

Article

Current Phylogeographic Structure of *Anemone altaica* (Ranunculaceae) on the Khamar-Daban Ridge Reflects Quaternary Climate Change in Baikal Siberia

Marina Protopopova ^{1,*} , Polina Nelyubina ^{1,2} and Vasiliy Pavlichenko ¹ 

¹ Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences, Lermontov St., 132, 664033 Irkutsk, Russia

² Faculty of Biology and Soil Sciences, Irkutsk State University, Karl Marx St., 1, 664003 Irkutsk, Russia

* Correspondence: marina.v.protopopova@gmail.com

Abstract: *Anemone altaica* Fisch. ex C. A. Mey., a component of the tertiary boreo-nemoral vegetation complex, exhibits a disjunct distribution from European Russia to Central China. The Khamar-Daban Ridge, extending along Lake Baikal's southern coast, has served as a refugium preserving mesophilic forest remnants in South Siberia since the Pleistocene. This study aimed to elucidate the phylogenetic relationships and historical biogeography of *A. altaica* within the Khamar-Daban refugium using plastid DNA markers (*trnL* + *trnL-trnF*). Phylogenetic and mismatch distribution analysis revealed polyphyly (more specifically diphyly) among *A. altaica* lineages, suggesting past hybridization events with related species followed by backcrossing. Estimation of isolation by distance effect, spatial autocorrelation analysis, PCoA, and AMOVA indicated a clear spatial genetic structure for *A. altaica* on the Khamar-Daban Ridge. The most reliable geographical model suggests that during periods of Pleistocene cooling, *A. altaica* persisted in at least six microrefugia within the ridge. Populations associated with these microrefugia formed western, central, and eastern genetic supergroups with limited gene flow among them. Gene flow likely occurred more easily during glaciations or early interglacials when the subalpine zone shifted closer to Lake Baikal due to the depression of the snow boundary, allowing adjacent populations to intermingle along the glacial edges and terminal moraines in mountain forest belt.



Academic Editor: James B. Innes

Received: 21 September 2024

Revised: 26 March 2025

Accepted: 3 April 2025

Published: 22 April 2025

Citation: Protopopova, M.; Nelyubina, P.; Pavlichenko, V. Current Phylogeographic Structure of *Anemone altaica* (Ranunculaceae) on the Khamar-Daban Ridge Reflects Quaternary Climate Change in Baikal Siberia. *Quaternary* **2025**, *8*, 20. <https://doi.org/10.3390/quat8020020>

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Keywords: climate change; glaciation; Cis-Baikal; disjunct distribution; hybrids; nemoral refugia; ice age; South Siberia; microrefugia; nemoral relict; tertiary relict; the Late Cenozoic

1. Introduction

Anemone altaica Fisch. ex C. A. Mey. refers to the tertiary boreo-nemoral vegetation complex and currently exhibits a strongly disjunctive distribution, stretching from the Arctic regions of European Russia to Central China. The species is occasionally regarded as infraspecific taxon of European *A. nemorosa* L. and has close related and vicariant taxa in East Asia and North America, e.g., *A. amurensis* (Korsh.) Kom. and *A. quinquefolia* L. However, comprehensive molecular phylogenetic reconstructions of *Anemone* L. have not yet fully resolved its phylogenetic relationships with these closely related species, primarily due to the limited sampling of these taxa. At present, at least half of *A. altaica* range is located in North and Northeast Asia. The range disjunction occurred due to dramatic changes in climatic condition during the Late Cenozoic, particularly progressive cooling and increased continentality, culminating in the Pleistocene. Although North Asia was not covered by a continuous ice shield during the Pleistocene, its vegetation underwent

significant alteration compared to its state in the Pliocene [1,2]. Specifically, the degradation of the Tertiary boreo-nemoral floristic complex finally resulted in the fragmentation of the trans-Palearctic broad-leaved forest zone into European and East Asian distinct segments, creating an expansive ‘gap’ spanning continental Eastern Siberia and Central Asia. The mountains of South Siberia played a crucial role as refugia in preserving mesophilous remnants of broad-leaved forests during the Pleistocene [1,2]. This highlights the great importance of these mountains in maintaining the biological diversity of the region. Within South Siberia, well-defined refugia are located in the mountainous areas and foothills of Gornaya Shoria, the Northeastern and Southwestern Altai, the Western Sayan and Eastern Sayan Mountains, and the Khamar-Daban Ridge [3–6]. The northern macroslope of the Khamar-Daban Ridge, which stretches approximately 320 km from west to east along the southern coast of Lake Baikal [5] and contributes to the formation of its catchment area, represents the easternmost concentration of nemoral relict species among the refugia areas mentioned above [4]. The bottom sediments analysis of Lake Baikal revealed that a nemoral dark coniferous–broad-leaved complex dominated Cis-Baikal region until approximately the midpoint of the Late Pliocene, acquiring more boreal features around the turn of the Pliocene and Eopleistocene [7]. The current climatic conditions here are determined by the influence of the water masses of Lake Baikal and the barrier role of the Khamar-Daban Ridge itself, which sets it apart from the adjacent regions in the south of Eastern Siberia. The climate along the northern macroslope of the ridge exhibits a milder character, featuring increased precipitation and a robust snow coverage, thereby approximating that of a temperate continental conditions [4,8,9]. The relatively even and humid climate contributes to the dominance of dark-coniferous forests, preserving nemoral relict species and allowing for their abundant distribution over a large area. The presence of a nemoral refugium on the northern macroslope of the Khamar-Daban Ridge was first demonstrated by N.A. Epova in 1956 [4,6]. The Pleistocene glaciations in this area had a mountain-valley character [5,7,10,11], which, combined with the fact that nemoral relicts are mostly confined to river and watercourses [12], resulted in the formation of microrefugia in certain valleys of major rivers within the Khamar-Daban Ridge. Originating from the dark coniferous-broad-leaved biome, *A. altaica* may be considered here as Tertiary nemoral relict species [13–15]. The present distribution of *A. altaica* on the ridge extends along its northern macroslope, expanding towards the west up to the Burovshina River [15] and towards the east up to the Timlyuy River near Kamensk settlement (this study). *Anemone altaica* grows here in floodplain forests and confines to riverbeds and streams. In the highlands, the species is often found in subalpine high-grass meadows. The phylogeographic structure of *A. altaica* has not been studied, and we know little about how populations survived the Pleistocene cold spells and glaciations on the Khamar-Daban Ridge and how they re-expanded during more favorable periods.

This study aimed to elucidate the phylogenetic relationships of *A. altaica* with closely related species and to shed light on its historical biogeography within the Khamar-Daban Ridge refugium during climate changes in the late Cenozoic, using molecular markers.

2. Materials and Methods

2.1. Sample Collection

From three to ten individuals were collected from each natural population, and single specimens per species were taken from herbarium collections. Each fresh sample was placed in an individual filter paper bag ($23\text{ g}\cdot\text{m}^{-2}$), dried, and stored in dry silica gel until DNA isolation. *Anemone altaica* plants were collected from 24 natural populations on the Khamar-Daban Ridge (KhD) and Western Sayan Mountains (WS) and respective vouchers were stored in herbarium of Irkutsk State University (IRKU, Irkutsk, Russia). The samples of *A. reflexa* Steph. & Willd. were collected from the Khamar-Daban Ridge as a reference. The sample of *Anemone nemorosa* L.

was collected from herbarium of V.F. Kuprevich Institute of Experimental Botany of the National Academy of Sciences of Belarus (MSK, Minsk, Republic of Belarus) and *A. quinquefolia* L. from the herbarium of the Federal Scientific Center of the East Asia Terrestrial Biodiversity of the Far Eastern Branch of the Russian Academy of Sciences (VLA, Vladivostok, Russia). A total of 228 specimens were collected and analyzed. The detailed list of samples is presented in Table 1.

Table 1. List of collected specimens of *A. altaica* and reference species.

Locality Abbr.	Voucher Information ¹	Coordinates, Altitude ²
<i>Anemone altaica</i>		
B1	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>B</u> abkha Riv., 6 June 2018, V. Chepinoga, A. Skornyakova, A. Yudinseva (IRKU094289)	51.52832° N 104.09334° E 493 m alt.
BN1	Russia, South Siberia, Krasnoyarskiy Kray, Ermakovskiy Rayon, the Western Sayan Mountains, <u>N</u> izhnyaya <u>B</u> uyba Riv., 11 June 2018, V. Pavlichenko, V. Chepinoga, M. Protopopova (IRKU042130–31)	52.824383° N 93.309001° E 1313 m alt.
BR1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>B</u> ol'shaya <u>R</u> echka Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094254)	51.95788° N 106.35214° E 469 m alt.
Bz1	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>B</u> ezymyannaya Riv., 3 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094248)	51.59373° N 103.90829° E 461 m alt.
E1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>E</u> lovka Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094252)	51.975154° N 106.504712° E 519 m alt.
HM1	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>K</u> hara- <u>M</u> urin Riv., 3 June 2022, M. Protopopova, V. Pavlichenko (IRKU094247)	51.455189° N 104.413624° E 479 m alt.
M1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the Bol'shoy <u>M</u> amay Riv., 3 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094246)	51.44989° N 104.78263° E 472 m alt.
M2	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the Bol'shoy <u>M</u> amay Riv., 27 June 2023, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU IRKU094271)	51.433755° N 104.798493° E 518 m alt.
M3	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the Bol'shoy <u>M</u> amay Riv., 27 June 2023, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU IRKU094272–74)	51.376564° N 104.868684° E 1208 m alt.
MM1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>M</u> aliy <u>M</u> amay Riv., 2 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094257)	51.450789° N 104.813297° E 466 m alt.
Mn1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>M</u> anturikha Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094242)	51.771462° N 105.984607° E 451 m alt.
MOs1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>O</u> sinovka Riv. (near <u>M</u> ishikha Riv.), 2 June 2024, M. Protopopova, P. Nelyubina, V. Pavlichenko (IRKU094278–80)	51.591755° N 105.384154° E 474 m alt.
Ms1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>M</u> ysovka Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094253)	51.67841° N 105.89572° E 513 m alt.
Msh1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>M</u> ishikha Riv., 1 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094255)	51.645528° N 105.542409° E 461 m alt.
N1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>N</u> ikitkina Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094243)	51.971705° N 106.487322° E 487 m alt.
Pr1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>P</u> ereemnaya Riv., 1 June 2024, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094275–77)	51.513294° N 105.207800° E 474 m alt.
S2	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>S</u> nezhnaya Riv., 2 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094251)	51.406766° N 104.642759° E 473 m alt.

Table 1. Cont.

Locality Abbr.	Voucher Information ¹	Coordinates, Altitude ²
S3	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>S</u> nezhnaya Riv., 29 June 2023, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094261-63)	51.35570° N 104.61346° E 936 m alt.
So1	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>S</u> olzan Riv., 3 June 2024, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094281-83,87)	51.488595° N 104.157478° E 534 m alt.
So2	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>S</u> olzan Riv., 3 June 2024, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094284-86)	51.498915° N 104.158083 E 513 m alt.
T1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>T</u> imlyuy Riv., 31 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094244)	51.965865° N 106.581702° E 516 m alt.
TOs1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>O</u> sinovka (near <u>T</u> ankhoy setl.) Riv., 1 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094256)	51.549228° N 105.094652° E 469 m alt.
Ut2	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>U</u> tulik Riv., 30 May 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094250)	51.54594° N 104.04675° E 453 m alt.
V1	Russia, South Siberia, the Republic of Buryatia, Kabanskiy Rayon, the Khamar-Daban Ridge, the <u>V</u> ydrinaya Riv., 1 June 2022, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094245)	51.485538° N 104.848959° E 457 m alt.
<i>Anemone nemorosa</i>		
	Republic of Belarus, Eastern Europe, Minskaya Oblast', Minskiy Rayon, 16 May 1979, G.A. Kim (MSK-V388)	Unknown
<i>Anemone reflexa</i>		
Bz1	Russia, South Siberia, Irkutskaya Oblast', Slyudyanskiy Rayon, the Khamar-Daban Ridge, the <u>B</u> ezymyannaya Riv., 30 June 2023, M. Protopopova, V. Pavlichenko, P. Nelyubina (IRKU094288)	51.59373° N 103.90829° E 461 m alt.
<i>Anemone quinquefolia</i>		
–	USA, North America, Massachusetts, Belchertown, 14 May 1989, C.R. Lombardi (VLA)	Unknown

Notes: ¹ Bold and underlined letters in the vouchers indicate those included in the locality abbreviations. ² The geographic coordinates and altitude data were referenced by combined GPS/GLONASS positioning, datum WGS84.

