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Waldsteinia within *Geum* s.l. (Rosaceae): Main Aspects of Phylogeny and Speciation History

Marina Protopopova [1,*], Vasiliy Pavlichenko [1], Victor Chepinoga [2], Alexander Gnutikov [3,4] and Renat Adelshin [2]

1 Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences, Lermontov St., 132, Irkutsk 664033, Russia
2 Faculty of Biology and Soil Sciences, Irkutsk State University, Karl Marx St., 1, Irkutsk 664003, Russia
3 Federal Research Center N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), B. Morskaya St., 42–44, Saint Petersburg 190000, Russia
4 Komarov Botanical Institute, the Russian Academy of Sciences, Prof. Popov St., 2, Saint Petersburg 197376, Russia
* Correspondence: marina.v.protopopova@gmail.com

Abstract: *Waldsteinia* is a small plant genus inhabiting the temperate regions of the Northern Hemisphere. According to the latest revisions, *Waldsteinia* is included in *Geum*. We have obtained a phylogenetic reconstruction based on the nuclear (ITS) and plastid (*trnL-trnF*) DNA to understand the phylogenetic structure of *Waldsteinia* and its relationships with other taxa of *Geum* s.l. Phylogenetic analysis based on the joint ITS + *trnL-trnF* dataset demonstrated *Waldsteinia* monophyly. The phylogenetic relationships of *Waldsteinia* species were better explained by their geographical distribution than their morphology. Hence, Euro-Siberian, Northeast Asian, and North American phylogeographic groups were distinguished, with East Asia having been suggested as the place of *Waldsteinia* origin. Considering the incongruence in *W. geoides* (a type species) position on the plastid and nuclear DNA trees, together with the discrepancy between the species morphology and its location on the plastid DNA tree, a hybrid origin was suggested for this species. Despite the fact that the position of *W. maximowicziana* is still not fully resolved, we support the point of view that claims it should be separated from the *W. ternata* aggregate (traditionally including *W. trifolia*, *W. ternata* s.str., and *W. maximowicziana*) and considered a separate species. The American *W. doniana*, *W. fragarioides*, and *W. lobata* belong to a single maternal lineage, but the observed genetic differences are too small to serve as a convincing argument for species segregation, so their relationships still remain unresolved.

Keywords: climate change; ITS concerted evolution; disjunct distribution; hybrids; the Holarctic; refugium; the Khamar-Daban Ridge; North Asia; reticulate evolution; tertiary relict

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1. Introduction

Rapid integration of molecular biology approaches into classic botany makes it possible to review the existing phylogenetic relationships based on the differences in DNA sequences. In these taxonomic revisions, small groups of related organisms are often considered, as their position on the tree of life is often debatable and sometimes too subjective. One of the examples can be *Waldsteinia* Willd., a small genus of herbaceous plants containing only a few species [1], which was joined to the genus *Geum* L. as a result of the latest revision [2]. *Waldsteinia* belongs to the Rosaceae family and inhabits the temperate zone of the Northern Hemisphere [3–19]. Before the recent taxonomical revision, it was divided into two subgenera: *Waldsteinia* and *Comaropsis* (Rich. ex. Nestl.) Teppner based on morphological differences [4,13], with all species except for *W. geoides* Willd. (the type species) belonging to the latter subgenus (Table 1). *Waldsteinia geoides* is a rather tall (15–25 cm) herb with an erect or shortly creeping rhizome and lobed basal leaves. The most

original features of this species are its well-developed leaf-like bracts, bowl-shaped receptacle, peltate petals, glabrous peduncles, which are concrescent in the lower half, and not distinctly shaped radicle of the embryo [4,14,15]. Species attributed to the *Comaropsis* are distinguished by small (reduced) bracts, flowers with a narrow conical receptacle, bifacial petals, long-haired, usually totally separate peduncles, and the embryos with a distinct radicle [4,14]. Except for *W. idahoensis* and *W. lobata* (15–40 cm high), *Comaropsis* plants are usually lower ((3)7–20 cm high) than *W. geoides,* with a creeping branched rhizome and rooting stolons, basal leaves that are mostly ternately compound/rarely deeply lobed (*W. doniana*, *W. fragarioides*, *W. maximowicziana, W. tanzibeica*, *W. ternata*, and *W. trifolia*) or shallowly lobed (*W. idahoensis* and *W. lobata*), usually gathered at the top of the rhizome (except for *W. tanzibeica*) [4,5,14–16,19]. In Eurasia, *Comaropsis* is represented by *W. ternata* s.l. Two other taxa, *W. trifolia* and *W. maximowicziana,* are often treated as subspecies of *W. ternata* s.l. *Waldsteinia tanzibeica* has never been considered an infraspecific taxon of *W. ternata*, although morphologically, it certainly belongs to the aggregate [10,16]. A similar situation is observed among the North American *Comaropsis*. In particular, *W. fragarioides* and *W. doniana* have overlapping ranges and are similar by having ternately compound or rarely deeply lobed leaves (not lobed). In this case, *W. doniana* may be treated as a subspecies or a variety of *W. fragarioides* [17] (Table 1). On the other hand, according to the classification concept based on floral characters (e.g., the relative size of the petals and sepals), *W. doniana* is more closely related to another North American species, *W. lobata* (with small petals and lobed leaves), than to *W. fragarioides* (with larger petals and trifoliolate leaves), and in this view, the former two species may form together a southern small-petaled clade. According to this view, *Waldsteinia* in eastern North America is represented by three species [18]. Moreover, a hypothesis that *W. doniana* originated as a hybrid between *W. fragarioides* and *W. lobata* was also proposed [19]. The other North American *Comaropsis* species is the large-petaled, lobed-leaved *W. idahoensis*, which is highly distinct from all other species by its morphology [19].

Table 1. The list of *Waldsteinia* samples collected for DNA analysis.

***Waldsteinia* Species**	**Species Name According to '*Geum*' Concept**	**Distribution [3–19]**
subgenus *Waldsteinia*		
++*W. geoides* Willd.	*Geum waldsteinia* Baill. =*G. waldsteiniae* Smedmark	C and E, incl. SE Europe (Bulgaria, Slovakia, Hungary, Macedonia, Serbia, Kosovo, Croatia, Romania, SW Ukraine)
subgenus *Comaropsis* (Rich. ex Nestl.) Teppner		
+*W. fragarioides* (Michx.) Tratt. ≡*W. fragarioides* subsp. *fragarioides*	*G. fragarioides* (Michx.) Smedmark	E of North America (from SE Canada: New Brunswick, Ontario and Quebec, and NE USA: from Maine west to Minnesota, to SE USA: Tennessee, North Carolina, and Arkansas)
+*W. doniana* Tratt. ≡*W. fragarioides* subsp. *doniana* (Tratt.) Teppner =*W. parviflora* Small =*W. fragarioides* var. *parviflora* (Small) Fernald	*G. donianum* (Tratt.) Weakley & Gandhi	SE of North America (USA: Alabama, Georgia, Kentucky, N Carolina, S Carolina, Pennsylvania (?), Tennessee, and Virginia)
W. idahoensis Piper	*G. idahoense* (Piper) Smedmark	W of North America (USA: Idaho, Montana)
+*W. lobata* (Baldwin) Torr. & A. Gray	*G. lobatum* (Baldwin) Smedmark	SE of North America (USA: Georgia, S Carolina, and N Carolina)
+*W. tanzybeica* Stepanov	*G. tanzybeicum* (Stepanov) Smedmark	South Siberia (the Western Sayan Mts.)
+*W. ternata* (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	South Siberia (the Western Sayan Mts., the Eastern Sayan Mts., and the Khamar-Daban Ridge)
+*W. trifolia* Rochel ex W.D.J.Koch ≡*W. ternata* subsp. *trifolia* (Rochel ex W.D.J.Koch) Teppner	*G. ternatum* (Stephan) Smedmark	C and SE Europe (SE Austria, Slovenia, Slovakia, Romania, and Serbia)
+*W. maximowicziana* (Teppner) Prob. ≡*W. ternata* subsp. *maximowicziana* Teppner	*G. ternatum* (Stephan) Smedmark	East Asia (NE China: S Jilin; Japan: Hokkaido, Honshu; North Korea, South Korea), SE of Russian Far East (the Low Amur: Khabarovsk and Primorsky regions; Sakhalin, and the Kuril Islands (?))

Notes: + taxa involved in the present study; ++ taxa involved both in the present study and in the study of J.E.E. Smedmark [2].

Waldsteinia is of great interest in terms of historical biogeography. *Waldsteinia* presumably dates back to the Neogene [20,21], having been widely distributed across the Northern Hemisphere in former times and now representing a remnant of the tertiary flora [1,4,22,23]. At least a few *Waldsteinia* species are considered true nemoral relicts with narrow and fragmented ranges [4,10,20,23]. Presently, all *Waldsteinia* species inhabit mixed mesophytic nemoral and hemiboreal forests in lowlands and the piedmonts of mountainous areas within the Holarctic [4,6,19,24]. The present geographical range of *Waldsteinia* is wide. However, it exhibits a discontinuous pattern, including the following clearly detached fragments: Central and Southeastern Europe (*W. geoides* and *W. trifolia*), South Siberia (*W. tanzybeica* and *W. ternata* s.str.), Eastern Asia (*W. maximowicziana*), eastern North America (*W. doniana*, *W. fragarioides*, and *W. lobata*), and the western part of North America bounded by Idaho and Montana states (*W. idahoensis*). *Waldsteinia tanzibeica* is the species with the narrowest range. It is considered a relict and the local endemic for the Western Sayan Mountains [16]. It is believed that even *W. fragarioides*, one of the species with the most extensive range, was much more widespread in the past than it is now. For this reason, this species may also be discussed as a relict plant from a more northern area [7,25]. This discontinuous range pattern of *Waldsteinia* correlates well with the long-known North American–Eastern Asian floristic relationship involving migration and interchange of Asian and American species via the region of the Bering Strait, followed by the disruption of the continuous ranges because of Land Bridge disappearance and the Pleistocene glaciations [23]. At the same time, no pollen records or any other reliable evidence confirming the former distribution range and migration pathways of *Waldsteinia* species are present. Comparatively low levels of morphological divergence together with fuzzy ploidy patterns within *Waldsteinia* do not allow for any clear answer either. It appears that the historical dynamics of this group have never been thoroughly studied, and only speculations and hypotheses exist on this subject.

