiation across sky island populations of *Lupinus alopecuroides* (Fabaceae) in the northern Andes. *Alp. Bot.* 126: 135–142. https://doi.org/10.1007/s00035-016-0165-7

Wang S.-Q. 2020. Genetic diversity and population structure of the endangered species *Paeonia decomposita* endemic to China and implications for its conservation. *BMC Plant Biol.* 20: 510. https://doi.org/10.1186/s12870-020-02682-z

Wu F. Q., Shen S. K., Zhang X. J., Wang Y. H., Sun W. B. 2015. Genetic diversity and population structure of an extremely endangered species: the world's largest *Rhododendron*. *AoB PLANTS* 7: plu082. https://doi.org/10.1093/aobpla/plu082

Xu Z., Zhang M.-L. 2015. Phylogeography of the arid shrub *Atraphaxis frutescens* (Polygonaceae) in Northwestern China: evidence from cpDNA sequences. *J. Hered.* 106(2):184–195. https://doi.org/10.1093/jhered/esu078

Yanina T. A. 2014. The Ponto-Caspian region: Environmental consequences of climate change during the Late Pleistocene. *Quat. Int.* 345: 88–99. https://doi.org/10.1016/j.quaint.2014.01.045

Yeh F. C. 1997. Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belg. J. Bot.* 129: 157.

Zachos F. E., Habel J. C. 2011. *Biodiversity hotspots. Distribution and protection of conservation priority areas.* Berlin: Springer-Verlag. 546 pp. https://doi.org/10.1007/978-3-642-20992-5

Zhang L., Sun F., Ma S., Wang C., Wei B., Zhang Y. 2022. Phylogeography of *Amygdalus mongolica* in relation to Quaternary climatic aridification and oscillations in northwestern China. *PeerJ.* 10: e13345. https://doi.org/10.7717/peerj.13345

Zhang M.-L., Wen Z.-B., Fritsch P. W., Sanderson S. C. 2015. Spatiotemporal evolution of *Calophaca* (Fabaceae) reveals multiple dispersals in Central Asian Mountains. *PLoS ONE* 10: e0123228. https://doi.org/10.1371/journal.pone.0123228

Zhao Y., Pan B., Zhang M. 2019. Phylogeography and conservation genetics of the endangered *Tugarinovia mongolica* (Asteraceae) from Inner Mongolia, Northwest China. *PLoS ONE* 14: e0211696. https://doi.org/10.1371/journal. pone.0211696