2.2. DNA Isolation, PCR, and Sequencing

Total DNA was isolated from silica gel-dried leaf tissue following the cetyltrimethylammonium bromide (CTAB) method [16] with some authors' modifications [17]. For phylogenetic reconstruction, sequences of *trnL* intron and *trnL-trnF* intergenic spacer of plastid DNA (ptDNA) were used as molecular markers. DNA region including both markers was amplified as an entire fragment using the combination of forward (c) and reverse (f) primers described in the study of P. Taberlet et al. [18]. Polymerase chain reaction (PCR) was performed in 20 µL reaction mixture containing 0.5 units of GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA), 1 × Green GoTaq Flexi Buffer, 2.5 mM of MgCl₂, 250 µM of each dNTP, and 250 nM of each primer. The amplification conditions were 95 °C for 5 min., followed by 35 cycles at 95 °C for 30 s; 57 °C for 30 s, and 72 °C for 1.5 min., with a final extension at 72 °C for 5 min. In the case of herbarium samples, insufficient amplification of the fragment, including both the *trnL* intron and *trnL-trnF* region was observed due to DNA degradation, probably caused by long-term storage and treatments involving freezing and/or insecticides. In this case, amplification of each of the two markers was carried out separately, using the primer combinations of c + d for *trnL* intron and e + f for *trnL-trnF* [18]. The reaction mixture and amplification condition for both markers were the same as described above, with the only difference being that extension time was reduced to 1 min in each cycle.

Amplicons were visualized in 1% agarose gel stained by ethidium bromide after electrophoresis, then gel-purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania), and Sanger sequenced using the BigDye Terminator Cycle

Sequencing Kit version 3.1 (Applied Biosystems, Waltham, MA, USA) in a 3500 Genetic Analyzer (Applied Biosystems and Hitachi, Tokyo, Japan). Each amplicon was sequenced using four different region-specific primers (c, d, e, and f).

2.3. Sequence Alignment and Phylogenetic Analysis

Raw sequencing data were edited using SnapGene Viewer software version 2.6.2 (GSL Biotech, San Diego, CA, USA) and MEGA software version 7.0.16 [19] and deposited in GenBank of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov>, accessed on 10 August 2024). For analysis, we aligned original DNA sequences of *A. altaica* with the sequences of closely related species (Table 2).

Table 2. The taxa and DNA sequences used for the phylogenetic reconstructions.

Taxon Name	Region ¹	Locality	Microrefugium ²	Supergroup ²	Plastotype ³	n ⁴	GenBank Accession Numbers		Ref.
							<i>trnL</i>	<i>trnL-trnF</i>	
<i>Anemone altaica</i>	EA *	—	—	—	P1	1	OK267274.1	OK267274.1	[20]
<i>Anemone altaica</i>	KhD	B1	I	W	P3	3	PQ660085	PQ660123	curr.
<i>Anemone altaica</i>	KhD	B1	I	W	P4	3	PQ660086	PQ660124	curr.
<i>Anemone altaica</i>	KhD	BR1	V	E	P1	10	PQ660087	PQ660125	curr.
<i>Anemone altaica</i>	KhD	Bz1	I	W	P3	6	PQ660088	PQ660126	curr.
<i>Anemone altaica</i>	KhD	E1	VI	E	P2	10	PQ660089	PQ660127	curr.
<i>Anemone altaica</i>	KhD	HM1	II	C	P2	6	PQ660090	PQ660128	curr.
<i>Anemone altaica</i>	KhD	M1	II	C	P2	8	PQ660091	PQ660129	curr.
<i>Anemone altaica</i>	KhD	M2	II	C	P2	10	PQ660092	PQ660130	curr.
<i>Anemone altaica</i>	KhD	M3	II	C	P2	10	PQ660093	PQ660131	curr.
<i>Anemone altaica</i>	KhD	MM1	III	C	P1	10	PQ660094	PQ660132	curr.
<i>Anemone altaica</i>	KhD	Mn1	V	E	P1	10	PQ660095	PQ660133	curr.
<i>Anemone altaica</i>	KhD	MOs1	IV	C	P2	5	PQ660096	PQ660134	curr.
<i>Anemone altaica</i>	KhD	MOs1	IV	C	P5	5	PQ660097	PQ660135	curr.
<i>Anemone altaica</i>	KhD	Ms1	V	E	P1	8	PQ660098	PQ660136	curr.
<i>Anemone altaica</i>	KhD	Ms1	V	E	P2	2	PQ660099	PQ660137	curr.
<i>Anemone altaica</i>	KhD	Msh1	IV	C	P2	7	PQ660100	PQ660138	curr.
<i>Anemone altaica</i>	KhD	Msh1	IV	C	P5	3	PQ660101	PQ660139	curr.
<i>Anemone altaica</i>	KhD	N1	V	E	P1	9	PQ660102	PQ660140	curr.
<i>Anemone altaica</i>	KhD	N1	V	E	P2	2	PQ660103	PQ660141	curr.
<i>Anemone altaica</i>	KhD	Pr1	IV(III)	C	P2	10	PQ660104	PQ660142	curr.
<i>Anemone altaica</i>	KhD	S2	II	C	P2	8	PQ660105	PQ660143	curr.
<i>Anemone altaica</i>	KhD	S2	II	C	P4	3	PQ660106	PQ660144	curr.
<i>Anemone altaica</i>	KhD	S3	II	C	P2	10	PQ660107	PQ660145	curr.
<i>Anemone altaica</i>	KhD	So1	I	W	P3	10	PQ660108	PQ660146	curr.
<i>Anemone altaica</i>	KhD	So2	I	W	P3	7	PQ660109	PQ660147	curr.
<i>Anemone altaica</i>	KhD	So2	I	W	P4	3	PQ660110	PQ660148	curr.
<i>Anemone altaica</i>	KhD	T1	VI	E	P2	10	PQ660111	PQ660149	curr.
<i>Anemone altaica</i>	KhD	TOs1	III	C	P1	2	PQ660112	PQ660150	curr.
<i>Anemone altaica</i>	KhD	TOs1	III	C	P2	4	PQ660113	PQ660151	curr.
<i>Anemone altaica</i>	KhD	TOs1	III	C	P4	4	PQ660114	PQ660152	curr.
<i>Anemone altaica</i>	KhD	Ut2	I	W	P3	8	PQ660115	PQ660153	curr.
<i>Anemone altaica</i>	KhD	Ut2	I	W	P4	2	PQ660116	PQ660154	curr.
<i>Anemone altaica</i>	KhD	V1	III	C	P1	2	PQ660117	PQ660155	curr.
<i>Anemone altaica</i>	KhD	V1	III	C	P2	10	PQ660118	PQ660156	curr.
<i>Anemone altaica</i>	WS	BN1	—	—	P6	3	PQ660119	PQ660157	curr.
<i>Anemone amurensis</i>	EA	—	—	—	—	1	EF139341.1	EF139341.1	[21]
<i>Anemone nemorosa</i>	EE	—	—	—	—	1	PQ660120	PQ660158	curr.
<i>Anemone reflexa</i>	EA	—	—	—	P1	1	MW043774	MW043774	[22]
<i>Anemone reflexa</i>	KhD	Bz1	—	—	P2	3	PQ660121	PQ660159	curr.
<i>Anemone quinquefolia</i>	NA	—	—	—	—	1	PQ660122	PQ660160	curr.
<i>Anemonastrum canadense</i> (L.) Mosyakin	—	—	—	—	—	1	AB248018.1	AB248018.1	[23]
<i>Anemonastrum narcissiflora</i> L.	—	—	—	—	—	1	AB248017.1	AB248017.1	[23]

Notes: ¹ EA—East Asia; EE—Eastern Europe; KhD—the Khamar-Daban Ridge; NA—North America; WS—the Western Sayan Mountains; * indicates the proposed region of sample due to the lack of detailed information in the original study. ² Association with microrefugium and/or supergroup is based on geographic models that are strongly supported by statistical evidence, as presented in the results section. ³ The identified plastotypes were continuously numbered for each species separately. ⁴ The number of sequences used in the analysis.

The *trnL* intron and the *trnL-trnF* markers were analyzed as combined dataset. The multiple alignments of nucleotide sequences by the MUSCLE application with a gap opening penalty of 500 and an extension penalty of 4 were conducted in MEGA, followed by manual editing. The generated insertion/deletion regions in alignments were considered one evolutionary event, were coded as binary characters (the presence '1' or absence '0' of the gap), and included as a separate binary data partition at the end of the matrix. In particular, 32 indels (site # 50–54, 68–83, 111, 112–114, 187–190, 198–201, 233, 253–266, 282–285, 292, 294–330, 331–333, 338–344, 582–583, 605–609, 610, 610–611, 610–612, 610–613, 610–614, 610–615, 610–617, 645–679, 708–717, 772–775, 779–788, 793–798, 799–804, 821–824, 832–839, 851, 851–854) from a total length of 915 alignment positions, were coded as binary data.

Phylogenetic reconstructions were obtained independently by the maximum likelihood (ML) method based on multiple nucleotide sequence alignments in MEGA and the Bayesian inference (BI) method based on the matrices combining the nucleotide alignments and binary (gaps) datasets in MrBayes version 3.2.5 [24]. The best-fit model of nucleotide substitutions based on the lowest Bayesian Information Criterion (BIC) calculated using the “find best DNA/protein models” tool in MEGA (Neighbor-Joining tree to use and ML as a statistical method were applied as the settings) was selected and then used to perform the analysis. Nucleotide frequencies calculated using the ‘find best DNA/protein models’ tool were also included to optimize the models implemented in MrBayes in the case of the Bayesian inference analysis.

For ML analysis, the Tamura 3-parameter model (T92, [25]) with no among-site rate variation was used for the entire *trnL* + *trnL-trnF* dataset and omitting the binary matrix. The initial tree for the heuristic search was obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. All aligned positions, including the indels, were used in the analysis. A bootstrap of 1000 replicates was used as a test of the phylogeny and the bootstrapped 50% majority-rule consensus trees were constructed.

The BI analysis of the nucleotide datasets was performed using the models implemented in MrBayes, with optimized parameters to better correspond with the model used in the ML analysis. The BI analysis was performed by specifying the model and parameters for each partition of the datasets separately using the ‘applyto’ option. For the DNA sequence data, the HKY-like model [26] was used, with the base frequencies optimized for the T92 model and fixed on the following values: *trnL* partition: A = 0.34, C = 0.16, G = 0.16, and T = 0.34; *trnL-trnF* partition: A = 0.35, C = 0.15, G = 0.15, and T = 0.35, with no evolutionary rate variation among the sites for both partitions. The indel partition was analyzed using the F81-like model [27] implemented in MrBayes, with equal stationary state frequencies to match the JC69 model [28].

For each dataset, two simultaneous and independent Markov chain Monte Carlo (MCMC) analyses were run with four parallel chains up to 10,000,000 generations, with sampling every 100 generations and diagnostic calculations every 1000 generations. The first 25% of samples from the cold chain were discarded. The standard deviation of split frequencies below 0.01 was regarded as sufficient convergence, and that value was considered chain stationarity being reached. The fluctuations of the cold chain likelihood in the stable range were also considered for the estimate of reaching stationarity. The sampled trees from both analyses were pooled, and 50% majority-rule consensus trees were constructed from 13,532 trees to estimate clade posterior probability values (PP). The final phylogenetic trees were edited in FigTree version 1.4.3 [29].

The final phylogenetic tree is presented as BI phylogram, with the additional indication of bootstrap values (BS) for the clades on the corresponding ML tree.

A network based on the *trnL* + *trnL-trnF* alignment was constructed using the TCS method [30] implemented in PopART software v. 1.7 [31].

The Analysis of Molecular Variance (AMOVA), nucleotide diversity (π), number of segregating sites (S), and Tajima’s D test of neutrality [32] were also calculated using PopArt. The

AMOVA was conducted to evaluate several geographical hypotheses regarding *A. altaica* on the Khamar-Daban Ridge utilizing ϕ -statistics [33] which is an optimized approach for haploid data. Specifically, we tested hypotheses concerning 23 distinct populations (without grouping) and explored the potential grouping of these populations into four to six groups, each associated with potential Pleistocene microrefugia, at least four of which were predicted in our previous studies [5,17]. The groups associated with microrefugia, as well as the corresponding microrefugia discussed in the text, are designated by Roman numerals (I–VI) and are numbered from west to east along the Khamar-Daban Ridge. The hypotheses we tested varied not only in the number of population groups but also in the boundaries of the zones associated with the putative microrefugia, specifically the population composition of these groups. This variation primarily arises from the fact that border populations tend to belong more significantly to one of the adjacent refugia than to the other. This issue was particularly relevant for the boundaries between Microrefugia II and III, as well as between III and IV. In the first case, this involved the locations of populations along the Maliy Mamay and Vydrinaya rivers (localities MM1 and V1), while in the second case, it pertained to the positioning of populations along the Pereemnaya River (locality Pr1). Additionally, we examined three geographical supergroups: western (W), central (C), and eastern (E), which encompassed Microrefugium I, Microrefugia II + III + IV, and Microrefugia V + VI, respectively (Table 2). A more detailed explanation of the hypotheses tested is provided in the results section.