As it was mentioned above, according to the latest revision based on the sequences of the internal transcribed spacer (ITS) and intergenic spacers of plastid DNA (*trnL-trnF*) of *Waldsteinia* together with the closely related *Coluria* R.Br. and *Taihangia* T.T.Yu & C.L.Li, are nested in the *Geum* genus [2]. *Geum* s.l. in this broad sense was previously considered the Geinae Schulze-Menz subtribe, including all herbaceous perennials. In its turn, Geinae (*Geum* s.l.), together with the woody genera *Fallugia* Endl. and *Sieversia* Willd., formed a clade corresponding to the Colurieae Rydb. tribe [26]. DNA-based phylogenetic reconstruction of Geinae [27,28] partly confirmed the hypothesis of reticulate evolution through hybridization and allopolyploidization [1,3]. Taking into account both the genetic interactions within species and the paraphyly of *Geum* (in the size suggested by W. Gajewski [1]), J.E.E. Smedmark [2] suggested that all herbaceous lineages belonging to the Geinae subtribe should be considered as a genus *Geum*. Despite the clear morphological segregation of *Waldsteinia* and *Coluria* by fruit types from other *Geum* s.l. [1,3], the broad generic concept was accepted by many taxonomists [18,26,29] and applied in global taxonomic databases, e.g., the Catalogue of Life ([11], accessed on 15 March 2023).

In the aforementioned phylogenetic reconstructions, only the type species of *Waldsteinia* and *Coluria* (*W. geoides* and *C. geoides* (Pall.) Bunge, respectively) were considered [2,30]. However, taking into account the low statistical support for some nodes, we speculate that the issue of the position of these groups on the tree and the relationships between them cannot be considered fully resolved. We suggest that a broader sampling of *Waldsteinia* and *Coluria* for the phylogenetic analysis can improve our understanding of evolutionary relationships within *Geum* s.l.

Our goals in this study were to: (1) specify the relationships between *Waldsteinia* and other taxa in *Geum* s.l. based on a broader sample; (2) estimate the inner phylogenetic structure of *Waldsteinia;* and (3) suggest the main historical pathways of species dispersal across the continents, which have never been investigated in detail.

To address these questions, we made a molecular phylogenetic reconstruction, adding more *Waldsteinia* species to those included in the previously reported phylogenies [2,30]

based on the ITS region of nuclear DNA and the *trnL-trnF* intergenic spacer of plastid DNA. We have also used these markers to shed light on *Waldsteinia* history, mainly focusing on the place of origin and the presumed migration patterns of this group during the late Cenozoic. To assess the role of polyploidization in speciation in *Waldsteinia* and during its dispersal across the continents, we collected the known data on chromosome numbers for all the species in the genus. Since the information on chromosome numbers was in some cases provided only in the original language (mainly Russian), the data on the ploidy distribution of North Asian species has so far been quite hard to obtain. Thus, our study contains the most comprehensive review of *Waldsteinia* chromosome numbers known to date. Moreover, except for *W. tanzybeica*, which has an extremely narrow range, *W. ternata* s.str. remained, until recently, the least studied species in terms of ploidy polymorphism among all Eurasian *Waldsteinia* species. Therefore, an important point was to screen additional populations of *W. ternata* to identify the dominant chromosome race as well as other possible types of ploidies in the species, including unknown diploids.

2. Materials and Methods

2.1. Plant Material Collection

For DNA sequencing, eight of nine *Waldsteinia* and two *Coluria* species were sampled. *Waldsteinia ternata* was collected from a natural population on the Khamar-Daban Ridge and the Eastern Sayan Mountains; *W. tanzybeica* from the Western Sayan Mountains; *W. maximowicziana* from the Lower Amur region; and *Coluria geoides* from the Altai Mountains. Samples of *W. geoides* and *W. trifolia* were collected from living collections in the botanical gardens. The samples of North American *Waldsteinia* species and *Coluria henryi* were collected from herbariums. The detailed list of samples is presented in Table 2. We did not have access to the narrowly distributed North American species *W. idahoensis*, and it was the only species that was not covered by our research.

Table 2. The list of DNA samples used for the phylogenetic reconstructions.

Taxon	Locality, Voucher, and Isolate Information	Field Specimen/ Life Collection/ Herbarium	Coordinates, Altitude [1]
Coluria geoides (Pall.) Bunge	Russia, South Siberia, the Altai Mts., the Katun Riv., 12 June 2022, *V. Chepinoga* and *N. Lashchinskiy* (NSK0092604), isolate A1	Field specimen	N 50.392198°, E 86.672328° 756 m alt.
Coluria henryi Batalin	China, Chongqing, 15 April 1938, *K.L. Chu* (PE01274689), isolate PE-Ch1	PE (China)	Unknown
Waldsteinia geoides Willd.	Europe, unknown locality, 5 August 2022, *M. Protopopova* and *V. Pavlichenko* (IRKU084896), isolate BGI1	Botanic garden of Irkutsk State University (Russia), life collection	Unknown
Waldsteinia doniana Tratt.	USA, Alabama, Winston County, the Sipsey Riv. near Addison, 10 April 1953, *J.W. Hardin* and *W.H. Duncan* (LE01182710), isolate LE-Wp1	LE (Russia)	Unknown
Waldsteinia fragarioides (Michx.) Tratt.	Canada, Ontario, Peterborough Country, near Douro-Dummer Township, 11 June 1948, *J. H. Soper* and *H. M. Dale* (LE01182708), isolate LE-Wf1	LE (Russia)	Unknown
Waldsteinia fragarioides (Michx.) Tratt.	USA, New York, Oatka Creek Park near Rochester, 20 May 1965, *H. Ernst* (LE01182709), isolate LE-Wf4	LE (Russia)	Unknown
Waldsteinia lobata (Baldwin) Torr. & A.Gray	USA, South Carolina, Oconee County, Brasstown Creek area, 11 May 1989, *S.R. Hill* and *C.N. Horn* (PE01683697), isolate PE-Wlo1	PE (China)	Unknown
Waldsteinia maximowicziana (Teppner) Prob.	Russia, the Far East, Lower Amur region, Sirenevka settlement, 11 September 2015, *E.A. Pimenova* (IRKU084897), isolate PK8	Field specimen	N 43°26′4.31″, E 131°58′59.02″
Waldsteinia tanzybeica Stepanov	Russia, South Siberia, the Western Sayan Mts., the Bolshoy Kebezh Riv., 11 June 2018, *V. Pavlichenko*, *V. Chepinoga*, *M. Protopopova* (IRKU084855), isolate BK1	Field specimen	N 53.071575°, E 093.132594° 406 m alt.

Table 2. *Cont.*

Taxon	Locality, Voucher, and Isolate Information	Field Specimen/ Life Collection/ Herbarium	Coordinates, Altitude [1]
Waldsteinia ternata (Stephan) Fritsch	Russia, South Siberia, the Khamar-Daban Ridge, the Bezymyannaya Riv., 31 May 2014, *V. Chepinoga*, *V. Pavlichenko*, *M. Protopopova*, and *S. Bystrov* (IRKU058136), isolate Bz1	Field specimen	N 51.59398°, E 103.90883° 496 m alt.
Waldsteinia ternata (Stephan) Fritsch	Russia, South Siberia, the Khamar-Daban Ridge, the Snezhnaya Riv., 2 June 2022, *V. Pavlichenko*, *M. Protopopova* (IRKU084895), isolate S1	Field specimen	N 51.418623°, E 104.631946° 476 m alt.
Waldsteinia ternata (Stephan) Fritsch	Russia, South Siberia, the Eastern Sayan Mts., the Zima Riv., 15 June 2015, *M. Protopopova*, *V. Chepinoga* (IRKU058083), isolate Z1	Field specimen	N 53.664800°, E 100.662747° 613 m alt.
Waldsteinia trifolia Rochel ex W.D.J.Koch	Europe, unknown locality, 28 September 2018, *V. Pavlichenko* (MSKH33328), isolate MSKH2	The Central Botanical Garden of the National Academy of Sciences of Belarus (Republic of Belarus), life collection	Unknown

[1] the geographic coordinates and altitude data were referenced by combined GPS/GLONASS positioning, datum WGS84.

At least six individuals were collected from each natural population, and single specimens per species were taken from botanical gardens and herbaria. Each sample was kept in an individual filter paper bag (23 g·m^{-2}), dried, and stored in silica gel until DNA isolation.

For new chromosome counts, the root tips of plants from natural populations were sampled and then fixed. For that, fresh tips up to 0.5 cm in length were retrieved and washed in distilled water, briefly dried on filter paper, followed by their pre-treatment in a 0.2% colchicine water solution for 2–4 h. The roots were washed of colchicine, briefly dried on filter paper, and placed in Klark's fixative (3:1 mixture of 96% ethanol-glacial [absolute] acetic acid) for at least 24 h. Samples were then washed five times and stored in 70% ethanol.

2.2. Counting Chromosome Numbers

The ploidy level was determined by a direct count of chromosomes on the metaphase plates of the root meristem, as described by M.S. Navashin [31] and L.I. Abramova and I.N. Orlova [32]. The root tips were macerated in 1 M hydrochloric acid for 10–15 s at 60 °C. Then, samples were washed three to five times in distilled water to eliminate residual hydrochloric acid and stained with 1% aceto-orcein for 8 h, followed by material squashing in 45% acetic acid. Metaphase plates were observed on an Axioscope 40 (Karl Zeiss, Oberkochen, Germany) under 100× magnification and captured by an AxiCam MRc 5 digital camera.

2.3. DNA Isolation and PCR

Total DNA was isolated from silica-dried leaf tissue following the cetyltrimethylammonium bromide (CTAB) method [33], with some authors' modifications [34].

For phylogenetic reconstruction, sequences of internal transcribed spacers (ITS1 and ITS2) of nuclear DNA (ncDNA) and *trnL-trnF* intergenic spacers of plastid DNA (ptDNA) were used as molecular markers. The ITS region was amplified using the forward ITS1-P2 [35] and the reverse ITS4 [36] primers, complementary to the flanking regions of the 18S and 26S rDNA genes. In order to reduce PCR-mediated recombination between ITS clones and to improve PCR accuracy, a proofreading polymerase was used together with lower initial template concentrations (not more than 5 ng per reaction) and PCR cycle numbers (not more than 30 cycles) as recommended by D.J.G. Lahr and L.A. Katz [37]. In particular, the PCR was performed in a reaction mixture of 20 µL containing 1x Q5 Reaction Buffer and 0.4 units of Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) with final concentrations of 2.0 mM of $MgCl_2$, 250 µM of each dNTP, and 500 nM of each primer. The conditions of amplification were 98 °C for 30 s; 30 cycles at 95 °C for 20 s; 58 °C

for 20 s; and 72 °C for 20 s, with a final elongation of 2 min at 72 °C. In the case of herbarium samples, insufficient amplification of the ITS region using Q5 polymerase was observed because of DNA degradation probably caused by long-term storage and treatment. GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA) was used to amplify the ITS region from herbarium samples and the *trnL-trnF* region from all the samples in the study. Using this polymerase, the ITS region could be successfully amplified using the primers indicated above. For the *trnL-trnF* region amplification, the combination of forward (e) and reverse primers (f) described in the study of P. Taberlet et al. [38] was used. The reaction mixture of 20 µL contained 1× Green GoTaq Flexi Buffer, 1 unit of GoTaq polymerase, and final concentrations of 2.5 mM of $MgCl_2$, 250 µM of each dNTP, and 250 nM of each primer in the final volume of 20 µL. The conditions of amplification for both DNA regions and primer pairs were 95 °C for 2 min; 35 cycles at 95 °C for 20 s; 52 °C (ITS) or 61 °C (*trnL-trnF*) for 30 s; and 72 °C for 1 min, with a final elongation of 5 min at 72 °C.

Amplicons were either directly purified from PCR mixtures (ITS) using the GeneJET Purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania) or visualized in 1% agarose gel stained by ethidium bromide after electrophoresis and then gel-purified (*trnL-trnF*) using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania).

2.4. Cloning and Sequencing

Purified amplicons were either directly sequenced (ITS, *trnL-trnF*) or additionally cloned in *Escherichia coli* cells (ITS only). For molecular cloning, amplicons were ligated into plasmid vectors pMiniT 2.0 (New England Biolabs, Ipswich, MA, USA) in the case of blunt-end products or into pTZ57R/T (Thermo Fisher Scientific, Vilnius, Lithuania) in the case of products with single 3′-A overhangs. Ligation was carried out according to the manufacturer's protocols using the insert-to-vector molar ratio of 3:1 in 5 µL of reaction mixture containing 12.5 ng of pMiniT 2.0 or 27.5 ng of pTZ57R/T. Further, 50 µL of One Shot TOP10 *E. coli* chemically competent cells (Invitrogen, Waltham, MA, USA) were heat shock transformed at 42 °C for 35 s using 2.5 µL of the ligation mixture. After transformation, cells were incubated in SOC liquid medium at 37 °C for 1.5 h and plated onto LB agar containing 100 $mg{\cdot}L^{-1}$ ampicillin. In the case of the pTZ57R/T vector, 40 µL (20 $mg{\cdot}L^{-1}$) of X-Gal solution were surface-spread over agar plates to enable blue-white screening for identification of the colonies carrying the insert. In the case of the pMiniT 2.0 vector carrying a toxic minigene in the cloning site, all grown colonies were considered to contain the insert. Eight colonies from each plate were picked with a sterile pipette tip and inoculated into 5 mL of liquid SOC medium containing 100 $mg{\cdot}L^{-1}$ of ampicillin. In the case of low transformation efficiency, all colonies were used for further analysis. Cells were grown overnight at 37 °C. Plasmids were isolated from overnight cultures using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Vilnius, Lithuania). Isolated plasmids and amplicons were Sanger sequenced in both forward and reverse directions using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Waltham, MA, USA) and M13(-20) (pTZ57R/T vector-based plasmids) or region-specific primers mentioned above (pMiniT 2.0 vector-based plasmids and PCR products) in a 3500 Genetic Analyzer (Applied Biosystems and Hitachi, Tokyo, Japan).

2.5. 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 deposited in GenBank of the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov, accessed on 10 March 2023). The ITS region was analyzed as the ITS1 and ITS2 combined set, excluding sequences for the 18S, 26S, and 5.8S genes of rRNA. Phylogenetic analysis based on the ITS ribotypes (R) was carried out in two variants: (a) using only the main ITS variants, which were found in all specimens of each *Waldsteinia* species using molecular cloning and corresponding to the total signal of the PCR product, and (b) using the broad sample of the ITS variants revealed in *Waldsteinia* by molecular cloning. The *trnL-trnF* region was analyzed, excluding parts of

the *trnL* and *trnF* genes, considering generally different evolutionary rates of coding and noncoding parts of DNA. The ITS1 + ITS2 and the *trnL-trnF* datasets were analyzed both separately and combined. For joint (ITS + ptDNA) analysis, the only main ITS ribotypes were used. For analysis, we aligned our original DNA sequences of *Waldsteinia* and *Coluria* with the sequences of other Colurieae published by J.E.E. Smedmark and T. Eriksson [30] and in some other research (Table 3).

Table 3. The taxa and DNA sequences used for the phylogenetic reconstructions.

Taxon Name [1]	Synonym by '*Geum*' Concept	Locality [2]	GenBank Accession Numbers				Ref. [4]
			Ribotype (R) [3]	ITS	Plastotype (P) [3]	*trnL-trnF*	
Acomastylis calthifolia (Sm.) F.Bolle	*Geum calthifolium* Sm.	–	–	AJ302338.1	–	AJ297324.1	[30]
A. elata (Wall.) F.Bolle	*G. elatum* Wall.	–	–	AJ302339.1	–	KY419976.1	[30,39]
A. rossii (R.Br.) Greene	*G. rossii* (R.Br.) Ser.	–	–	AJ302340.1	–	AJ297326.1	[30]
A. sikkimensis (Prain) F.Bolle	*G. sikkimense* Prain	–	–	AJ302341.1	–	AJ297327.1	[30]
Coluria geoides (Pall.) Bunge.	*G. geoides* (Pall.) Smedmark	A1	R1	MN478378	P1	MN478380	curr.
C. geoides (Pall.) Bunge.	*G. geoides* (Pall.) Smedmark	–	R2	AJ302343.1	–	–	[30]
C. henryi Batalin	*G. henryi* (Batalin) Smedmark	PE-Ch1	–	–	P1	MN478381	curr.
Erythrocoma triflorum (Pursh) Greene	*G. triflorum* Pursh	–	–	AJ302344.1	–	AJ297330.1	[30]
Fallugia paradoxa (D.Don) Endl. ex Torr.	*Fallugia paradoxa* (D.Don) Endl. ex Torr.	–	–	AJ302345.1	–	AJ297331.1	[30]
Geum aleppicum Jacq.	*G. aleppicum* Jacq.	–	–	KX645654.1	–	–	[40]
G. andicola (Phil.) Reiche	*G. andicola* (Phil.) Reiche	–	–	AJ302346.1	–	AJ297332.1	[30]
G. bulgaricum Pančić	*G. bulgaricum* Pančić	–	–	AJ302347.1	–	AJ297333.1	[30]
G. canadense Jacq.	*G. canadense* Jacq.	–	–	DQ006033.1	–	–	[41]
G. geniculatum Michx.	*G. geniculatum* Michx.	–	–	AJ302348.1	–	AJ297334.1	[30]
G. heterocarpum Boiss.	*G. heterocarpum* Boiss.	–	–	AJ302349.1	–	AJ297335.1	[30]
G. japonicum Thunb.	*G. japonicum* Thunb.	–	–	–	–	AY818238.1	[42]
G. montanum L.	*G. montanum* L.	–	–	AJ302350.1	–	AJ297336.1	[30]
G. reptans L.	*G. reptans* L.	–	–	AJ302351.1	–	AJ297337.1	[30]
G. rivale L.	*G. rivale* L.	–	–	AJ302352.1	–	AJ297338.1	[30]
G. schofieldii Calder & Roy L.Taylor	*Geum schofieldii* Calder & Roy L.Taylor	–	–	AJ302353.1	–	AJ297339.1	[30]
Geum sp. [5]	*Geum* sp.	–	–	AJ302342.1	–	AJ297328.1	[30]
G. speciosum Albov	*G. speciosum* Albov	–	–	AJ302354.1	–	AJ297340.1	[30]
G. urbanum L.	*G. urbanum* L.	–	–	AJ302337.1	–	AJ297323.1	[30]
G. vernum (Raf.) Torr. & A.Gray	*G. vernum* (Raf.) Torr. & A.Gray	–	–	AJ302355.1	–	AJ297341.1	[30]
Novosieversia glacialis (Adams ex Fisch.) F.Bolle	*G. glaciale* Adams ex Fisch.	–	–	AJ302356.1	–	AJ297342.1	[30]
Oncostylus cockaynei F.Bolle	*G. cockaynei* (F.Bolle) Molloy & C.J.Webb	–	–	AJ302357.1	–	AJ297343.1	[30]
O. leiospermus (Petrie) F.Bolle	*G. leiospermum* Petrie	–	–	AJ302358.1	–	AJ297344.1	[30]
Rosa persica J.F.Gmel.	*Rosa persica* J.F.Gmel.	–	–	AJ416468.1	–	AJ416466.1	[30]
Sanguisorba officinalis L.	*Sanguisorba officinalis* L.	–	–	AY635041.1	–	AY634774.1	[43]
Sieversia pentapetala (L.) Greene	*G. pentapetalum* (L.) Makino	–	–	AJ302359.1	–	AJ297345.1	[30]
S. pusilla (Gaertn.) Hultén	*Geum selinifolium* (Fisch. ex F. Schmidt) Hultén	–	–	AJ302360.1	–	AJ297346.1	[30]
Taihangia rupestris T.T.Yu & C.L.Li	*G. rupestre* (T.T.Yu & C.L.Li) Smedmark	–	–	AJ302361.1	–	AJ297347.1	[30]
Waldsteinia doniana Tratt.	*G. donianum* (Tratt.) Weakley & Gandhi	LE-Wp1	R1	MK616360	P1	MK616367	curr.
W. fragarioides (Michx.) Tratt.	*G. fragarioides* (Michx.) Smedmark	LE-Wf1	R1	MK616358	P1	MK616366	curr.
W. fragarioides (Michx.) Tratt.	*G. fragarioides* (Michx.) Smedmark	LE-Wf4	R2	MK616359	P1	OQ632997	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R1	MK616352	P1	MK616361	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R2	OQ625814	–	–	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R3	OQ625815	–	–	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R4	OQ625816	–	–	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R5	OQ625817	–	–	curr.
W. geoides Willd.	*G. waldsteinia* Baill.	BGI1	R6	OQ629840	–	–	curr.
W. lobata (Baldwin) Torr. & A.Gray	*G. lobatum* (Baldwin) Smedmark	PE-Wlo1		–	P1	MN478379	curr.
W. maximowicziana (Teppner) Prob.	*G. ternatum* (Stephan) Smedmark	PK8	R1	MK616357	P1	MK616365	curr.
W. maximowicziana (Teppner) Prob.	*G. ternatum* (Stephan) Smedmark	PK8	R2	OQ625818	–	–	curr.
W. maximowicziana (Teppner) Prob.	*G. ternatum* (Stephan) Smedmark	PK8	R3	OQ625819	–	–	curr.
W. maximowicziana (Teppner) Prob.	*G. ternatum* (Stephan) Smedmark	PK8	R4	OQ625820	–	–	curr.
W. tanzybeica Stepanov	*G. tanzybeicum* (Stepanov) Smedmark	BK1	R1	MK616354, MK616355	P1	MK616363	curr.
W. tanzybeica Stepanov	*G. tanzybeicum* (Stepanov) Smedmark	BK1	R2	OQ625821	–	–	curr.
W. tanzybeica Stepanov	*G. tanzybeicum* (Stepanov) Smedmark	BK1	R3	OQ625822	–	–	curr.
W. tanzybeica Stepanov	*G. tanzybeicum* (Stepanov) Smedmark	BK1	R4	OQ625823	–	–	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	BZ1	R1	OQ625824	P1	OQ632998	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	S1	R1	MK616353	P1	MK616362	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	Z1	R1	OQ625825	P1	OQ632999	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	BZ1	R2	OQ625826	–	–	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	BZ1	R3	OQ625827	–	–	curr.
W. ternata (Stephan) Fritsch	*G. ternatum* (Stephan) Smedmark	BZ1	R4	OQ625828	–	–	curr.
W. trifolia Rochel ex W.D.J.Koch	*G. ternatum* (Stephan) Smedmark	MSKH2	R1	MK616356	P1	MK616364	curr.
W. trifolia Rochel ex W.D.J.Koch	*G. ternatum* (Stephan) Smedmark	MSKH2	R2	OQ625829	–	–	curr.
W. trifolia Rochel ex W.D.J.Koch	*G. ternatum* (Stephan) Smedmark	MSKH2	R3	OQ625830	–	–	curr.
W. trifolia Rochel ex W.D.J.Koch	*G. ternatum* (Stephan) Smedmark	MSKH2	R4	OQ625831	–	–	curr.
W. trifolia Rochel ex W.D.J.Koch	*G. ternatum* (Stephan) Smedmark	MSKH2	R5	OQ625832	–	–	curr.