Calculations of genetic diversity metrics, including the Information Index for genetic diversity (equivalent to the Shannon-Weaver Index) and ϕ' -indexes (standardized ϕ -statistics), Principal Coordinates Analysis (PCoA), and tests of Wright's Isolation by Distance (IBD) hypothesis [34], as well as estimations of global and local spatial autocorrelation, were performed using GenAlEx v.6.5. [35,36]. The PCoA of all samples was performed using a distance matrix with data standardization. In this case, pairwise distances were calculated following the method outlined by D.R. Huff et al. [37]. Additionally, the PCoA on populations was conducted using a covariance matrix with data standardization based on pairwise Nei unbiased genetic distances [38]. The impact of isolation by distance (IBD) was evaluated by correlating the matrix of geographic distances between populations with the matrix of their genetic differentiation values (ϕ -statistics). This analysis was performed using a Mantel test [39,40], which involved statistical testing through 10,000 random permutations. Global spatial autocorrelation was estimated using the method developed by P.E. Smouse & R. Peakall [41], as implemented in GenAlEx. Statistical testing for spatial autocorrelation was performed based on 1000 random permutations, along with bootstrap resampling using 1000 replicates. The significance of the overall correlogram was assessed using the heterogeneity test as outlined by P.E. Smouse et al. [42]. Additionally, the 2-Dimensional Local Spatial Autocorrelation (2D LSA) approach proposed by M.C. Double et al. [43] was employed to examine local patterns of spatial genetic autocorrelation across a two-dimensional landscape. Significance testing was conducted using one-tailed or two-tailed tests with 1000 permutations. Analysis of mismatch distribution was performed in Microsoft Excel [44] based on a pairwise genetic distance matrix of all samples calculated using GenAlEx. All graphs were also created using Microsoft Excel tools.

3. Results

3.1. Phylogenetic Relationships Between *Anemone altaica* and Close Related Species

The phylogenetic analysis using ptDNA revealed a clear topology, distinguishing at least six plastotypes (P1–P6) of *A. altaica* (Figure 1). The topology of the tree suggested a curious polyphyletic nature of *A. altaica* (Figure 1a). In particular, two genetic lineages of *A. altaica* were found to be significantly associated with different plastogroups (PG). PG1 was exclusively represented by lineage I of *A. altaica*, while PG2 combined the plastotypes representing lineage II of *A. altaica* as well as plastotypes of other species, i.e., *A. reflexa*, *A.*

quinquefolia, and most likely *A. amurensis*. The lineage I embraced the plastotypes P1 and P6 representing *A. altaica* from the Western Sayan Mountains, East Asia, and partially from the Khamar-Daban Ridge. The lineage II represents all remaining plastotypes observed exclusively on the Khamar-Daban Ridge populations (Figure 1b). Within PGII, P3 of *A. altaica* clustered with the plastotypes of other *Anemone* species such as P2 of *A. reflexa* and *A. quinquefolia*. Remarkably, our analysis revealed that both plastotypes of *A. reflexa* used in the study were identical to either P3 or P4 of *A. altaica* from the Khamar-Daban Ridge (shown in one color in Figure 1a,b). Notably, the variations among plastotypes of lineage II (P2–P5) were exclusively expressed as indel polymorphisms rather than mismatches, while the distinction between plastotypes of two distinct lineages involved both mismatches and indels.

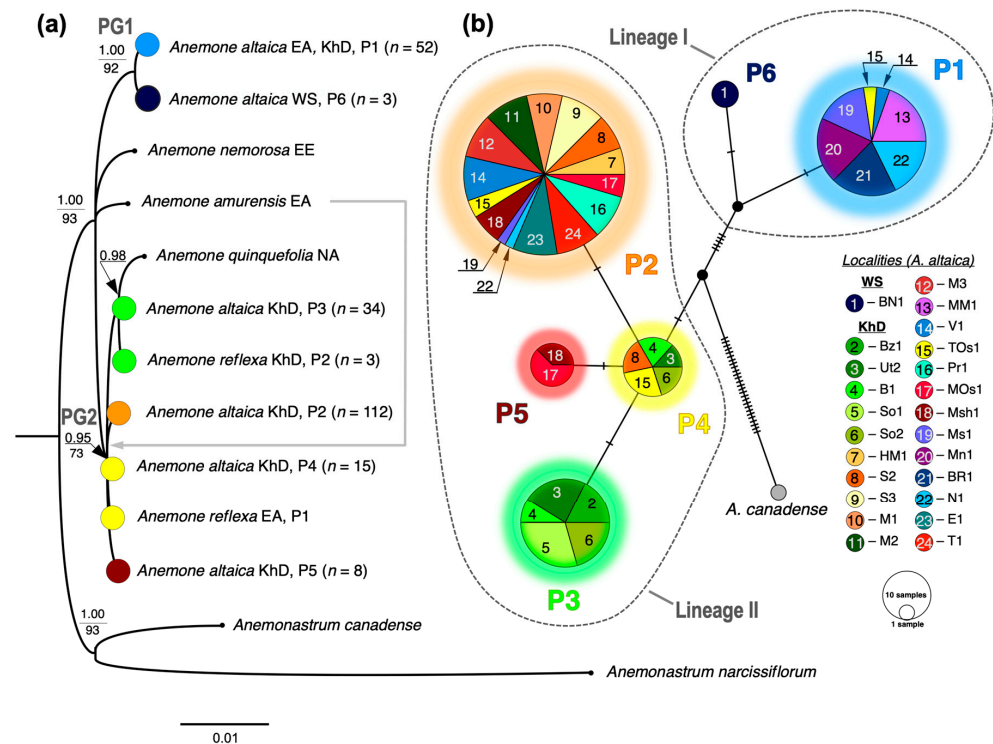


Figure 1. Diversity and phylogenetic relationships of plastotypes (P) in *Anemone altaica* from various localities. (a) BI phylogram of plastotypes of *A. altaica* and closely related species. Posterior probabilities (PP) are displayed above the dividing line in proximity to the nodes, Bootstrap values (BS) for the respective clades on the ML tree shown below this line. The gray arrow indicates the supported position of the taxon on ML 50% majority-rule consensus tree compared to the BI tree. The scale bar denotes the number of expected changes (substitutions and/or indels) per site, corresponding to a unit of branch length. The color pattern represents the distinct plastotypes (P1–P6) identified within *A. altaica* populations. Plastotypes of *A. reflexa* that correspond to those in *A. altaica* are color-coded in the same way. The number of identical sequences found for each plastotype is indicated in brackets next to the branch names. Absence of information regarding the number of sequences indicates that there is only one sequence per plastotype. The plastogroups (PG) discussed in the text indicated above the branches (b) TCS network constructed using only plastotypes from South Siberian populations of *A. altaica*. *Anemonastrum canadense* was used as an outgroup. Plastotypes P1–P6 in *A. altaica* are depicted as colored circles interconnected by lines, with hatch marks indicating the number of evolutionary events (substitutions/indels). The size of each circle reflects the count of identical sequences sampled (refer to the circular scale). The colors of circle sectors and accompanying numbers represent *A. altaica* populations. Unidentified black dots denote network vertices. Abbreviated regions and locality names correspond to those listed in Tables 1 and 2.

3.2. Phylogeographical Patterns of *A. altaica* on the Khamar-Daban Ridge Based on *ptDNA* Data

The dominant plastotype of *A. altaica* on the Khamar-Daban Ridge was P2, identified in 112 specimens across 15 populations out of a total of 220 individuals and 23 populations

(Figure 1b). Plastotype P1 was subdominant, occurring in 51 specimens belonging to 7 populations from the Khamar-Daban Ridge. It was also identical to the one deposited in GenBank which likely belongs to *A. altaica* from the People's Republic of China or another part of its East Asian range [20], although the exact origin of the sample is unknown due to insufficient information provided in the original study. In general, the plastotype intrapopulation diversity on the Khamar-Daban Ridge was found to be poor, with most populations (13 out of 23) carrying only one of the five plastotypes, nine populations carrying at least two plastotypes, and only one population carrying three plastotypes.

To evaluate the potential phylogeographic structure of *A. altaica*, we conducted a Principal Coordinates Analysis (PCoA) based on genetic distances (Figure 2).

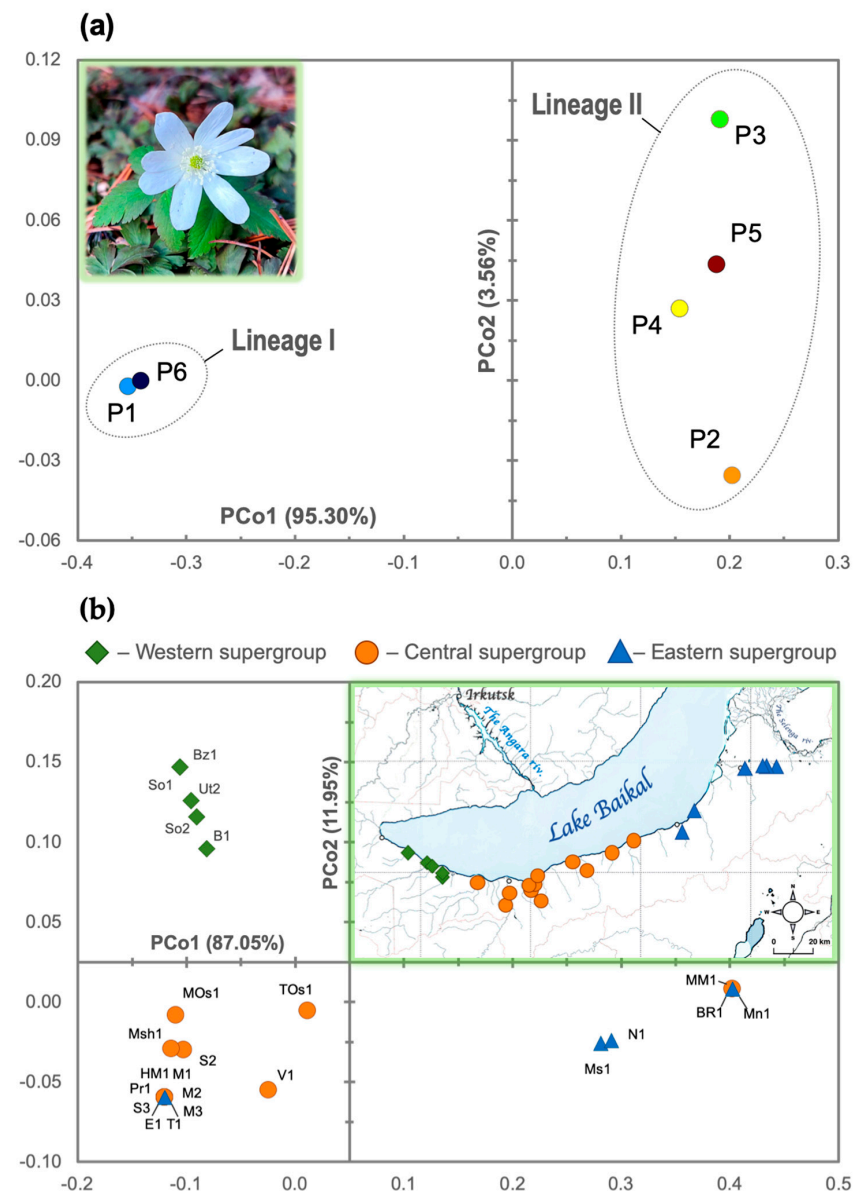


Figure 2. Patterns of genetic structure of *Anemone altaica* visualized using Principal Coordinate Analysis (PCoA) based on (a) pairwise genetic distances among all samples. The colors and corresponding abbreviations indicate the plastotypes, following a format similar to that shown in Figure 1a; (b) pairwise Nei unbiased genetic distances for the Khamar-Daban population only. Populations abbreviated consistently with Tables 1 and 2 and Figure 1b, and are colored according to their geographical supergroups, matching the map shown in insert in the upper right section of the graph field.

As anticipated, the PCoA, which plotted all samples based on their pairwise genetic distance matrix, revealed clustering patterns corresponding to six distinct plastotypes (Figure 2a). The plastotypes grouped into clusters representing two distinct lineages, a pattern consistent with both the phylogenetic analysis and the TCS network (Figure 1). The PCoA results, derived from Nei genetic distances among populations, identified at least three distinct genetic supergroups of populations on the Khamar-Daban Ridge (Figure 2b). This finding allowed us to propose a geographical grouping of populations based on their distribution along the northern macroslope of the ridge, considering the dominance of specific plastotypes. Based on the PCoA results, we conditionally categorized the populations into three supergroups: Western (W), Central (C), and Eastern (E). However, the potential for additional divisions within these supergroups cannot be entirely dismissed (Figure 2b). A detailed geographic distribution of plastotypes among the populations on the Khamar-Daban Ridge is illustrated in Figure 3.