Notes: [1] The species names are mainly presented as cited in the study of J.E.E. Smedmark and T. Eriksson [30], the *Waldsteinia* names as given in Table 2, and the other taxon names as given in the studies where the sequences were originally mentioned. [2] Locality names are abbreviated as given in Table 2. [3] The identified ribotypes and plastotypes were continuously numbered for each species separately. [4] In the studies in which the sequences were mentioned, 'curr.' equals to the sequences obtained in the current study. [5] The species was incorrectly mentioned as *Coluria elegans* Cardot in the original study of J.E.E. Smedmark and T. Eriksson [30], but later recognized as not belonging to *Coluria* at all [27].

For *W. lobata* and *C. henryi*, only the *trnL-trnF* region was used in the analysis because of insufficient amplification of the ITS1-ITS2 region from the available herbarium specimens. In total, 37 sequences belonging to 36 taxa were included in the analysis of the main ITS ribotypes (R), and 53 sequences belonging to 34 taxa of ITS ribotypes obtained by molecular cloning were also included in the analysis. In the case of the *trnL-trnF* region (plastotypes, P), 37 sequences of 37 taxa were included in the main analysis. Some species for which the ptDNA- and ncDNA-based phylogenies were not congruent were excluded from the joint ITS + *trnL-trnF* phylogeny because of disturbances in the clustering (see below). Therefore, in the joint ITS + *trnL-trnF* analysis, only 34 sequences from 33 taxa were included.

The multiple alignments of nucleotide sequences by the MUSCLE application with a gap opening penalty of 500 and an extension penalty of 4.01 were conducted in MEGA software version 7.0.16 [44], 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 the case of the analysis of the main ITS ribotypes 46 indels (site # 24, 36, 52, 55, 60, 62, 65, 66–69, 94, 95, 96, 100, 101–109, 114, 117, 118–136, 137–171, 172–178, 179, 186–200, 216, 217–218, 267, 280, 291, 326, 342, 349, 352, 359–360, 396, 414, 422, 442, 444, 476, 490, 498, 501, 508–511, 512, 516, 533–534, 534, 539, and 537–540) from the total length of 540 positions of the alignment were coded as binary data. In the case of the analyses of a broadened sample of ITS ribotypes obtained by molecular cloning, 31 indels (site # 24, 36, 52, 59, 65–68, 94, 95, 108–126, 127–161, 162, 184, 257, 292, 307, 308, 315, 318, 325–326, 362, 383, 388, 410, 456, 464, 469, 478, 482, 499, 500, 505, and 503–506) from the total length of 506 positions in the alignment were coded as binary data. The differences in indels coding between two ITS analyses were due to the expanded dataset of intragenomic variants of ITS in the case of molecular cloning and applying the different outgroup strategies. For *trnL-trnF* analysis, 36 indels (site # 1–3, 1–19, 4–5, 31–37, 38–41, 44–48, 82, 110–111, 112–117, 122, 125–137, 138, 139, 140, 141, 142–145, 163–167, 180–202, 207, 208–213, 214–220, 221–224, 253–258, 259, 274–281, 293–300, 304, 309–317, 336–340, 359–364, 389, 418–420, 421, 454–460, 461–464, and 465–474) from a total length of 507 alignment positions, were coded as binary data. For *trnL-trnF* plastotype network analysis based on a reduced sample of taxa 13 indels (site # 31–37, 77, 105–112, 117, 120–132, 133, 134, 133–138, 156–160, 178–183, 248, 271–275, and 380–389) from the total length of 422 alignment positions, the data were coded as binary.

Phylogenetic reconstructions were obtained independently by the Bayesian inference method (BI) based on the matrices combining the nucleotide alignments and binary (gaps) datasets in MrBayes version 3.2.5 [45] and the maximum likelihood method (ML) based on multiple nucleotide sequence alignments in MEGA independently. 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.

A BI analysis of nucleotide datasets was performed using the models implemented in MrBayes with optimized parameters to better correspond with the models used in the ML analysis (see below). The analyses were performed by specifying the model and parameters for each partition of the DNA datasets using the 'applyto' option. In particular, for the ITS dataset, the HKY-like model [46] with fixed equal stationary state frequencies, gamma distribution, or no rate variation was applied to get the K80 or K80+G models depending on the dataset (see below). For the *trnL-trnF* sequences, we also used a HKY-like model with the base frequencies optimized for the T92 model and fixed on values 0.35, 0.15, 0.15, and 0.35 of A, C, G, and T, respectively, with no evolutionary rate variation among the sites. Analysis of the ITS + *trnL-trnF* datasets was performed by applying separated parameters for ITS and *trnL-trnF* partitions as described above using 'applyto' option. Binary data

(indels + inversion) were analyzed using the F81-like model [47] implemented in MrBayes with equal stationary state frequencies to match the JC69 model [48].

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 taken into account for the estimate of reaching stationarity. The sampled trees from both analyses were pooled, and 50% majority-rule consensus trees were constructed from 62,146 (joint dataset of ITS and *trnL-trnF* regions) to at least 139,000 (ITS, *trnL-trnF*) trees to estimate clade posterior probability values (PP). The final phylogenetic trees were edited in FigTree version 1.4.3 [49].

For ML analysis of the ITS region, the Kimura 2-parameter model (K80, [50]) was applied, and for the dataset of the main ITS ribotypes, the model was additionally optimized with gamma-distribution of substitution rate variation among sites (+G, 4 categories). For ML analysis of the *trnL-trnF* region, the Tamura 3-parameter model (T92, [51]) with no among-site rate variation was used. For ML analysis of the ITS + *trnL-trnF* joint dataset, the T92 +G (4 categories) was used. For all analyses, 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. In this study, ML-cladograms are presented as condensed trees computed in MEGA and based on the original tree with the highest log-likelihood and collapsing branches with bootstrap confidence levels (BS) lower than 50%. The bootstrapped 50% majority-rule consensus trees for each dataset were constructed to compare topology with the highest log likelihood tree.

To assess the matrilineal genealogical relationship between *Waldsteinia* species and closely related taxa, a network based on *trnL-trnF* plastotypes was constructed using the integer neighbor-joining method (IntNJ, with a reticulation tolerance parameter equal to '0') implemented in PopART software version 1.7 [52]. The network was built based on the combined nucleotide alignment and binary (gaps) matrix of *Waldsteinia* taxa, *C. geoides*, *C. henryi*, and *T. rupestris,* with *S. pusilla* + *F. paradoxa* as outgroups.

3. Results

3.1. Phylogenetic Analysis Based on Nuclear DNA

The evolutionary relationships between the nuclear genomes of the species under study were inferred based on the polymorphisms in the main ITS ribotypes (Figure 1, the left side).

The phylogenetic trees based on ML and BI methods consisted of the well-supported clade of Colurieae (node A, BS, 100; PP, 1.00), which included node C (BS, 85; PP, 0.99) with the woody *Fallugia* and *Sieversia,* and a well-supported clade of herbaceous perennials (node B, BS, 100; PP, 1.00), i.e., earlier known as subtribe Geinae [30] and later as *Geum* s.l. [2].

The subtribe Geinae (*Geum* s.l.) was rather poorly structured and contained only a few clades, including but not limited to the clade formed by *G. schofieldii* and New Zealand *Oncostylus* spp. (node E, BS, 76; PP, 0.99) and the clade formed by *G. andicola*, *G. bulgaricum*, and *N. glacialis* (node H, BS, 88; PP, 1.00). Most taxa, including *C. geoides* and *T. rupestris,* remain unresolved. In what regards *Waldsteinia*, the main ITS ribotype (R1) sequence for *W. geoides* obtained in the present study was identical to the one published by J.E.E. Smedmark and T. Eriksson [30], which explains why only one sequence was used in the main phylogeny reconstruction (Table 3). The main *W. ternata* ribotype (R1) was shared by the specimens from different localities (Bz1, S1, and Z1, see Table 3). For this reason, only a single sequence for this species was used in the phylogeny reconstruction. On the phylogenetic tree, all studied *Waldsteinia* species were grouped together,

although with only moderate support (node F, BS, 66). Within the clade F, *W. geoides* was a sister to the group including *Comaropsis* species (node L, BS, 90). This latter group contained a clade combining ribotypes from European (*W. trifolia*) and Siberian (*W. ternata* and *W. tanzybeica*) species (node K, PP, 0.96) and a North American group including *W. fragarioides* and *W. doniana* (node M, PP, 1.00). The unresolved branch corresponded to the Northeast Asian *W. maximowicziana* ribotype.

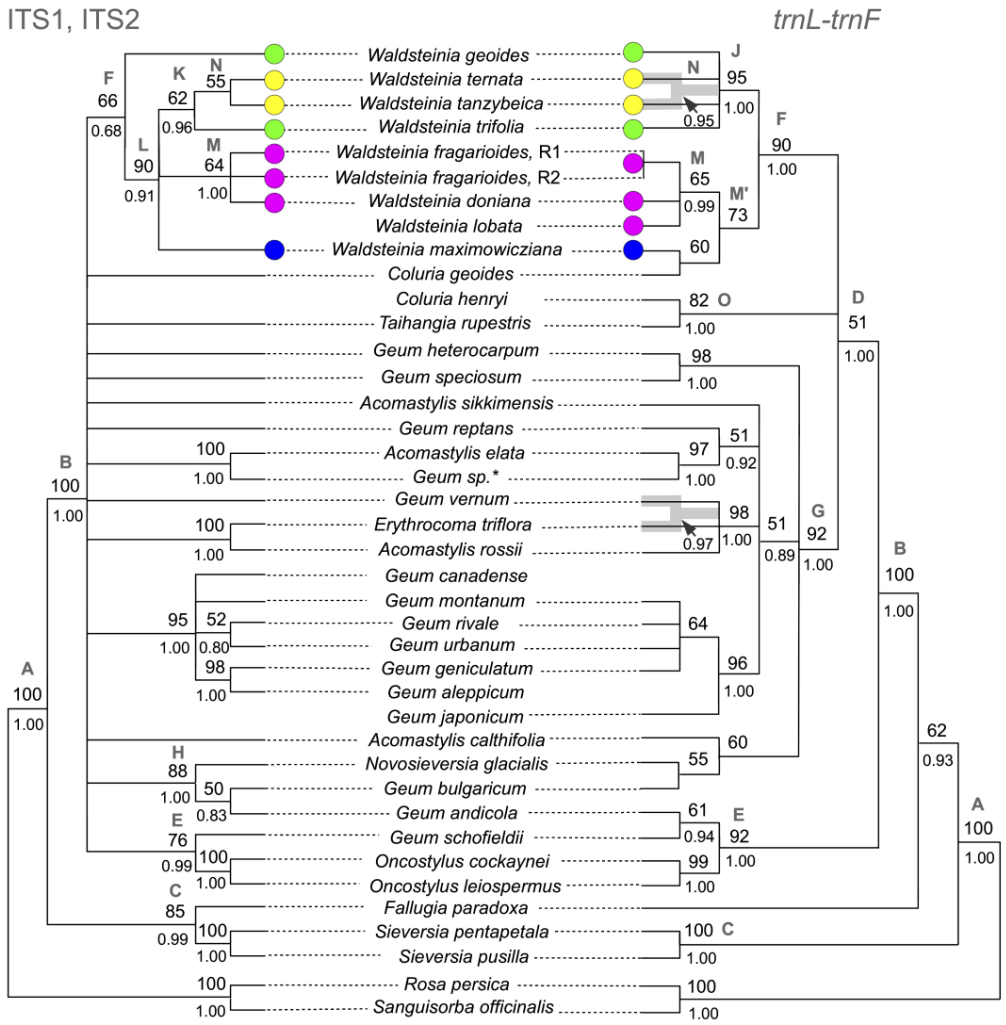

Figure 1. Phylogenetic trees based on the ML 50% bootstrap confidence level condensed tree (the tree with the highest log likelihood): tree based on the main ITS ribotypes (R), on the left side; *trnL-trnF* tree, on the right side. The BI analysis was carried out independently. Thick gray lines indicate additional supported nodes (PP $\geq$ 0.95) on the BI 50% majority-rule consensus tree compared to the ML tree. The branch leading to the clade combining *Rosa persica* and *Sanguisorba officinalis* was used to root the trees. Bootstrap values are indicated above the branches, and posterior probabilities of the corresponding clades on the BI tree (if relevant) are indicated below the branches. The capital letters at the nodes correspond to the groups discussed in the text. Species names are given as listed in Table 3 ('Taxon Name' column). The geographical pattern of *Waldsteinia* species distribution is indicated by different colors: Europe is light-green, North America is light purple, Northeast Asia is dark blue, and South Siberia is yellow. Note: * indicates the species incorrectly mentioned as *Coluria elegans* in the original study of J.E.E. Smedmark and T. Eriksson [30], but later recognized as not being *Coluria* [27].

To detect the low-copy ITS variants that may bear evidence of the probable hybridization events between Eurasian *Waldsteinia* suggested based on the incongruent inheritance

of ncDNA and ptDNA in *W. geoides* (see below), molecular cloning of this DNA region was additionally carried out for these species (Figure 2).

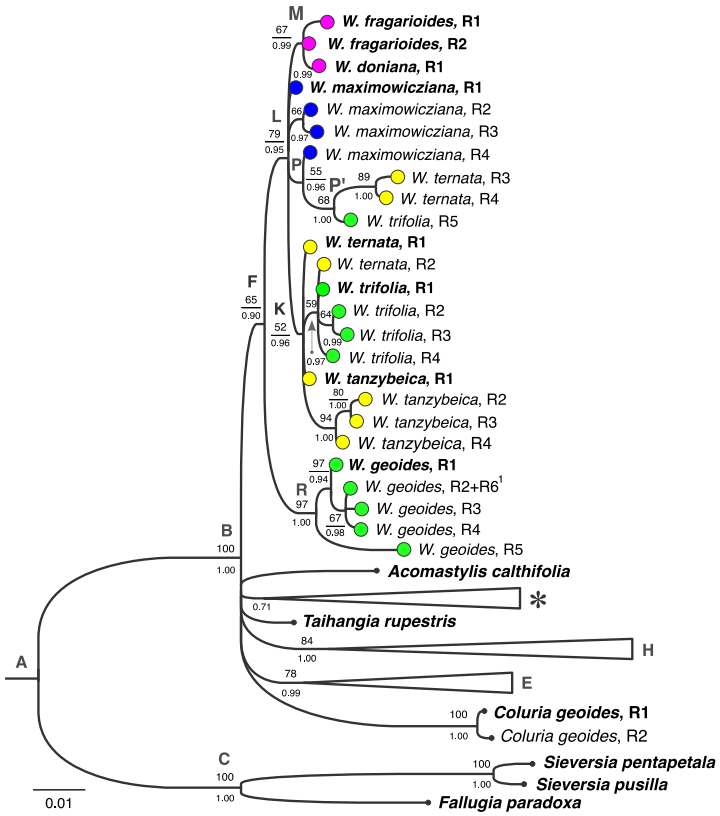

Figure 2. BI phylogram of multiple ITS ribotypes (R) of *Waldsteinia* and related species based on the 50% majority-rule consensus tree. The ITS ribotypes used in the associated phylogenetic analyses (Figure 1 and subsequent ones) are shown in bold. Posterior probabilities are indicated below the branches and bootstrap values of the respective clades on the independent ML tree (if relevant) above the branches. Capital letters at the nodes indicate the groups (including the collapsed clades) discussed in the text and correspond to those in Figure 1. The color pattern also corresponds to that in Figure 1. The asterisk indicates the collapsed clade, which includes the following taxa: *Acomastylis elata*, *A. rossii*, *A. sikkimensis*, *Erythrocoma triflorum*, *Geum aleppicum* Jacq., *G. canadense*, *G. geniculatum*, *G. heterocarpum*, *G. montanum*, *G. reptans*, *G. rivale*, *Geum* sp., *G. speciosum*, *G. urbanum*, and *G. vernum*. The scale bar indicates the number of expected changes (substitutions and/or indels) per site, corresponding to the unit of branch length. Note: [1] Since R2 and R6 of *W. geoides* are identical in their ITS1 and ITS2 partitions (they differ by only a single mismatch in the 5.8S part), the two ribotypes were combined for the phylogenetic analysis, which was based on the ITS dataset excluding the 5.8S part (see Section 2.5).

The analysis revealed additional ribotypes in the ITS region. All ribotypes in *W. geoides* (R1–R6) showed high affinity (Figure 2, node R, BS, 97; PP, 1.00). The phylogenetic structure of *Comaropsis* (node L, BS, 79; PP, 0.95) reconstructed based on the extended ribotype sample in general corresponded with the results of the analysis of the main ITS ribotypes (Figure 1). In particular, the clade combining Euro-Siberian *Comaropsis* (node K, PP, 0.96) ribotypes and the North American species (node M, PP, 0.99) haplogroup were presented on the tree as they were in the case with the main ribotypes. The North American species showed extremely high affinity for each other, and not more than one or two mismatches were found in the ITS region between *W. doniana* (R1) and the ribotypes belonging to *W. fragarioides* (R2 and R1, respectively). Molecular cloning revealed an additional haplogroup combining the minor ribotypes of Euro-Siberian species and Northeast Asian *W. maximowicziana* (node P, PP, 0.96). The branch lengths in the phylogram suggested that the main ribotype of

W. maximowicziana (R1) has the shortest distance to the node L, which corresponds to the most recent common ancestor of *Comaropsis*.

The results of the multiple alignment also showed that there was only one site (site # 395, dark green) that may be a synapomorphy for the *Waldsteinia* group. However, this site may also be considered a homoplasy or even plesiomorphy if we take the outgroups (*Rosa* and *Sanguisorba*) into account (Figure 3).