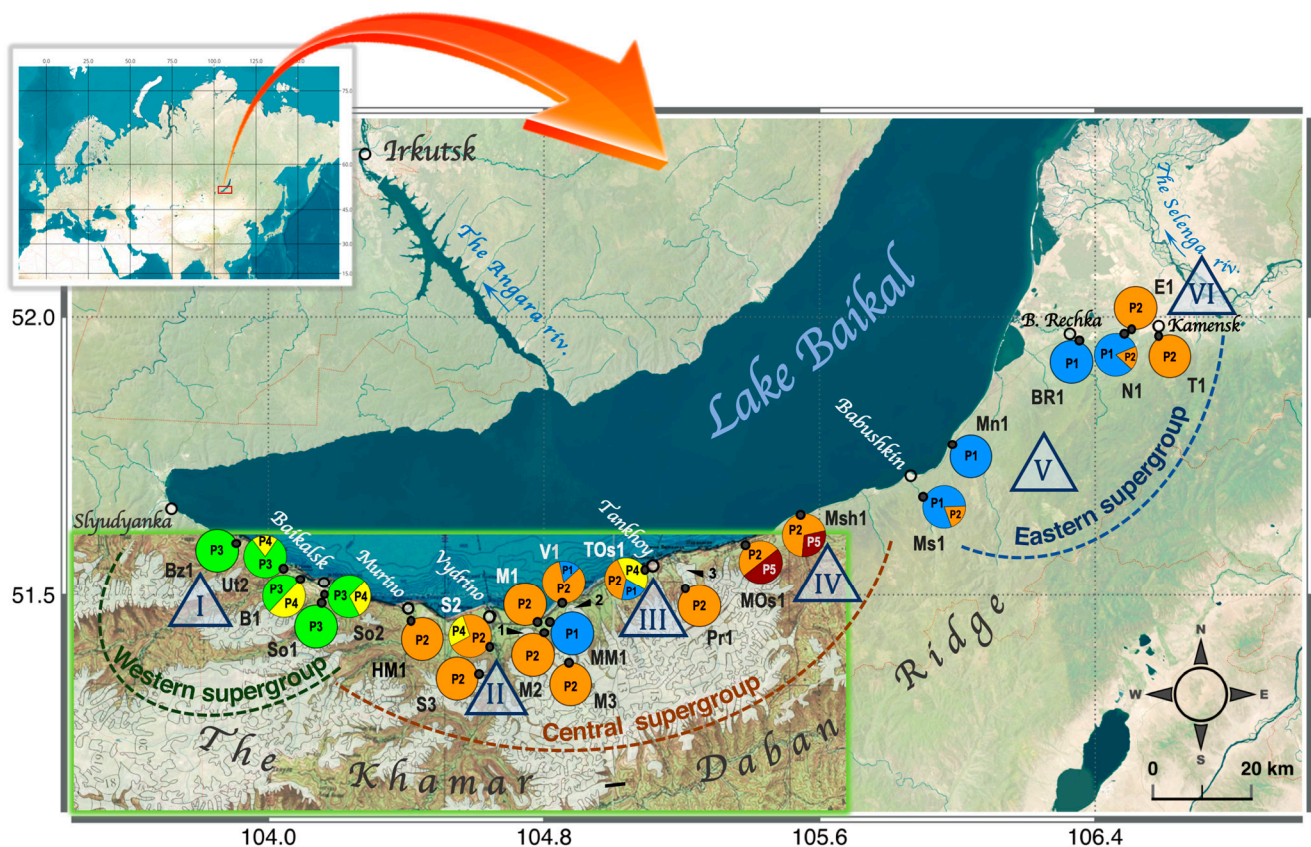


Figure 3. The phylogeographical patterns of *Anemone altaica* on the Khamar-Daban Ridge. The inset in the lower left corner is a superimposed image displaying the location of glaciers and their nourishment areas (showed as brighten areas with glacier isolines) during the Sartan (the last) glaciation, as referenced from [10]). Each colored circle represents distinct populations, abbreviated in a manner consistent with Table 1 and Figure 1b. The precise location of each population is marked by a small dark dot at the edge of the pie chart. The colors of the circle sectors, along with their corresponding abbreviations, indicate the plastotypes present in each population, following a format similar to that shown in Figure 1a. The size of the sectors reflects the proportion of individual plastotypes within the total sample from the population. Roman numerals within triangles schematically denote hypothesized Pleistocene microrefugia on the northern macroslope of the Khamar-Daban Ridge. Geographic supergroups of the populations discussed in the text are represented by arcuate dashed lines. Additionally, small numbered arrows highlight glacier tongues with maximum descent: 1—the Osinovka River (near Vydrino village), 2—the Vydrinaya River, 3—the Pereemnaya River. The map was generated using QGIS v. 3.36.0 [45], utilizing a map projection of WGS 84-Pseudo-Mercator and datum WGS84.

Specifically, within the Khamar-Daban Ridge, Plastotype P1 was dominant in the E supergroup, although it also appeared in other supergroups (Figure 3). Its maximum extent to the west reached the Maliy Mamay River (MM1 locality) in the central region of the macroslope. Plastotype P2 was dominant within the C supergroup and exhibited the most extensive distribution among the Khamar-Daban populations, being present from the Timlyuy River (T1 locality) near Kamensk settlement (E supergroup) in the east to the Khara-Murin River (HM1 locality) near Murino settlement (C supergroup) in the west. Plastotype P3 was exclusively found in the W supergroup, with its occurrence limited to the Solzan River (So1 and So2 localities) near Baikalsk city in the eastward direction. Plastotype P4 was extended from the C to the W supergroup of the populations, at least within the forest belt. Plastotype P5 was identified in the easternmost populations belonging to the C supergroup (MOs1 and Msh1 localities).

To evaluate the detailed phylogeographic structure of *A. altaica* on the Khamar-Daban Ridge, several geographical hypotheses were proposed and subsequently tested using the Analysis of Molecular Variance (AMOVA). The most representative of these hypotheses are presented in Table 3 and illustrated in Figure 3. The AMOVA for Hypothesis 1, which posits no spatial grouping, revealed a strong genetic differentiation within the Khamar-Daban Ridge populations ($\phi_{ST} = 0.81161$, $p < 0.001$). Notably, the majority of the genetic variation was attributed to interpopulation polymorphism (81.16%) rather than intrapopulation differences (18.84%) (Table 3).

Table 3. AMOVA statistics for different structure hypothesis of *Anemone altaica* on the Khamar-Daban Ridge.

Source of Variation	d.f.	Sum of Squares	Variation, %	ϕ -Statistics ¹	p -Value ²
Hypothesis 1: 23 distinct populations (without grouping)					
Among populations	22	2124.583	81.16	$\phi_{ST} = 0.81161$	<0.001
Within populations	197	431.576	18.84		
Hypothesis 2: Population supergroups: Western, Central, and Eastern					
Among groups	2	811.977	36.69	$\phi_{CT} = 0.36693$	=0.006
				$\phi'_{CT} = 0.81955$	=0.001
Among populations within groups	20	1312.607	47.61	$\phi_{SC} = 0.75199$	<0.001
				$\phi'_{SC} = 0.68006$	=0.001
Within populations	197	431.576	15.70	$\phi_{ST} = 0.84299$	<0.001
Hypothesis 3: Population groups associated with 4 microrefugia according to [5]					
Among groups	3	493.425	12.08	$\phi_{CT} = 0.12082$	=0.138
Among populations within groups	19	1631.158	70.36	$\phi_{SC} = 0.80028$	<0.001
Within populations	197	431.576	17.56	$\phi_{ST} = 0.82441$	<0.001
Hypothesis 4: Population groups associated with 5 microrefugia: I, II, III, IV, and V + VI					
Among groups	4	1112.208	39.22	$\phi_{CT} = 0.39223$	=0.011
				$\phi'_{CT} = 0.54290$	=0.001
Among populations within groups	18	1012.376	44.00	$\phi_{SC} = 0.72231$	<0.001
				$\phi'_{SC} = 0.69092$	=0.001
Within populations	197	431.576	16.88	$\phi_{ST} = 0.83123$	<0.001
Hypothesis 5: Population groups associated with 6 microrefugia (main): I, II, III, IV, V, and VI					
Among groups	5	1812.855	70.98	$\phi_{CT} = 0.70983$	<0.001
				$\phi'_{CT} = 0.86459$	=0.001
Among populations within groups	17	311.728	12.71	$\phi_{SC} = 0.43817$	<0.001
				$\phi'_{SC} = 0.36053$	=0.001
Within populations	197	431.576	16.30	$\phi_{ST} = 0.83698$	<0.001
Hypothesis 6: Population groups associated with 6 microrefugia (alternative): I, II, III, IV, V, and VI					
Among groups	5	1717.003	66.45	$\phi_{CT} = 0.66449$	<0.001
				$\phi'_{CT} = 0.85700$	=0.001
Among populations within groups	17	407.580	17.93	$\phi_{SC} = 0.51245$	<0.001
				$\phi'_{SC} = 0.39447$	=0.001
Within populations	197	431.576	16.36	$\phi_{ST} = 0.83642$	<0.001

Table 3. Cont.

Source of Variation	d.f.	Sum of Squares	Variation, %	ϕ -Statistics ¹	p -Value ²
Within (super)groups					
Western supergroup (W):					
Among populations	4	1.276	10.79	$\phi_{ST} = 0.10791$	=0.058
Within populations	37	5.200	89.21		
Central supergroup (C):					
Among populations	11	586.786	69.77	$\phi_{ST} = 0.69769$	<0.001
Within populations	105	219.248	30.23		
Eastern supergroup (E):					
Among populations	5	724.545	75.44	$\phi_{ST} = 0.75442$	<0.001
Within populations	55	207.127	24.56		
Microrefurium (group) I:					
Among populations	4	1.276	10.79	$\phi_{ST} = 0.10791$	=0.058
Within populations	37	5.200	89.21		
Microrefurium (group) II:					
Among populations	5	0.655	14.99	$\phi_{ST} = 0.14992$	=0.020
Within populations	49	2.182	85.01		
Microrefurium (group) III:					
Among populations	2	280.833	54.92	$\phi_{ST} = 0.54926$	<0.001
Within populations	29	198.667	45.07		
Microrefurium (group) IV:					
Among populations	2	5.067	15.34	$\phi_{ST} = 0.15337$	=0.019
Within populations	27	18.400	84.66		
Microrefurium (group) V:					
Among populations	3	23.897	3.00	$\phi_{ST} = 0.03002$	=0.227
Within populations	37	207.127	97.00		
Microrefurium (group) VI:					
Among populations	1	0	NaN	NaN	<0.001
Within populations	18	0	NaN		

Notes: ¹ ϕ -statistics were derived from an individual-by-individual genetic distance matrix, which was derived from the total number of locus differences. Additionally, ϕ' -values are presented for hypotheses that are statistically supported. The ϕ' -values were obtained by dividing the ϕ -values by the maximum theoretical ϕ -values. In this context, ϕ' -values were calculated using an alternative approach based on genetic distances matrix derived from differences in plastotype composition rather than from the number of locus differences; significant ϕ_{CT} values, which indicate differentiation among the tested groups, are highlighted in bold. ² Statistical testing was conducted through 1000 random permutations.

Hypothesis 2 was formulated based on the PCoA results (Figure 2), which identified three phylogeographical supergroups (W, C, and E) of *A. altaica* on the Khamar-Daban Ridge. AMOVA results (Table 3) revealed significant ϕ_{CT} (0.367, $p = 0.006$) and ϕ'_{CT} (0.820, $p = 0.001$) values, coupled with the high ϕ'_{CT} estimates (reflecting differentiation among supergroups) relative to ϕ'_{SC} -value (reflecting differentiation within supergroups). These results robustly support the three-supergroup structure, rejecting the null hypothesis of genetic homogeneity and panmixia. At the same time, the elevated ϕ_{SC} -value (0.722) relative to ϕ_{CT} (0.367) suggests potential finer-scale genetic substructure within the identified supergroup.

All remaining hypotheses were based on different numbers of potential Pleistocene microrefugia that constituted the revealed supergroups. According to these hypotheses, the main refugium located on the northern macroslope of the Khamar-Daban Ridge was divided into population groups that corresponded to several microrefugia and their associated zones of recolonization. In particular, Hypothesis 3 consisted of dividing into groups of populations associated with four refugia as previously suggested [5]. According to this division, the first group was the westernmost, the eastern boundary of which included localities on the Solzan River (So1 and So2), the eastern boundary of the second microrefugium included the locality on the Vydrinaya River (V1), and the eastern boundary of the third microrefugium passed along the Pereemnaya River (Pr1). The fourth group included all the remaining populations located to the east (Figure 3, [5]). The results of the AMOVA not supported this structure hypothesis because ϕ_{CT} not exceeding zero ($\phi_{CT} = 0.121$, $p = 0.138$).

Since the hypothesis of four microrefugia proved untenable, our subsequent strategies involved dividing the populations into additional groups on the eastern side of the range and adjusting the boundaries of the potential groups. This approach is based on two main reasons. Firstly, the initial allocation of four microrefugia was derived from a simultaneous analysis of the distribution of 27 relict plants [5], most of which have significantly narrower ranges—especially in the eastern part of the ridge—compared to that of *A. altaica*. Consequently, it is plausible that there are more than four microrefugia for *A. altaica*. Therefore, we also tested the hypotheses of five and six microrefugia. Secondly, assessing the affiliation of border populations was challenging due to shared plastotypes with specimens belonging to the adjacent microrefugia (Figure 3). This issue was particularly pertinent at the boundaries between Microrefugia II and III, III and IV, as well as V and VI, due to the close proximity of populations and their shared plastotypes. In the first case, this involved populations along the Maliy Mamay and Vydrinaya rivers (localities MM1 and V1). In the second case, it pertained to the positioning of populations along the Pereemnaya River (locality Pr1), and in the latter case, it involved locality on the Nikitkina River (N1).