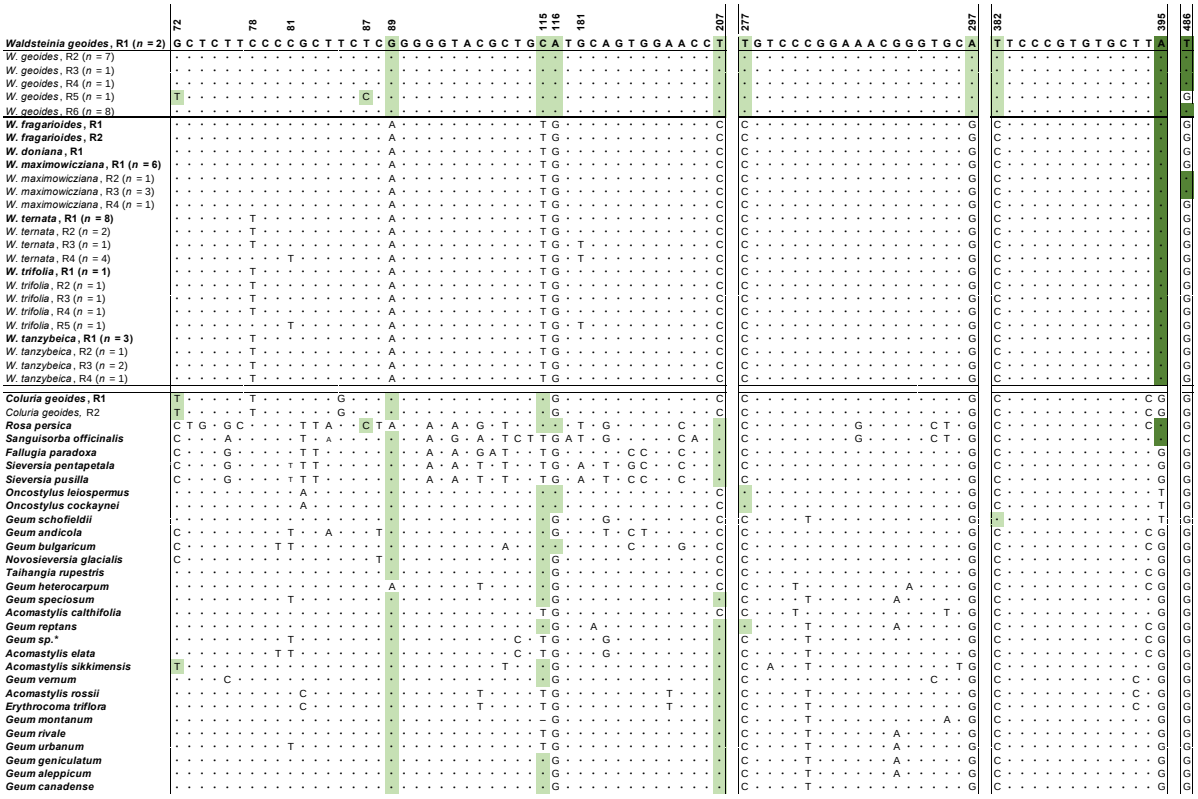

Figure 3. Parts of the multiple alignment of the ITS ribotype (R) sequences showing the mismatches between *W. geoides* and other species. ITS region variants used for the main phylogenetic reconstruction (Figure 1) are given in bold. The number of ribotype copies found by molecular cloning is provided in brackets. Dots indicate the same base as in the reference sequence (*W. geoides*, R1); characters indicate the differences. Numbers indicate the positions in the multiple alignments, including the indel regions. The indels themselves and missing data are mostly not shown in the picture. Light green indicates the mismatch positions between the ribotypes belonging to *W. geoides* and other *Waldsteinia* species. The dark green indicates possible synapomorphies for *W. geoides* and other *Waldsteinia* species. The species names are provided as they are presented in Figure 1. Note: * indicates the species incorrectly mentioned as *Coluria elegans* in the original study of J.E.E. Smedmark and T. Eriksson [30], but later recognized as not being *Coluria* [27].

Another position (site # 486, dark green) was identical in most *W. geoides* ribotypes and the two ribotypes from only one of the *Comaropsis* species. At the same time, there were at least nine sites by which *W. geoides* ribotypes differed from the sequences of other *Waldsteinia* species (Figure 3, light green). One site seems to be an autapomorphy for *W. geoides* (site # 297), and the others were shared by *W. geoides* and different sets of taxa.

3.2. Phylogenetic Analysis Based on Plastid DNA

The phylogenetic tree based on the *trnL-trnF* plastid DNA region (Figure 1, the right side) was better structured than the ITS tree and had similar topology to those presented by J.E.E. Smedmark and T. Eriksson [30]. Colurieae received good support (node A, BS, 100; PP, 1.00) and included well-supported clades of the subtribe Geinae (*Geum* s.l.) (node

B, BS, 100; PP, 1.00), monophyletic *Sieversia* (node C, BS, 100; PP, 1.00), and *Fallugia paradoxa.* However, the latter was included in the common clade with *Sieversia* on the ITS phylogenetic tree. In turn, herbaceous Geinae (node B) consisted of a big clade comprised of a majority of species (node D, PP, 1.00) and a small, well-supported clade (node E, BS, 92; PP, 1.00) of monophyletic *Oncostylus* spp. together with *G. andicola* and *G. schofieldii*. Except for *G. andicola*, the clade defined by node E had a structure similar to the corresponding clade on the ITS tree. The clade defined by the node D consisted of (1) the well-supported *Waldsteinia* clade (node F, BS, 90; PP, 1.00) in which *C. geoides* was now found as compared with the corresponding clade on the ITS tree, (2) the clade made up by *C. henryi* and *T. rupestris* (node O, BS, 82; PP, 1.00), and (3) the well-supported clade embracing the rest of *Geum* s.l. (node G, BS, 92; PP, 1.00) and characterized by additional well-supported structure.

In the *Waldsteinia* species, for which specimens from several populations were used in the analysis, we did not observe any intraspecies polymorphism in the *trnL-trnF* region. In particular, the plastotypes of *W. ternata* specimens from all three studied localities were identical (P1, see Table 3). Three North American species, including two populations of *W. fragarioides,* also had identical plastotypes. Moreover, the sequences of the *trnL-trnF* region in *W. geoides* and *C. geoides* obtained in the present study were identical to those reported by J.E.E. Smedmark and T. Eriksson for the mentioned species [30]. Given this identity of plastotypes, we used only one sequence per each *Waldsteinia* taxon and *C. geoides* to perform phylogenetic reconstructions and designated them as P1 in Table 3 for each species individually. On the phylogenetic tree, all Siberian and European *Waldsteinia* species formed a separate subclade (node J, BS, 95; PP, 1.00), to which the European *W. geoides* was added in comparison with the corresponding clade on the ITS tree (the left side, node K). Inside the clade, *W. ternata* and *W. tanzybeica* combined on the BI (node N, PP, 0.95) but not on the ML tree. Three North American plastotypes being identical were grouped in a single clade (node M, PP, 0.99), similar to that on the ITS tree. The relationships between Northeast Asian *W. maximowicziana*, *C. geoides,* and the aforementioned *Waldsteinia* groups of species remained unresolved. However, we have found that these species tended to form a single clade together with the North American plastotypes (node M', BS, 73).

3.3. Combined Phylogenetic Analysis

The topology of the phylogenetic tree built based on the joint dataset (ITS + *trnL-trnF*) was well-structured and similar to the topology of the *trnL-trnF* tree. In particular, subtribe Geinae (*Geum* s.l.) consisted of a well-supported *Waldsteinia* clade (clade I, BS, 96; PP, 1.00), a clade containing most of the *Geum* s.l. species (clade IV, BS, 76; PP, 1.00), and a clade comprising of *Oncostylus* and *G. schofieldii* (clade V, BS, 98; PP, 1.00) (Figure 4). We excluded *G. andicola* from the analysis of the joint dataset because of the serious discrepancy between its position on the ITS and the *trnL-trnF* tree, as it showed affinity for both a subgroup within the clade IV and the group V (see Figure 1), which disturbed the normal clusterization of all other species. *Coluria geoides* (branch II) appeared to be sister to *Waldsteinia,* according to the BI analysis (PP, 0.95). The second analyzed *Coluria* species, *C. henryi*, was missing on the tree because of the insufficient amplification of the ITS1-ITS2 region. The relationships between *T. rupestris* (branch III) and any of the aforementioned taxa and clades remained unresolved because of the poor support observed for the I+II+III group. *Fallugia* (branch VI) and *Sieversia* (clade VII) were outgroup taxa for Geinae, and together with the latter they made the Colurieae.

The clade I, which contained all *Waldsteinia* species, was monophyletic and well supported by both ML and BI analyses. Within *Waldsteinia*, the group combining the European and Siberian taxa was well supported (BS, 78; PP, 1.00) and formed by a nested clade of three *Comaropsis* species (*W. tanzybeica*, *W. ternata*, and *W. trifolia*) and *W. geoides* as a sister. Within the latter clade, two Siberian species (*W. ternata* and *W. tanzybeica*) clustered together (BS, 75; PP, 0.99). The North American haplotypes H1 (R1 + P1) and H2 (R2 + P1) of *W. fragarioides* and the haplotype of *W. doniana* formed a common clade with high support (BS, 86; PP, 1.00). The relationships between Northeast Asian *W. maximowicziana* and the

aforementioned groups within *Waldsteinia* remained unresolved. However, we found that this species tends to assemble with the North American haplotypes with high support (BS, 82) if the ML consensus tree instead of the highest log likelihood tree is built (Figure 4, a dashed line).