In total, 12 alternative hypotheses to Hypothesis 3 were tested, each featuring a different number of microrefugia and varying boundaries positions. The most statistically supported hypotheses are presented in Table 3. Notably, none of the hypotheses proposing a division into groups associated with four microrefugia, in addition to Hypothesis 3, received statistical support. The ϕ_{CT} indexes exceeded zero for the hypotheses involving five (including Hypothesis 4 in Table 3) and six microrefugia (including Hypotheses 5 and 6 in Table 3). Importantly, the AMOVA results for structural Hypotheses 5 and 6 indicated that ϕ_{CT} surpassed ϕ_{SC} , suggesting their reliability. The distinction between Hypotheses 5 and 6 lies not in the number of microrefugia but in the positioning of the boundary between population groups associated with Microrefugia III and IV. In particular, in Hypothesis 6, unlike in Hypothesis 5, the locality near the Pereemnaya River (Pr1) was associated with Microrefugium III rather than IV. Although this division aligns more closely with the original hypothesis [5], Hypothesis 6 was deemed less optimal due to a lower ϕ_{CT} value compared to that of Hypothesis 5. Moreover, Hypothesis 5 exhibited the greatest excess of ϕ_{CT} over ϕ_{SC} (2.4 times) compared to all other tested hypotheses, indicating it represents the most optimal structural division.

According to Hypothesis 5, the populations were clustered into six groups associated with the proposed microrefugia as follows:

- Microrefugium I: Bz1, Ut2, B1, So1, and So2 localities
- Microrefugium II: HM1, S2, S3, M1, M2, and M3 localities
- Microrefugium III: MM1, V1, and TOs1 localities
- Microrefugium IV: Pr1, MOs1, and Msh1 localities
- Microrefugium V: Ms1, Mn1, Br1, and N1 localities
- Microrefugium VI: E1 and T1 localities

The approximate locations of microrefugia are indicated by Roman numerals within triangles in Figure 3. The associations between localities and microrefugia, based on the most supported hypotheses, are detailed in Table 2. According to this model of genetic-geographical division and consistent with Hypothesis 2, Microrefugium I corresponds to the W supergroup, Microrefugia II through IV constitute the C supergroup, while Microrefugia V and VI correspond to the E supergroup.

The estimation of ingroup structures using AMOVA revealed that Φ_{ST} values among populations within (super)groups were predominantly greater than zero, indicating genetic segregation among populations (Table 3). Notably, groups associated with Microrefugia I (=W supergroup), V, and VI showed no significant variation between populations. Conversely, groups associated with Microrefugia II and IV displayed moderate Φ_{ST} values,

while the C and E supergroups, along with Microrefugium III, exhibited high Φ_{ST} levels, highlighting significant genetic differentiation among populations, even across short geographical distances.

To estimate the correlation between geographic distance and genetic differentiation among populations, the Mantel test was applied (Figure 4). The results indicated an isolation by distance (IBD) effect among the populations from the Khamar-Daban Ridge (p -value < 0.005). However, the strength of the linear relationship between the two variables varied depending on the sample composition analyzed. In particular, the IBD analysis based on the complete sample comprising all studied populations revealed only a moderate correlation ($r = 0.287$), indicating that only approximately 8% of genetic variance ($r^2 = 0.083$) can be explained by geographic distance (Figure 4a).

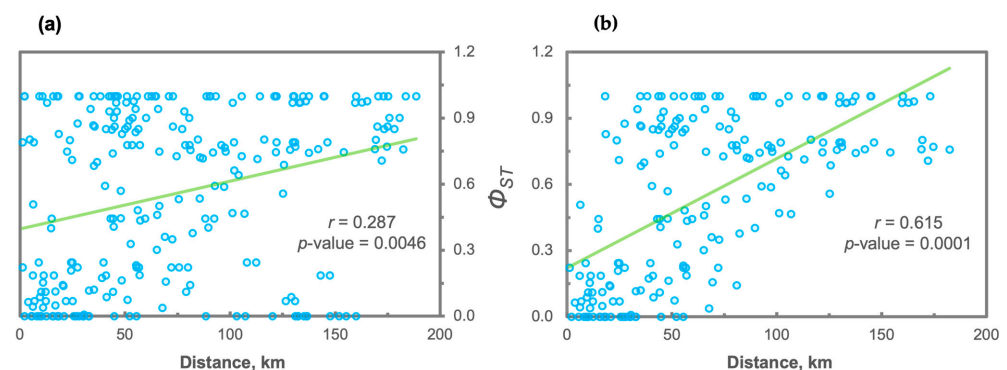


Figure 4. Mantel test for the isolation by distance (IBD) effect conducted on (a) the complete sample comprising all studied populations; and (b) the reduced sample obtained by excluding specimens from MM1, E1, and T1 localities. Significance testing was performed using 10,000 permutations. The regression line is indicated in green.

When the sample for IBD analysis was reduced by excluding three populations (MM1, E1, and T1), which, according to the PCoA analysis (Figure 2b), clustered within genetic groups predominantly represented by populations from other geographic supergroups, there was a significant increase in correlation, reaching $r = 0.615$ (Figure 4b). This suggests that when outliers are excluded, about 38% of the genetic variability ($r^2 = 0.378$) can be attributed to geographic distance. Consequently, in general, nearby populations tend to be genetically more similar than would be expected by chance, and genetic differences increase linearly with geographic distance.

To conduct a more detailed investigation into which sections of the macroslope are most influential in shaping the spatial-genetic structure of *A. altaica*, we performed an analysis of spatial autocorrelation (Figure 5). The Global Spatial Autocorrelation analysis involved dividing the data into six distance classes, with distances between compared individuals ranging from 0 to 200 km (Figure 5a). The first distance class (0 km) corresponded to the genetic structure observed within local populations, while the 20 km and 40 km classes reflected distances between populations within refugia or between neighboring refugia. The 80 km class pertained to distances between specimens in non-adjacent refugia. Notably, the longest microrefugium (Microrefugium V), which has a maximum distance between populations of just over 50 km, also falls within this category. The remaining two classes represented specimens from the most distant refugia. In a manner similar to Moran's I criteria, the autocorrelation coefficient (r) utilized here [41,43] conveys the following interpretations. A value of $r = 1$ indicates perfect positive spatial autocorrelation, where high values or low values of the assessed criteria cluster together; a value of $r = -1$ suggests perfect negative spatial autocorrelation, where adjacent observations exhibit contrasting

values, resulting in a checkerboard pattern; and a value of $r = 0$ signifies perfect spatial randomness [35,36,46].

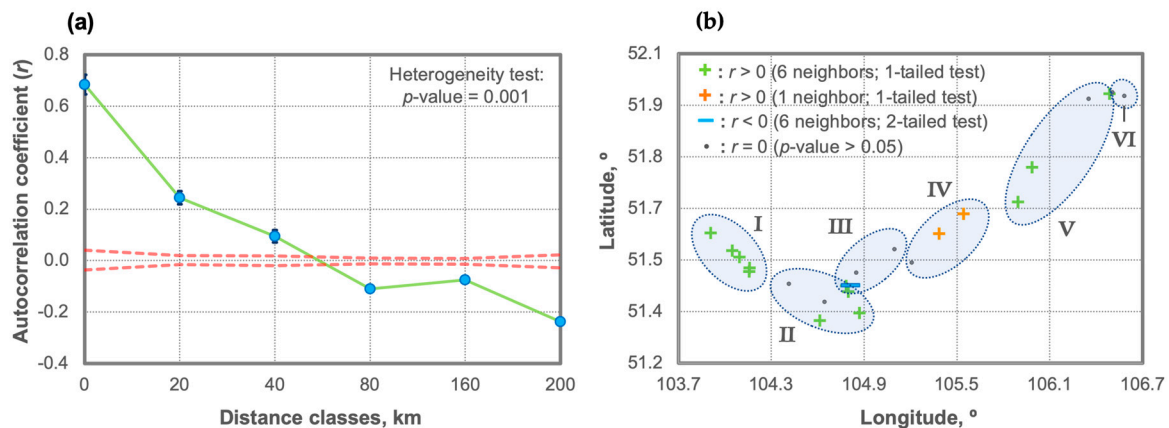


Figure 5. Spatial Autocorrelation for *A. altaica* on the Khamar-Daban Ridge. (a) The correlogram resulting from Global Spatial Autocorrelation analysis based on a pair-wise genetic distance matrix derived from differences in plastotype composition, along with a geographic distance matrix for all sampled individuals. The autocorrelation coefficient r measures the genetic similarity between pairs of individuals whose geographic separation falls within the specified distance class. Dashed red curves indicate 95% confidence intervals derived from 1000 permutations, while the error bars bound the 95% confidence interval calculated using bootstrap method with 1000 replicates. (b) 2-Dimensional Local Spatial Autocorrelation (2D LSA) analysis based on the pairwise genetic distance matrix and geographic distance matrix for the studied populations. Populations demonstrating significant relationships with their surroundings are marked with ‘plus’ or ‘minus’ symbols. Significance testing was conducted using one-tailed or two-tailed tests with 1000 permutations. Roman numerals and ellipses denote the approximate outlines of Pleistocene microrefugia according to Hypothesis 5.

The results revealed systematic spatial variation in genetic distances among the specimens ($r \neq 0$). Specifically, significant positive spatial autocorrelation was observed in the first distance classes, indicating that adjacent specimens share more similar plastotypes than those that are farther apart. In contrast, within the higher distance classes (80 km, 100 km, and 200 km), negative spatial autocorrelation was noted, suggesting that adjacent specimens within these particular distance classes tend to exhibit contrasting plastotypes. The heterogeneity test also indicated the overall correlogram significance (p -value = 0.001).

The local spatial autocorrelation was also estimated, focusing on the relationships between each population and its surrounding context, rather than on the genetic structure across the entire study site, as is done in global analyses. The results revealed that certain populations from Microrefugia I, II, IV, and V demonstrate high similarities to their nearest neighbors, as indicated by positive autocorrelation values (represented by a ‘plus’ sign in the graph). These populations are likely to play a significant role in shaping the spatial genetic structure. In contrast, the negative r -value for the MM1 locality (represented by a ‘minus’ sign in the graph) suggests that it is a spatial outlier, as it significantly deviates from the values of its neighboring localities. Importantly, the outlier status of the MM1 locality aligns with previous findings from PCoA and IBD (Figures 2 and 4).

Historical demography was inferred from various diversity metrics, neutrality test, and observed mismatch distribution. The overall population exhibited moderately high nucleotide diversity (π) and plastotype diversity (h), along with a genetic diversity index (I) (Table 4). Notably, all subdivisions displaying the highest genetic diversity metrics included both genetic lineages of *A. altaica*, represented by plastogroups PGI and PGII. In

contrast, subdivisions that contained only one genetic lineage or where one lineage was dominant exhibited significantly lower levels of genetic diversity.

Table 4. Diversity metrics and test of neutrality hypothesis for (super)groups of *A. altaica* on the Khamar-Daban Ridge.

Group/Supergroup	N _{pop}	P	h	N _e	I	S	π	Tajima's D (p-Value)
Western supergroup (W)	5	2	0.316	1.446	0.487	1	0.00040	0.5060 (0.305)
Central supergroup (C)	12	4	0.415	1.700	0.820	9	0.00256	0.4412 (0.321)
Eastern supergroup (E)	6	2	0.485	1.913	0.670	8	0.00498	3.3435 (0.000)
Microrefurium (group) I	5	2	0.316	1.446	0.487	1	0.00040	0.5060 (0.305)
Microrefurium (group) II	6	2	0.105	1.115	0.212	1	0.00013	−0.6810 (0.735)
Microrefurium (group) III	3	3	0.621	2.510	0.983	8	0.00522	3.1312 (0.000)
Microrefurium (group) IV	3	2	0.405	1.642	0.580	2	0.00103	1.2349 (0.119)
Microrefurium (group) V	4	2	0.180	1.214	0.320	8	0.00185	−0.6464 (0.722)
Microrefurium (group) VI	2	1	0.000	1.000	0.000	0	0.00000	0.0000 (0.000)
The general population	23	5	0.660	2.917	1.275	10	0.00429	2.2914 (0.017)

Notes: N_{pop}—Number of local populations in the sample, P—Number of plastotypes, h—Unbiased plastotype (haplotype) diversity, N_e—effective number of plastotypes, I—Information index for genetic diversity (equivalent to the Shannon-Weaver Index) calculated based on plastotype frequencies, S—Number of segregating sites, π—Nucleotide diversity. Significant D-values are indicated in bold.

Simultaneously, a limited absolute number of plastotypes (P) and their effective number (N_a) were observed. The slight excess of the total number of plastotypes over their effective number indicates a scarcity of rare plastotypes in nearly all studied subdivisions (Table 4). The excess of nucleotide diversity over the expected number of segregating sites coupled with their distribution across a limited number of highly represented plastotypes resulted in a shift in the drift–mutation equilibrium, as evidenced by positive Tajima's D value in the E supergroup and Microrefugium III, as well as in the overall sample. Furthermore, deviations from the neutral model of evolution were found only for subdivisions, where both genetic lineages of *A. altaica* co-occurred with equal or nearly equal frequencies. The remaining (super)groups did not exhibit significant deviations from drift-mutation equilibrium (Table 4).

The mismatch distribution graph exhibited a clear bimodal pattern for *A. altaica* on the Khamar-Daban Ridge (Figure 6).

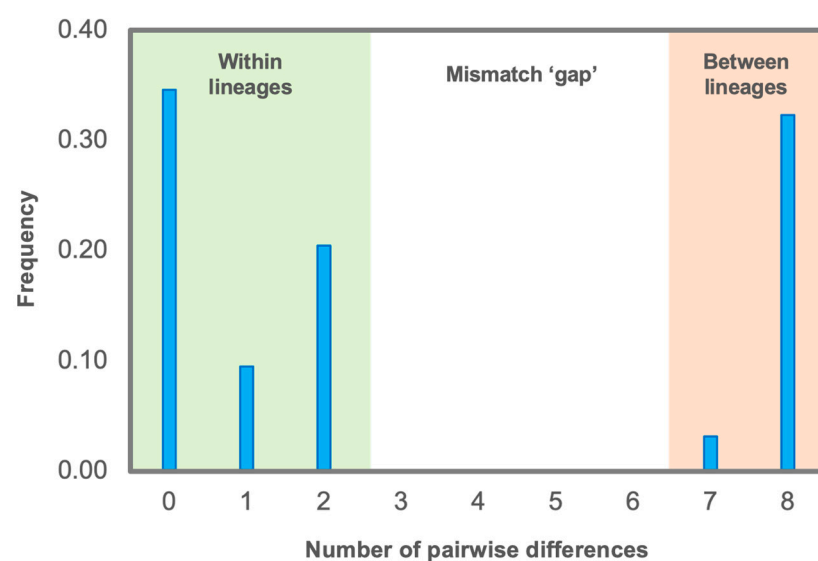


Figure 6. Mismatch distributions for *trnL* + *trnL-trnF* ptDNA region in *A. altaica* on the Khamar-Daban Ridge.

Specifically, the first peak on the graph, representing low mismatch numbers, reflects the frequency of pairwise genetic differences within genetic lineages. Conversely, the second peak, found at higher mismatch numbers, suggests significant genetic differentiation between lineages. A substantial ‘gap’ separating these two peaks may explain the positive Tajima’s D statistic.

4. Discussion

4.1. Reevaluating the Phylogenetic Relationships of *Anemone altaica* and Closely Related Species

Anemone altaica [$\equiv$ *Anemone nemorosa* subsp. *altaica* (Fisch. ex C.A. Mey.) Korsh.], along with several other *Anemone* species, such as *A. quinquefolia* [$\equiv$ *A. nemorosa* var. *quinquefolia* (L.) Pursh] and *A. amurensis* ($\equiv$ *A. nemorosa* subsp. *amurensis* Korsh.), are considered closely related to each other and to the European *A. nemorosa* [47–49]. However, the previous molecular phylogenetic reconstructions of the *Anemone* genus ever did not cover *A. altaica* and the mentioned close related species together [21,50,51]. Although our reconstruction, using two plastid markers, did not further resolve relationships among closely related taxa beyond previous findings, this was not the primary objective of our study. At the same time, our phylogenetic analysis suggested a curious diphyletic nature of *A. altaica* based on ptDNA data. Specifically, our analysis identified at least two distinct genetic lineages within *A. altaica*. We hypothesize that Lineage I represents the authentic line of *A. altaica* (Figure 1). This hypothesis is based on the fact that this lineage is represented by a monophyletic clade (PG1) that includes plastotypes of *A. altaica* from diverse geographical regions. In contrast, plastotypes belonging to Lineage II have, to date, only been observed in *A. altaica* from the Khamar-Daban Ridge. This lineage appears to be shared with the closely related species *A. reflexa*, *A. quinquefolia*, and likely *A. amurensis*, which together form a common plastogroup (PG2) in the phylogenetic tree. Within this group of taxa, *A. reflexa* is consistently considered related to *A. altaica* and the other aforementioned species belonging to the same section, although it was previously classified in a separate series, Reflexae. [48,49]. Notably, *A. reflexa* is clearly morphologically distinct from other species in the group, characterized by very narrow, reflexed tepals that run parallel to the peduncle [52]. Here, we present evidence suggesting the relationship between *A. reflexa* and the other mentioned species may be closer than previously thought. In this key, our results are consistent with recent data using genomes of several *Anemone* species [51].

Returning to the genetic divergence between the two *A. altaica* lineages, it is considerable, with at least seven mismatches, including indels, representing approximately 1% of their alignment length (Figures 1b and 6). This level of divergence approaches that typically observed between species. Furthermore, mismatch distribution analysis revealed a clear bimodal pattern, with a substantial gap in frequencies between within-lineages and between-lineages values, further supporting the divergence of these two lineages. The significant nucleotide divergence of *A. altaica* plastotypes and the diphyletic nature of its lineage cannot be easily explained by incomplete lineage sorting with closely related species. We believe the best hypothesis may involve incidents of hybridization of *A. altaica* with related species and backcrossing, resulting in past plastid capture events. Specifically, plastotypes of *A. altaica* belonging to Lineage II may have arisen in situ on the Khamar-Daban Ridge through this process. We propose that *A. reflexa* is the most likely candidate for hybridization with *A. altaica*, as the two species share identical plastotypes. Moreover, these two species are often found growing together on the Khamar-Daban Ridge, though modern hybrids have not been documented in this territory. The lack of observed modern hybrids in the ridge may be tied to differences in flowering time rather than physiological mechanisms of reproductive isolation. This hypothesis can be confirmed by the presence of

narrowly localized modern hybrids between these two species, which have been previously described [53].

4.2. Present Phylogeographic Patterns of *Anemone altaica* on the Khamar-Daban Ridge and Potential Pleistocene Distribution Scenarios

The geographic distribution of identified plastotypes across the Khamar-Daban Ridge, combined with AMOVA results, revealed significant genetic differentiation among *A. altaica* populations ($\phi_{ST} = 0.812$). This pattern aligns with the species' fragmented distribution along the ridge. As a mesophilic species, *Anemone altaica* is primarily confined to riverbeds, similar to other nemoral relicts. The observed genetic isolation likely arises from the barrier effect of riverbeds, particularly in foothill zones, which restrict gene flow. These biogeographic constraints collectively reinforce limited genetic connectivity across the species' range. The northern macroslope of the Khamar-Daban Ridge exhibits a relief intensely dissected by rivers and streams that drain into Lake Baikal, forming part of its catchment basin. Consequently, high-altitude interfluvial areas consist of mountain spurs extending northward from the ridge's central axis.

Our grid mapping results, previously conducted in a model area situated between two interfluvial areas of the Bolshoy Mamay River (corresponding to M1–M3 and MM localities in Figure 3), indicated that within the forest belt, populations of *A. altaica* were predominantly found along the riversides and streams [12]. In interfluvial areas, populations were sporadic and primarily restricted to temporary watercourses. The species is also found in subalpine wet and high-grass meadows, where it can exhibit high population densities, however, it struggles in drier regions of watershed ridges. Consequently, there exists a partial geographic isolation of populations between neighboring rivers. It is noteworthy that the current distribution of nemoral relicts on the Khamar-Daban Ridge is significantly influenced by their proximity to Lake Baikal. Specifically, at a distance exceeding 10–15 km from the shoreline, the climatic conditions become harsher, leading to a decrease in the presence of many nemoral relicts, with some species disappearing entirely [14]. For instance, *A. altaica* thrives in the subalpine meadows of rivers with relatively short courses and in the watersheds of low order located close to Lake Baikal. An illustrative case is the Bolshoy Mamay River, where the distance from the shore to its upper reaches is under 10 km. In contrast, for lengthy rivers that flow from the axial part of the ridge, where the forest boundary may lie 30 km or more from Lake Baikal, the subalpine zone tends to be drier and unsuitable for *A. altaica*. Consequently, the major rivers of the Khamar-Daban Ridge, such as the Utulik, Snezhnaya, Vydrinnaya, Pereemnaya, Mishikha, and others, act as significant geographic barriers hindering the species from spreading over extensive distances along the ridge. In pre-Pleistocene times, characterized by a warmer and more humid climate featuring expansive broad-leaved forests and smaller mountain ranges compared to the current landscape, it is likely that the isolation of populations was less pronounced than it is in modern times. The examination of Lake Baikal's bottom sediments reveals the dominance of a nemoral dark coniferous–broad-leaved complex in the Cis-Baikal region until the middle of the Late Pliocene. Subsequently, a transition towards more boreal characteristics occurred towards the end of the Pliocene and into the early Pleistocene.

This climate shift indicates that as the climate cooled and dried during the Pleistocene, populations likely began to become more isolated, congregating along river floodplains near the shores of Lake Baikal. In the southern Baikal area, glaciation was predominantly observed as a mountain-valley phenomenon. The massive volume of water in Lake Baikal and the interception of moist 'western' air masses by the Khamar-Daban Ridge have helped mitigate the impact of global climate changes on the southern shore of the lake. These factors played a crucial role in preserving elements of the broad-leaved complex on the

northern macroslope of the Khamar-Daban Ridge and its foothills, establishing it as the most significant nemoral refugium within the Baikal region.

Previously, we introduced the concept of several microrefugia on the Khamar-Daban Ridge, identified based on the species richness of nemoral relicts [5]. Four possible microrefugia were pinpointed on the Khamar-Daban Ridge, showcasing the highest concentration of relict species. These presumed microrefugia were exclusively located within the floodplains of major rivers, as indicated by triangles in Figure 3: Microrefugium I (corresponding to B1 and Ut2 localities) along the Babkha and Utulik rivers, Microrefugium II (corresponding to S2 and S3 localities) along the Snezhnaya river, Microrefugium III (corresponding to TOs1 and Pr1 localities) along the Osinovka (near Tankhoy settlement) and Pereemnaya rivers, and Microrefugium IV (corresponding to the Msh1 locality) along the Mishikha river. Therefore, some large rivers, in addition to their barrier role in limiting the gene flow along populations, also played a crucial role as Pleistocene microrefugia, allowing relict species to survive in their floodplains.

The positioning of these microrefugia aligns with a recently established model detailing the location of glaciers and their nourishment areas on the ridge during the Sartan glaciation, corresponding to the Last Glacial Maximum (LGM) in Siberia (see inset on Figure 3, adapted from [10]). Analysis of the glacier layout reveals that the floodplains of the Utulik River together with lower course of the Babkha River (corresponding to Microrefugium I), the Snezhnaya River (corresponding to Microrefugium II), and the Mishikha River (corresponding to Microrefugium IV) remained unglaciated during the Last Glacial Maximum. The central ridge area exhibited higher susceptibility to snow cover due to various local factors. This led to Microrefugium III being notably closer to the Baikal shoreline due to glacial activity, resulting in its geographical isolation from Microrefugia II and IV. This isolation was caused by glacier tongues extending towards the lake along the Osinovka (near Vydrino village), Vydrinnaya, and Pereemnaya rivers, as indicated by arrows 1, 2, and 3 in Figure 3. The positioning of the glaciers suggests that the Pereemnaya River was unable to serve as a microrefugium, contrary to our previous assumptions [5], as it was covered by a glacier. Furthermore, the results of the AMOVA indicated that populations from the Pereemnaya River (Pr1 locality) were even more closely associated with Microrefugium IV than with Microrefugium III. This suggests that the Osinovka River and its adjacent areas (near Tankhoy settlement, corresponding to the TOs1 locality) likely played a primary role in Microrefugium III. Conversely, the riverbanks of the Pereemnaya River appear to have been recolonized by *A. altaica* from Microrefugium IV, or partially from Microrefugium III. It is worth noting that, *A. altaica* exhibits one of the most elongated ranges from west to east along the Khamar-Daban Ridge among nemoral relicts [5,15]. Its range stretches far eastward beyond the boundaries of the indicated microrefugia. Considering the absolute elevation of the snow line on the Khamar-Daban Ridge during the LGM [10], it is plausible that the area east of Microrefugium IV did not experience substantial glacial coverage, given the lower absolute mountain altitudes compared to the central and western parts of the ridge. Therefore, we hypothesized that the eastern populations likely did not originate from re-colonization from Microrefugium IV but were sustained in situ due to the presence of some eastern microrefugia. In support of the existence of more than four Pleistocene microrefugia for *A. altaica*, the results of the AMOVA indicate that the hypothesis of six, rather than four or five, population groups provides the most optimal scheme for partitioning the observed genetic diversity (Table 3). Specifically, high estimates of ϕ_{CT} relative to ϕ_{SC} indicate that nucleotide diversity was partitioned among hierarchical levels, in which variance in nucleotide diversity is maximized among groups and minimized within groups [54]. Consequently, the evidence supports the hypothesis of population groups associated with at least to six microrefugia as the most representative

model for the geographic structure of genetic diversity among the sampled populations of *A. altaica*. More concretely, the geographic locations of four of these microrefugia closely correspond to those previously discussed. Additionally, two further microrefugia are characteristic of *A. altaica* and are located further east than the others (Figure 3).