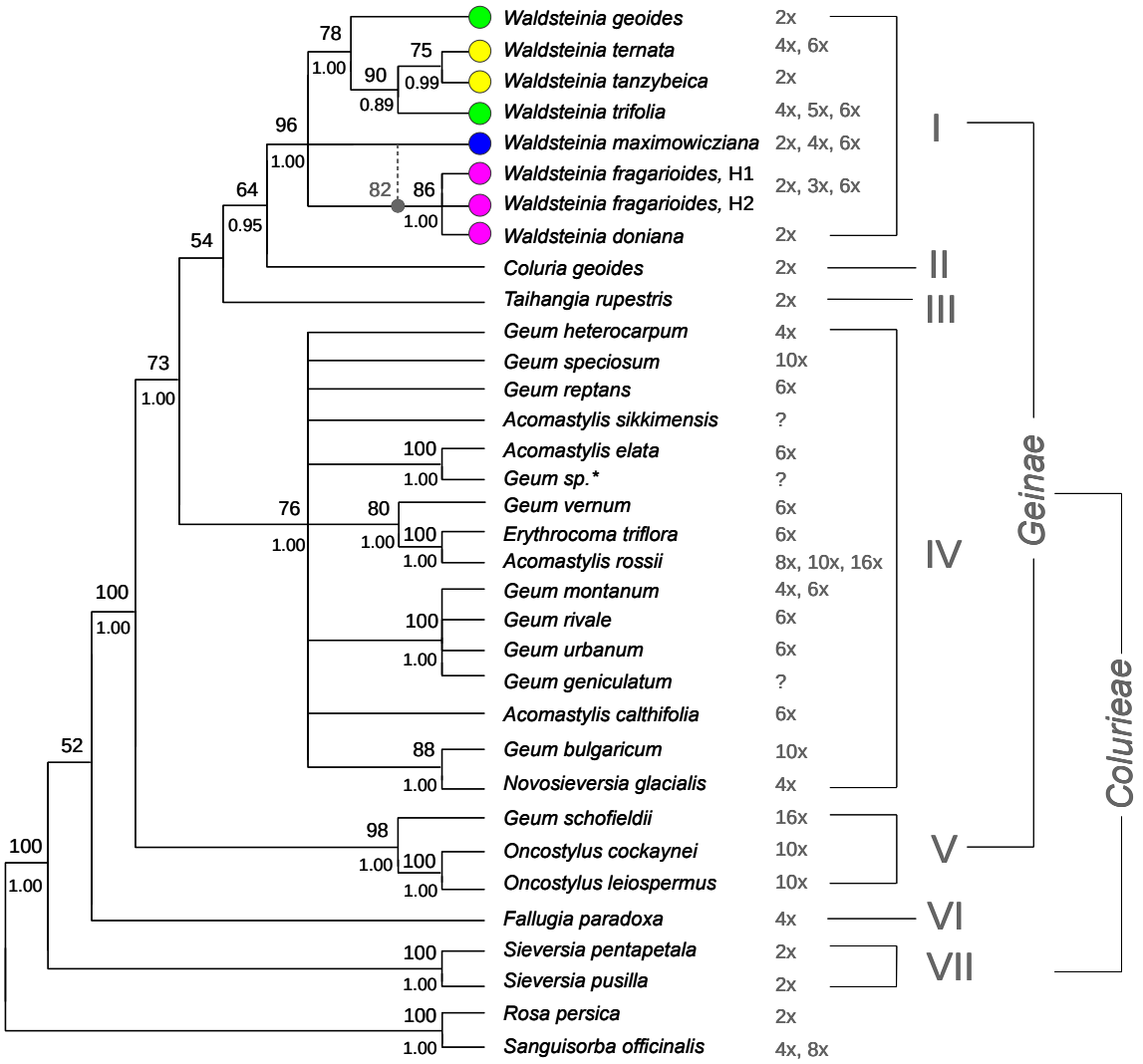

Figure 4. Phylogenetic tree based on the ML 50% bootstrap confidence level condensed tree constructed using the joint ITS + *trnL-trnF* dataset. The dashed line shows the extra node with high support on the ML bootstrap 50% majority-rule consensus tree. The BI analysis was carried out independently. The branch leading to the clade combining *Rosa persica* and *Sanguisorba officinalis* was used to root the trees. Bootstrap values are shown above the branches, and posterior probabilities of the corresponding clades on the BI tree (if relevant) are shown below the branches. The clades and branches discussed in the text are indicated by Roman numbers. Ploidy levels are presented in the column on the right side (including the literature data [30]). The species names are given as they are presented in Figure 1. The color pattern also corresponds to that in Figure 1. Note: * indicates the species incorrectly mentioned as *Coluria elegans* in the original study of J.E.E. Smedmark and T. Eriksson [30], but later recognized as not being *Coluria* [27].

3.4. Chromosome Numbers

The original data ('curr.') and the summary of the published information on chromosome numbers are presented in Table 4 as well as in Figure 4 and the next one.

Table 4. The chromosome data ($2n$) of the *Waldsteinia* species, $x = 7$.

The Part of Range	Locality	Region [1]	Coordinates (If Known)	Voucher [2]	$2n$	Ref.
		I. Waldsteinia ternata				
The Khamar-Daban Ridge (Southern Siberia)	the Bezymannaya riv.	Irk	N 51.59373°, E 103.90829°, 461 m alt.	C1533, IRKU	28	curr.
	the Utulik riv.	Irk	N 51.54594°, E 104.04675°, 453 m alt.	C1549, IRKU	28	curr.
	the Khara-Murin riv.	Irk	N 51.45202°, E 104.41242°, 468 m alt.	C1540, IRKU	28	curr.
	the Snezhnaya riv., # 1	Irk	N 51.43906°, E 104.63385°, 474 m alt.	C1521, IRKU	28	curr.
	the Snezhnaya riv., # 2	Irk	N 51.3833°, E 104.6333°, 492 m alt.	C0958, IRKU	42	[53]
	the Bolshoi Mamai riv., # 1	Bur	N 51.44864°, E 104.77549°, 472 m alt.	C1546, IRKU	28	curr.
	the Bolshoi Mamai riv., # 2	Bur	N 51.45546°, E 104.78033°, 456 m alt.	C1518, IRKU	28	curr.
	the Vydrinaya riv.	Bur	N 51.48181°, E 104.85162°, 457 m alt.	C1510, IRKU	28	curr.
	the Anosovka riv.	Bur	N 51.5167°, E 104.9501°, 470 m alt.	C1067, IRKU	28	[53]
	the Dulikha riv.	Bur	N 51.53376°, E 105.02878°, 474 m alt.	C1514, IRKU	28	curr.
	Unknown	–	–	–	42	[54]
The Eastern Sayan Mts. (Southern Siberia)	the Zima riv.	Irk	N 53.66476°, E 100.66254°, 613 m alt.	C1561, IRKU	28	curr.
The Western Sayan Mts. (Southern Siberia)	the Kaldar riv.	Krs	N 53.02776°, E 092.39216°, 379 m alt.	C1683, IRKU	28	curr.
		II. Waldsteinia tanzibeica				
The Western Sayan Mts.	the Bolshoy Kebezh riv., # 1	Krs	N 53°04′, E 93°08′	KRSU	14	[55]
	the Bolshoy Kebezh riv., # 2	Krs	N 53.071575°, E 93.132594°, 406 m alt.	C1677, IRKU	14	curr.
		III. Waldsteinia maximowicziana				
The Russian Far East	Akademgorodok # 1	Prk (Vla)	–	07607, VLA	14	[56]
	The Mal. Sedanka riv.	Prk (Vla)	–	09514, VLA	14	[56]
	The Bogataya Griva	Prk (Vla)	–	10968, VLA	14	[57]
	Taiozhny settl.	Prk	–	07266, VLA	14	[56]
	Akademgorodok # 2	Prk (Vla)	–	11387, VLA	28	[58]
	Russky island	Prk (Vla)	–	12242, VLA	28	[59]
	Vtoraya rechka, # 1	Prk (Vla)	–	05697, VLA	28	[60]
	Vtoraya rechka, # 2	Prk (Vla)	–	10548, VLA	28	[57]
	Chernaya rechka	Prk (Vla)	–	10985, VLA	28	[58]
	Partizan settl.	Prk	–	10177, VLA	28	[57]
	Razdolnoe settl.	Prk	–	11314, VLA	28	[58]
	Razdolnoe settl.	Prk	–	12759, VLA	28	[61]
	Tigrovy settl.	Prk	–	09515, VLA	28	[56]
	The Tigrovaya riv.	Prk		13637, VLA	14	[62]
	Komsomolsk-on-Amur	Khk	–	08808, VLA	28	[56]
	Palevo settl.	Sakh	–	08885, VLA	28	[63]
	–	–	–	–	42	[13,54]
The Japanese archipelago	Arasmyama, near Asahikawa	Hokk	–	–	28	[64]
		IV. Waldsteinia trifolia				
The South-Eastern Alps and the Carpatians (Central and Eastern Europe)	Bleiburg, northern (Carinthia)	Aus	–	–	28	[4]
	Meža (Ranve)	Sln	–	–	28	[4]
	Paka riv. (Valenje)	Sln	–	–	28	[54]
	Tisovec	Slk	–	SLO	28	[65]
	Revúca	Slk	–	SLO	28	[65]
	Strelníky	Slk	–	SLO	28	[66,67]
	Frantschach (Carinthia)	Aus	–	–	35	[4]
	Wolfsberg (Carinthia)	Aus	–	–	35	[4]
	Lavamünd (Carinthia)	Aus	–	–	35	[4]
	Bleiburg, southern (Carinthia)	Aus	–	–	35	[4]
	Lippitzbach (Carintia)	Aus	–	–	35	[68]
	Nevljica riv. (Kamnic)	Sln	–	–	35	[69]
	Hliník nad Hronom	Slk	–	GZU	35	[4]
	Transylvania	Rom	–	–	42	[1,4]
		V. Waldsteinia geoides				
The Carpathians (Central and Eastern Europe)	Unknown	Hun	–	–	14	[70]
	Unknown (cult., Kiel)	–	–	–	14	[71]
	Unknown	–	–	–	14	[17,72]
	Jablonov nad Turňou	Slk	–	SLO	14	[65]
	Unknown (cult.)	Pol	–	–	14	[73,74]

Table 4. *Cont.*

The Part of Range	Locality	Region [1]	Coordinates (If Known)	Voucher [2]	2*n*	Ref.
		VI. Waldsteinia fragarioides				
The Appalachian Mts. and the Great Lakes region (North America)	Greater Napanee	Ont	–	–	14	[13]
	Ottawa	Ont	–	3552, DAO	14	[75]
	Fitzroy Provincial Park # 1	Ont	–	3553, DAO	14	[17,75]
	Fitzroy Provincial Park # 2	Ont	–	3554, DAO	14	[17,75]
	Kutztown	Penn	–	3556, DAO	14	[17,75]
	Otter Lake Sanctuary	Ont	–	3550, DAO	21	[75]
	Gatineau Park	Que	–	3551, DAO	21	[75]
	Ottsville	Penn	–	3555, DAO	21	[17,75]
	George Landis Arboretum	NY	–	–	21	[13]
	Smart View, Blue Ridge parkw.	Va	–	–	42	[54]
		VII. Waldsteinia doniana				
The Piedmont of the Appalachian Mts. (North America)	Uwharrie National Forest	NC	–	GZU	14	[17]
		VIII. Waldsteinia idahoensis				
The Bitterroot Mts. region (North America)	Lochsa River	Ida	–	GZU	28	[17]
		IX. Waldsteinia lobata				
The Southern Appalachian Mts. (North America)	Brasstown Creek	SC	–	GZU	14	[17]

Notes: [1] the administrative region/country of the localities: **Aus**, Austria; **Bur**, Republic of Buryatia, Russia; **Hokk**, Hokkaido, Japan; **Ida**, Idaho, US; **Irk**, Irkutskaya Oblast', Russia; **Krs**, Krasnoyarsky Krai, Russia; **Khk**, Khabarovsky Krai, Russia; **NC**, North Caroline, USA; **NY**, New York, USA; **Ont,** Ontario, Canada; **Penn**, Pennsylvania, USA; **Prk,** Primorsky Krai, Russia; **Que**, Quebec, Canada; **Rom**, Romania; **Sakh**, Sakhalinskaya Oblast', Sakhalin island, Russia; **SC**, South Caroline, USA; **Sln**, Slovenia; **Slk**, Slovakia, **Va**, Virginia, USA; **Vla**, Vladivostok, Russia. [2] The voucher ID (if known) and herbarium codes are given: **DAO**, Department of Agriculture (Ottawa, Canada); **GZU**, Karl Franzes University of Graz (Graz, Austria); **IRKU,** the herbarium of the Department of Botany and Genetics, Irkutsk State University (Irkutsk, Russia); **KRSU**, the herbarium of Siberian Federal University (Krasnoyarsk, Russia); **SLO**, Comenius University (Bratislava, Slovakia); **VLA**, the herbarium of Institute of Biology and Soil Science (Vladivostok, Russia).