It is worth noting that our assumptions are based on glacial reconstructions solely from the LGM period [10]. While it is generally believed that each successive glaciation in the Pleistocene was colder than the previous one, the drier climate linked to Arctic ice accumulation suggests that glaciers from earlier glaciations in Siberia may have been larger than those during the LGM [55]. Therefore, we posit that the distribution patterns of microrefugia and population isolation during the LGM likely hold true and for earlier Pleistocene glaciations, which might be just more extensive in certain places of the Khamar-Daban Ridge.

Furthermore, both the PCoA and standardized ϕ' -statistics support the distribution of proposed six microrefugia among three geographic supergroups (W, C, and E), with limited gene flow between them. The results of autocorrelation analysis also confirmed a strong spatial genetic structure of *A. altaica* on the Khamar-Daban Ridge at both global and local scales. Specifically, the geographical distances between classes, which exhibited significant autocorrelation values in the distribution of spatial variation in genetic distances among specimens, generally aligned with those observed among supergroups and among microrefugia distances (Figure 5). Local spatial autocorrelation, focusing on relationships between each population and its surrounding context, revealed high similarities between populations and their nearest neighbors in most groups associated with microrefugia. High values of ϕ_{ST} and $\phi(\phi')_{CT}$ in the supergroups and microrefugia models confirm that while some small watersheds on the Khamar-Daban Ridge may have acted as geographic barriers, the major rivers extending into the axial region of the ridge serve as the primary obstacles to gene flow over significant distances, both in the past and present.

At the same time, the IBD assessment revealed that not all differences between individuals and their groups can be attributed to geographic distance alone (Figure 4). For example, the results from PCoA and 2D LSA demonstrated the presence of clear outliers among populations that carry plastotypes poorly correlated with their genetic surroundings (Figures 2 and 5). Specifically, removing these outliers from the IBD analysis significantly strengthened the correlation. The mentioned outliers are exemplified by populations from Microrefugium VI (E1 and T1 localities), which genetically align more closely with the C supergroup rather than the E supergroup to which they belong geographically (Figure 2). Similarly, this applies to a population from Microrefugium III (MM1 locality), which geographically belongs to the C group but are genetically grouped with those from the E supergroup (Figures 2 and 5b).

Mismatches between the geographical location of certain populations and their affiliation with specific genetic groups or supergroups can be attributed to past periods when the *A. altaica* range was more contiguous, followed by fragmentation events during the Pleistocene. Periodic fragmentation of the range likely led to the preservation of random plastotypes due to bottlenecks and independent genetic drift. This resulted in the retention of similar plastotypes, albeit at different frequencies, in distinct, non-adjacent populations and groups. This process is now evident because some distant populations still carry similar plastotypes. It suggested such plastotypes likely remained in situ from past times rather than arising through recent long-distance migration. Supergroups C and E exhibit greater commonality and appear to have maintained contact for a longer duration, as reflected by their sharing of plastotypes P1 and P2 (Figure 3). These plastotypes dominate the general population, although they occur at different frequencies in the two supergroups. In contrast, W supergroup seems to be more distinct from two mentioned, as it does not share the

dominant plastotypes with the other groups. The genetic isolation of the western group of populations has also been demonstrated in our previous studies of other relict species on the ridge, such as *Eranthis sibirica* DC. [17]. Similar patterns observed across different species likely indicate the existence of temporary or permanent geographic barriers that impede effective gene flow between the western and central parts of the ridge.

On the other hand, sharing of identical plastotypes over short distances, such as between adjacent populations or microrefugia, suggests that genetic isolation is incomplete and that contact between groups persists or has occurred periodically in the past. We believe that the best opportunity for gene flow occurred not during the re-expansion of confined populations during warming periods, but rather during glaciations or the beginning of interglacials. Specifically, as mentioned above, the wide riverbeds in the forest belt, along with the lengthy areas of interfluves exhibiting boreal features, currently serve as significant barriers to population mixing. In the mountain-forest belt, the population density in the interfluvial area increases [12], possibly due to the higher density of watercourses. This, combined with a decrease in the barrier function of the river itself due to the narrowing of the main channel, may contribute to the partial mixing of populations from different watersheds. Subalpine meadows provide a favorable biome for *A. altaica*, where it can form large aggregations, and the barrier function of rivers practically disappears. However, the restriction of population spread in the subalpine belt is mainly caused by the dry watersheds. Therefore, a significant limitation on the mixing of populations in the mountain-forest and subalpine belts, as noted above, is their remoteness from Lake Baikal. Consequently, gene flow is currently possible only within short distances on the watersheds of short rivers. During the Pleistocene cooling periods, due to the depression of the snow line, the upper limit of the forest belt also experienced depression [3]. The shift of the subalpine zone closer to the bowl of Lake Baikal should have contributed to the spread of *A. altaica*. Consequently, adjacent populations may have had the chance to intermingle across the subalpine region as they expanded along the glacial edge. Furthermore, the mixing of populations from opposite river banks could have taken place via glacial terminal moraines which penetrated the mountain forest belt and served as connecting bridges during that period. Thus, we propose that despite the decrease in air humidity and temperature during Pleistocene cold spells, which appears unfavorable for *A. altaica*, these periods created conditions conducive to population migration. This was facilitated by the formation of landscape prerequisites during glaciations or early interglacial periods. Additionally, it is hypothesized that the cold spells were more favorable for the development and expansion of forest formations at the expense of steppe landscapes [3]. Consequently, this, combined with the downward shift of the subalpine zone, suggests an expansion of biome zones suitable for *A. altaica* during these cold periods.

At the same time, if we consider the ‘hybridization hypothesis’, the current phylogeographic patterns of *A. altaica* may partially reflect the history of another taxon, *A. reflexa*, a possible plastid donor. Although hybridization incidents likely occurred long ago, the different environmental preferences of the two species could still drive the present phylogeographic patterns of *A. altaica*. In particular, while *A. reflexa* can be considered a nemoral-boreal species [56], it may have more pronounced boreal properties than *A. altaica*. This could explain why, although the species often co-occur, *A. reflexa* is more frequently found in biomes with stronger boreal features where *A. altaica* is never present. Consequently, the patterns of genetic and actual divergence of *A. altaica* populations may not always align precisely due to a trace of introgression.

The overall population of *A. altaica* on the Khamar-Daban Ridge, along with some of its subdivisions, exhibited moderately high genetic diversity metrics, including nucleotide diversity (π) (Table 4). Generally, the level of genetic variability is positively correlated with

effective population size [57]. However, in our case, the diversity indices may not fully capture the underlying dynamics. Notably, the highest values were observed in subdivisions that included two genetic lineages of *A. altaica*, with both exhibiting high frequencies (Table 4). The mismatch distribution graph (Figure 6) clearly indicates that the differences between lineages are significantly greater than the differences within lineages, which may account for the observed increase in diversity metrics. Considering the ‘hybridization hypothesis’ regarding the origin of one of the lineages, the revealed diversity may actually stem from genetic introgression rather than mutation processes. This suggests that these metrics may not reliably reflect effective population size or demographic processes. Interestingly, subdivisions containing only one genetic lineage, or where one lineage was dominant, exhibited significantly lower levels of genetic diversity. Furthermore, we believe that using only a limited number of markers in this study is insufficient to draw reliable conclusions about effective population size or demographic processes based on calculated diversity metrics. Therefore, we do not find it necessary to continue speculating based on this dataset.

A similar situation was encountered during the calculation of Tajima’s D test of neutrality. Specifically, positive Tajima’s D values were found in the E supergroup, Microrefugium III, and the general population, mirroring the previous case where these groups contained both genetic lineages as subdominant components. The positive value of Tajima’s D test suggests a deviation from the neutral model of evolution, which is characterized by a balance between mutations and genetic drift. A positive Tajima’s D value is often associated with either balancing selection or sudden population contraction. However, we propose that the modern phylogeographic structure of *A. altaica* on the Khamar-Daban Ridge most likely originated via genetic introgression events, offering the most plausible explanation for the observed positive Tajima’s D value. In this scenario, what appears to be balancing selection is, in fact, the result of the emergence of certain plastotypes via plastid capture from closely related species, rather than through mutations and subsequent divergence. Nevertheless, the effects of recent population contractions cannot be excluded, especially considering the dramatic climatic changes during the Pleistocene-Holocene.

5. Conclusions

Based on the polyphyletic (more specifically diphyletic) nature and significant nucleotide divergence of plastotypes in *A. altaica*, we propose that past hybridization events with related species, followed by subsequent backcrossing, have ultimately led to plastid capture. The current phylogeographic patterns of *A. altaica* may partially reflect the historical influence of *A. reflexa*, which is the most reliable candidate for the donor of plastid DNA during these introgression processes. Genetic differentiation among populations of *A. altaica* indicates that even small watersheds may impede gene flow within the Khamar-Daban Ridge. However, major rivers flowing from the axial part of the ridge have historically served as the main barriers to gene flow during the Pleistocene and continue to do so today. The most reliable geographical model suggests that during periods of Pleistocene cooling, *A. altaica* persisted in at least six microrefugia within the ridge. Populations associated with these microrefugia formed western, central, and eastern genetic supergroups with limited gene flow among them. The geographic locations of four of six microrefugia closely correspond to our earlier predictions regarding most of the relict species on the Khamar-Daban Ridge. Additionally, two further microrefugia are characteristic of *A. altaica* and are located further east than the others. The presence of common plastotypes across different populations and groups suggests that genetic isolation is not complete; rather, contact between these groups has either persisted or occurred periodically in the past. Gene flow likely took place during glaciations or early interglacial periods when the subalpine zone

shifted closer to Lake Baikal, facilitating intermingling among adjacent populations as they expanded along glacial edges and through terminal moraines in the mountain forest belt.

Author Contributions: Conceptualization, M.P.; methodology, M.P. and V.P.; validation, M.P.; formal analysis, M.P. and P.N.; investigation, M.P., P.N. and V.P.; resources, M.P. and V.P.; data curation, M.P.; writing—original draft preparation, M.P.; writing—review and editing, M.P., V.P. and P.N.; visualization, M.P. and V.P.; supervision, M.P.; project administration, M.P.; funding acquisition, M.P., V.P. and P.N. All authors have read and agreed to the published version of the manuscript.

Funding: The study was financed by the grant of Russian Science Foundation No. 23-24-00501, <https://rscf.ru/project/23-24-00501/> (studying Khamar-Daban Ridge populations of *A. altaica*) and carried out within the state assignment of Ministry of Science and Higher Education of the Russian Federation for Siberian Institute of Plant Physiology and Biochemistry of the Siberian Branch of RAS (Project State Registration No. 125021902487-9) (studying populations of *A. nemorosa*, *A. reflexa*, and *A. quinquefolia*) and the project of Irkutsk State University to support junior scientists (P.N.) No. 091-24-317 (studying the BN1 population of *A. altaica*).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Original sequence data are available at GenBank (<http://www.ncbi.nlm.nih.gov/genbank>, accessed on 10 August 2024) by their accession numbers presented in Table 2.

Acknowledgments: We thank the herbaria of Institute of Experimental Botany named after V.F. Kuprevich of the National Academy of Sciences of Belarus (MSK, Minsk, Republic of Belarus) and especially Dmitriy Dubovik and the Federal Scientific Center of the East Asia Terrestrial Biodiversity of the Far Eastern Branch of the Russian Academy of Sciences (VLA, Vladivostok, Russia) for allowing the collection of samples; the Baikal State Nature Biosphere Reserve for assistance in the field studies carried out on its territory and the protected area; herbarium of Irkutsk State University (IRKU, Irkutsk, Russia) and especially Nadezhda Stepantsova for assistance allowing us to deposit the vouchers, and for help with mounting the herbarium vouchers, Natalya Shvetsova for her help with lab assistance, Nikolay Stepanov and Victor Chepinoga for assistance us with field work during 2018, Pavel Zherebtsov for transportation during fieldwork on the Khamar-Daban Ridge; the Ministry of Natural Resources and Environment of the Republic of Buryatia for the permission for the plant sampling. The research was conducted using the equipment of the Core Facilities Center ‘Bioanalitika’ (Irkutsk, Russia).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Krestov, P.V.; Omelko, A.M.; Nakamura, Y. Phytogeography of Higher Units of Forests and Krummholz in North Asia and Formation of Vegetation Complex in the Holocene. *Phytocoenologia* **2010**, *40*, 41–56. [\[CrossRef\]](#)
2. Krestov, P.V.; Barkalov, V.Y.; Omelko, A.M.; Yakubov, V.V.; Nakamura, Y.; Sato, K. Relic Vegetation Complexes in the Modern Refugia of Northeast Asia. *Komar. Chtenia [V. L. Komar. Meml. Lect.]* **2009**, *56*, 5–63. (In Russian)
3. Malyshev, L.I.; Peshkova, G.A. *Osobennosti I Genezis Flory Sibiri (Predbaikalye I Zabaikalye) [Particularities and Genesis of the Flora of Siberia (Prebaikalia and Transbaikalia)]*; Nauka: Novosibirsk, Russia, 1984. (In Russian)
4. Polozii, A.V.; Krapivkina, E.D. *Relikty Tretichnyh Shirokolistvennyh Lesov vo Flore Sibiri [Relics of Tertiary Deciduous Forests in the Flora of Siberia]*; Tomsk University Press: Tomsk, Russia, 1985. (In Russian)
5. Chepinoga, V.V.; Protopopova, M.V.; Pavlichenko, V.V. Detection of the Most Probable Pleistocene Microrefugia on the Northern Macroslope of the Khamar-Daban Ridge (Southern Prebaikalia). *Contemp. Probl. Ecol.* **2017**, *10*, 38–42. [\[CrossRef\]](#)
6. Epova, N.A. Relics of Tertiary Broad-Leaved Forests in the Siberian Fir Taiga of the Khamar-Daban Ridge. In *Izvestiia Biologo-Geograficheskogo Nauchno-Issledovatel'skogo Instituta pri Irkutskom Gosudarstvennom Universitete Imeni A. A. Zhdanova*; Institute of Biology and Geography, A.A. Zhdanov Irkutsk State University: Irkutsk, Russia, 1956; Volume 16, pp. 47–61. (In Russian)
7. Belov, A.V.; Bezrukova, E.V.; Sokolova, L.P.; Abzayeva, A.A.; Letunova, P.P.; Fisher, E.E.; Orlova, L.A. Vegetation of the Baikal Region as an Indicator of Global and Regional Changes in Natural Conditions of North Asia in the Late Cainozoic. *Geogr. Nat. Resour.* **2006**, *6*, 5–18. (In Russian)
8. Krasnopevtseva, V.M.; Namzalov, B.B.; Krasnopevtseva, A.S. 3. Peculiarity of the Biology and Rhythm Flowering Ephemeral Plants of Khamar-Daban Mountain Range (Southn Prebaikalye). *Bull. Buryat State Univ.* **2008**, *4*, 107–111. (In Russian)

9. Gamova, N.S.; Protopopova, M.V.; Pavlichenko, V.V.; Korotkov, Y.N. First Reliable Finding of *Galium odoratum* (L.) Scop. And New Findings of *Galium Paradoxum* Maxim. In the Republic of Buryatia. *Trans. Karelian Res. Cent. RAS* **2024**, *1*, 1–10. (In Russian) [CrossRef]
10. Eriakeev, F. Paleogeography of the Sartan Glaciation of the Hamar-Daban Ridge (Southern Baikal Region). *Transbaikial State Univ. J.* **2020**, *26*, 17–32. [CrossRef]
11. Mel'nikov, Y.I. Pleistocene Gaps in the Areas of Birds of Eastern Siberia and Their Filling in the Modern Period of Climate Warming. *IOP Conf. Ser. Earth Environ. Sci.* **2023**, *1154*, 012065. [CrossRef]
12. Chepinoga, V.V.; Protopopova, M.V.; Pavlichenko, V.V.; Dudov, S.V. Habitat Distribution Patterns of Nemoral Relict Plant Species on the Khamar-Daban Ridge (the South of Eastern Siberia) according to Grid Mapping Data. *Russ. J. Ecol.* **2021**, *52*, 212–222. [CrossRef]
13. Krasnoborov, I.M. *Flora Alpina Montium Sajanesium Occidentalium*; Nauka: Novosibirsk, Russia, 1976. (In Russian)
14. Krasnopevtseva, V.M.; Krasnopevtseva, A.S. Relict Plants of the Tertiary Nemoral Complex in Southern Transbaikalia. *Tr. Tigirekskogo Zapov. [Proc. Tigirek State Nat. Reserve]* **2010**, *3*, 194–196. (In Russian) [CrossRef]
15. Chepinoga, V.V.; Mishina, A.V.; Protopopova, M.V.; Pavlichenko, V.V.; Bystrov, S.O.; Vilor, M.A. New Data on Distribution of Several Nemoral Relict Plant Species on the Foothills of the Khamar-Daban Ridge (Southern Baikal). *Bot. Zhurnal* **2015**, *100*, 478–489. (In Russian)
16. Doyle, J.J.; Doyle, J.L. A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.
17. Protopopova, M.V.; Pavlichenko, V.V. *Eranthis salisb* (Ranunculaceae) in South Siberia: Insights into Phylogeography and Taxonomy. *Diversity* **2022**, *14*, 779. [CrossRef]
18. Taberlet, P.; Gielly, L.; Pautou, G.; Bouvet, J. Universal Primers for Amplification of Three Non-Coding Regions of Chloroplast DNA. *Plant Mol. Biol.* **1991**, *17*, 1105–1109. [CrossRef] [PubMed]
19. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
20. Zhang, N. Direct Submission in GenBank. Available online: <https://www.ncbi.nlm.nih.gov/nuccore/OK267274.1> (accessed on 18 July 2024).
21. Lee, C.S.; Lee, N.S.; Yeau, S.H. Molecular Phylogenetic Study of *Anemone pendulisekala* (Ranunculaceae). *Korean J. Plant Taxon.* **2006**, *36*, 263–277. [CrossRef]
22. Zhang, N.; Lu, Y.; Zhang, Z. The Complete Chloroplast Genome Sequence of *Anemone reflexa* (Ranunculaceae). *Mitochondrial DNA Part B Resour.* **2021**, *6*, 304–305. [CrossRef] [PubMed]
23. Fujii, N.; Senni, K. Phylogeography of Japanese Alpine Plants: Biogeographic Importance of Alpine Region of Central Honshu in Japan. *Taxon* **2006**, *55*, 43–52. [CrossRef]
24. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef]
25. Tamura, K. Estimation of the Number of Nucleotide Substitutions When There Are Strong Transition-Transversion and G+C-Content Biases. *Mol. Biol. Evol.* **1992**, *9*, 678–687. [CrossRef]
26. Hasegawa, M.; Kishino, H.; Yano, T. Dating of the Human-Ape Splitting by a Molecular Clock of Mitochondrial DNA. *J. Mol. Evol.* **1985**, *22*, 160–174. [CrossRef]
27. Felsenstein, J. Evolutionary Trees from DNA Sequences: A Maximum Likelihood Approach. *J. Mol. Evol.* **1981**, *17*, 368–376. [CrossRef] [PubMed]
28. Jukes, T.H.; Cantor, C.R. *Evolution of Protein Molecules*; Academic Press: New York, NY, USA, 1969; Volume 3, pp. 21–132.
29. Rambaut, A. FigTree: Tree Figure Drawing Tool, Version 1.4.3. Available online: <http://tree.bio.ed.ac.uk/software/figtree/> (accessed on 15 July 2022).
30. Templeton, A.R.; Crandall, K.A.; Sing, C.F. A Cladistic Analysis of Phenotypic Associations with Haplotypes Inferred from Restriction Endonuclease Mapping and DNA Sequence Data. III. Cladogram Estimation. *Genetics* **1992**, *132*, 619–633. [CrossRef]
31. Leigh, J.W.; Bryant, D. PopART: Full-Feature Software for Haplotype Network Construction. *Med. Ecol. Evol.* **2015**, *6*, 1110–1116. [CrossRef]
32. Tajima, F. Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics* **1989**, *123*, 585–595. [CrossRef] [PubMed]
33. Excoffier, L.; Smouse, P.E.; Quattro, J.M. Analysis of Molecular Variance Inferred from Metric Distances among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. *Genetics* **1992**, *131*, 479–491. [CrossRef] [PubMed]
34. Wright, S. Isolation by Distance. *Genetics* **1943**, *28*, 114–138. [CrossRef]
35. Peakall, R.; Smouse, P.E. GENALEX 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research. *Mol. Ecol. Notes* **2006**, *6*, 288–295. [CrossRef]

36. Peakall, R.; Smouse, P.E. GenAlEx 6.5: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research—An Update. *Bioinformatics* **2012**, *28*, 2537–2539. [CrossRef]
37. Huff, D.R.; Peakall, R.; Smouse, P.E. RAPD Variation within and among Natural Populations of Outcrossing Buffalograss [Buchloë Dactyloides (Nutt.) Engelm.]. *Theor. Appl. Genet.* **1993**, *86*, 927–934. [CrossRef]
38. Nei, M. Genetic Distance between Populations. *Am. Nat.* **1972**, *106*, 283–292. [CrossRef]
39. Mantel, N. The Detection of Disease Clustering and a Generalized Regression Approach. *Cancer Res.* **1967**, *27*, 209–220.
40. Smouse, P.E.; Long, J.C.; Sokal, R.R. Multiple Regression and Correlation Extensions of the Mantel Test of Matrix Correspondence. *Syst. Zool.* **1986**, *35*, 627–632. [CrossRef]
41. Smouse, P.E.; Peakall, R. Spatial Autocorrelation Analysis of Individual Multiallele and Multilocus Genetic Structure. *Heredity* **1999**, *82*, 561–573. [CrossRef] [PubMed]
42. Smouse, P.E.; Peakall, R.; Gonzales, E. A Heterogeneity Test for Fine-Scale Genetic Structure. *Mol. Ecol.* **2008**, *17*, 3389–3400. [CrossRef] [PubMed]
43. Double, M.C.; Peakall, R.; Beck, N.R.; Cockburn, A. Dispersal, Philopatry and Infidelity: Dissecting Local Genetic Structure in Superb Fairy-Wrens (*Malurus cyaneus*). *Evolution* **2005**, *59*, 625–635. [CrossRef]
44. Microsoft Corporation. Microsoft Excel. Available online: <https://office.microsoft.com/excel> (accessed on 31 December 2016).
45. QGIS Development Team. QGIS Geographic Information System. Open Source Geospatial Foundation Project. Available online: <http://qgis.osgeo.org> (accessed on 6 April 2024).
46. Fu, W.; Zhao, K.; Zhang, C.; Tunney, H. Using Moran's I and Geostatistics to Identify Spatial Patterns of Soil Nutrients in Two Different Long-Term Phosphorus-Application Plots. *J. Plant Nutr. Soil. Sci.* **2011**, *174*, 785–798. [CrossRef]
47. Dutton, B.E.; Keener, C.S.; Ford, B.A. *Anemone quinquefolia* Linnaeus. In 1993+ *Flora of North America North of Mexico*; 25+ volumes, volume 3; Flora of North America Editorial Committee, Ed.; Oxford University Press: New York, NY, USA; Oxford, UK, 1997. Available online: http://floranorthamerica.org/Anemone_quinquefolia (accessed on 11 July 2024).
48. Tamura, M. Angiospermae: Ordnung Ranunculales, Fam. Ranunculaceae, Anemoneae. In *Die Natürlichen Pflanzenfamilien*; Hiepko, P., Ed.; Duncker and Humblot: Berlin, Germany, 1995; Volume 17a IV, pp. 324–349.
49. Ziman, S.N.; Bulakh, E.V.; Kadota, Y.; Keener, C.S. Modern View on the Taxonomy of the Genus *Anemone* L. Sensu Stricto (Ranunculaceae). *J. Jpn. Bot.* **2008**, *83*, 127–155.
50. Hoot, S.B.; Meyer, K.M.; Manning, J.C. Phylogeny and Reclassification of *Anemone* (Ranunculaceae), with an Emphasis on Austral Species. *Syst. Bot.* **2012**, *37*, 139–152. [CrossRef]
51. Hu, S.; Shi, W.; Huang, Y.; Zhang, Z.; Lin, Q.; Shi, C. Comparative Analysis of Complete Chloroplast Genomes of Five *Anemone* Species and Phylogenetic Analysis within Tribe *Anemoneae* (Ranunculaceae). *J. Plant Biochem. Biotechnol.* **2024**, *33*, 271–287. [CrossRef]
52. Yuzepchuk, S.V. *Anemone* L. In *Flora of the USSR*; Komarov, V.L., Ed.; Academy of Sciences of the USSR: Moscow-Leningrad, Russia, 1937; Volume 7, pp. 236–282. (In Russian)
53. Popov, M.G. *Flora Sredney Sibiri [Flora of Central Siberia]*; Academy of Sciences of the USSR: Moscow-Leningrad, Russia, 1957; Volume 1. (In Russian)
54. Boyd, O.F.; Philips, T.K.; Johnson, J.R.; Nixon, J.J. Geographically Structured Genetic Diversity in the Cave Beetle *Darlingtonia kentuckensis* Valentine, 1952 (Coleoptera, Carabidae, Trechini, Trechina). *Subterr. Biol.* **2020**, *34*, 1–23. [CrossRef]
55. Velichko, V.V. Oledneniya Chetvertichnogo Perioda [the Quaternary Glaciations]. Great Russian Encyclopedia. Electronic Version 2019. Available online: <https://old.bigenc.ru/geology/text/5556825> (accessed on 11 May 2024). (In Russian).
56. Gorchakovskii, P.L.; Shurova, E.A. *Redkie I Ischezayushchie Rastenia Urala I Priural'ya Rare and Endangered Plants of the Ural and Cis-Ural Region*; Nauka: Moscow, Russia, 1982. (In Russian)
57. Chen, T.; Wang, X.; Tang, H.; Chen, Q.; Huang, X.; Chen, J. Genetic Diversity and Population Structure of Chinese Cherry Revealed by Chloroplast DNA TrnQ-Rps16 Intergenic Spacers Variation. *Genet. Resour. Crop Evol.* **2013**, *60*, 1859–1871. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.