The basic chromosome number for all *Waldsteinia* species is a constant: $x = 7$. Most species investigated more than once exhibited the existence of different ploidy levels. *Waldsteinia geoides* and *W. tanizbeica* were exceptions that apparently were stable diploids ($2x$; $2n = 14$). In addition to this species, diploids were revealed in two other species, i.e., *W. doniana* and *W. lobata*. However, because they were studied only once, their stability as diploids still needs to be confirmed. Diploids also occurred in the East Asian *W. maximowicziana* and in the North American *W. fragarioides*.

In addition to diploids, tetraploid ($4x$; $2n = 28$) races were found to be one of the most common within the genus, especially for Eurasian species. This included *W. ternata*, *W. trifolia* (simultaneously with the pentaploid race), *W. maximowicziana* (simultaneously with the diploid race), and North American *W. idahoensis*. Hexaploids ($6x$; $2n = 42$) occurred occasionally in different species and were reported mainly by H. Teppner [4,13,54]. The repeated attempts to find hexaploids for *W. ternata*, once revealed in previous research on the Khamar-Daban Ridge [53], were unsuccessful. All new counts showed only tetraploids (Table 4). A similar situation was also observed for *W. maximowicziana*. Hexaploid variants of this species could not be consistently found in the Russian Far East [62]. Chromosome races, including triploids ($3x$) and pentaploids ($5x$), were found to be codominant for *W. fragarioides* and *W. trifolia*, respectively.

3.5. Geographical Patterns of Plastotype Distribution

The phylogeographical analysis of *Waldsteinia* performed using the phylogenetic relationships based on the *trnL-trnF* plasotypes indicated maternal inheritance (Figure 5).

The plastotype network (Figure 5a) was prepared based on the well-supported clade F combining *Waldsteinia* species and *C. geoides* on the plastid DNA phylogram (Figure 5b, BS, 90; PP, 1.00), the sequences of closely related species (*T. rupestris* and *C. henryi*), and outgroup taxa (*F. paradoxa* and *S. pusilla)*. The plastotype of *W. maximowicziana* appeared to be that belonging to the most recent common maternal ancestor of *C. geoides* and *Waldsteinia*

species. The plastotypes of *C. geoides*, North American (*W. doniana*, *W. fragarioides*, and *W. lobata*), and European (*W. geoides* and *W. trifolia*) species of *Waldsteinia* were derivatives of that of *W. maximowicziana*. The youngest plastotypes within *Waldsteinia* belonged to the Siberian taxa (*W. tanzybeica* and *W. ternata*). The current geographical location of plastotypes is schematically shown in Figure 5c, where arrows indicate our hypothetical model of the most probable scenario of intercontinental species migrations (see Discussion).

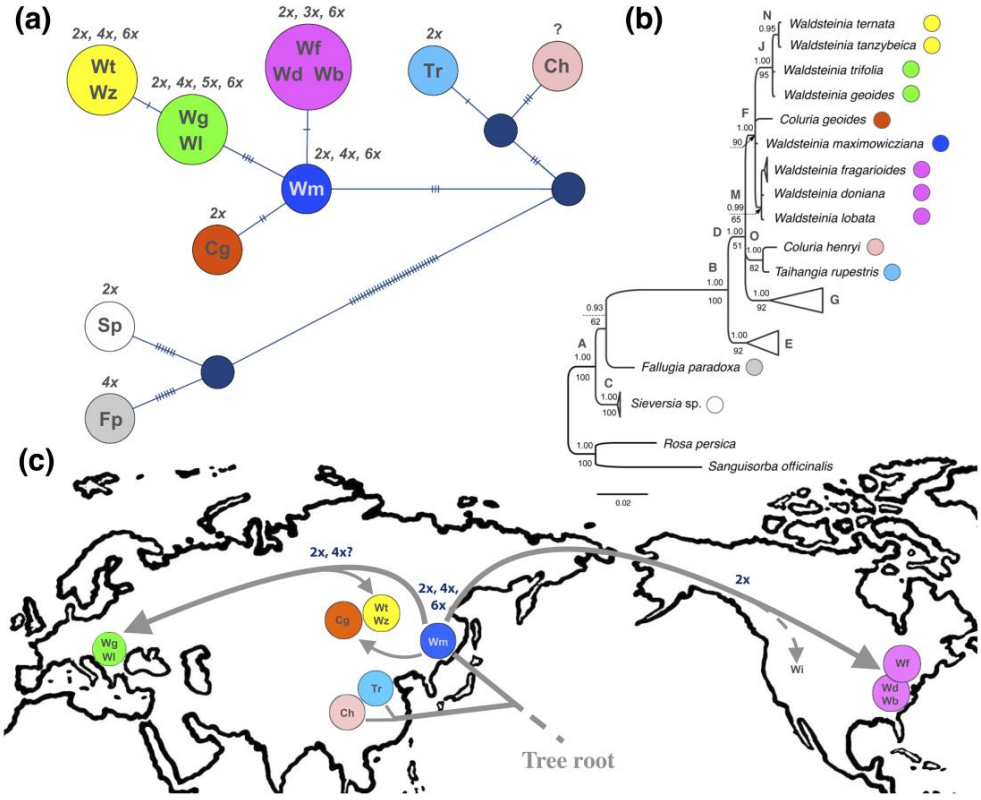

Figure 5. Plastotype network of *Waldsteinia* and several related species constructed using the IntNJ method (**a**); phylogram resulting from the BI analysis of the *trnL-trnF* region (**b**); and suggested scenarios of the historical pathways of *Waldsteinia* species dispersal across the continents based on their present phylogeographical structure (**c**). (**a**): Different haplotypes are presented as colored circles, with species abbreviations provided inside the circles, and are connected by lines, where hatch marks correspond to the number of evolutionary events (substitutions and/or indels). The color pattern of *Waldsteinia* taxa corresponds to that in Figure 1, where unlabeled dark blue dots show network vertices. Modern ploidy levels corresponding to each plastotype are indicated above the circles. (**b**): The BI phylogenetic tree; the posterior clade probabilities are shown above the branches, and bootstrap values of the respective clades on the independent ML tree are below the branches. Capital letters indicate the nodes (including the collapsed clades) discussed in the text and correspond to those in Figure 1. The color pattern also corresponds to that in Figure 1. The scale bar indicates the number of expected changes (substitutions and/or indels) per site per unit of branch length. (**c**): The curved gray arrows schematically show the supposed distribution directions of species carrying different plastotypes within Holarctic during the Cenozoic. The dashed arrow shows the hypothetical pathway that is not based on molecular genetic data. The dominant chromosome races participating in *Waldsteinia* dispersal are indicated by curved arrows. The straight lines schematically indicate the branches on the phylogenetic tree corresponding to the closely related taxa (*Coluria henryi* and *Taihangia rupestris*). Notes: The species names were abbreviated by the following: **Cg**, *Coluria geoides*; **Ch**, *C. henryi*; **Fp**, *Fallugia paradoxa*; **Sp**, *Sieversia pusilla*; **Tr**, *Taihangia rupestris*; **Wb**, *Waldsteinia lobata*; **Wd**, *W. doniana*; **Wf**, *W. fragarioides*; **Wg**, *W. geoides*; **Wi**, *W. idahoensis*; **Wl**, *W. trifolia*; **Wm**, *W. maximowicziana*; **Wt**, *W. ternata*; **Wz**, *W. tanzybeica*.

4. Discussion

4.1. The Updated Phylogenetic Reconstruction of Colurieae

Our reconstruction based on the joint ITS + *trnL-trnF* dataset (Figure 4) did not generally contradict the results obtained by J.E.E. Smedmark and T. Eriksson [30]. However, there were some differences in *Fallugia* and *Sieversia* relationships. In the study of J.E.E. Smedmark and T. Eriksson [30], *Fallugia* was sister to the combined clade of *Sieversia* and Geinae (*Geum* s.l.). In our study, the relationships between *Fallugia* and *Sieversia* appeared to be unresolved in the final reconstruction. However, the ITS and *trnL-trnF*-based phylogenetic trees revealed different interactions between these two groups, but neither of them matched the result obtained in the work of J.E.E. Smedmark and T. Eriksson [30]. We believe that the observed incongruence may be explained by the differences in taxon sampling between the two studies, considering the broad sampling of outgroup taxa in the study of J.E.E. Smedmark and T. Eriksson [30]. Within Geinae, the clade V (Figure 4; bootstrap, 98; PP, 1.00) mainly formed by *Oncostylus* was sister to the well-supported clade (I+II+III+IV; BS, 73; PP, 1.00), including all the other representatives of the group. The relationship between the *Oncostylus* clade and the other Geinae was not fully resolved on the phylogenetic tree obtained by J.E.E. Smedmark and T. Eriksson ([30]: Figure 5, node K). In both studies, nesting of North American *G. schofieldii* in the New Zealand *Oncostylus* clade (V) was observed. The *Oncostylus* clade additionally included *G. andicola* on the plastid DNA tree but not on the nuclear DNA tree (Figure 1). That incongruence between the ncDNA and ptDNA trees was first detected and discussed by J.E.E. Smedmark and T. Eriksson [30], who suggested that it might be evidence of the hybrid origin of *G. andicola*. We believe that using additional data for the aforementioned taxa as well as their relatives to avoid the misidentified species effect could clarify the situation within the *Oncostylus* lineage. Nevertheless, our results showed that the New Zealand *Oncostylus* and probably a few American *Geum* species linked by a common origin stood out from the other Geinae and were a sister group to the latter.

A sister group to *Oncostylus* consisted of two well-supported clades and two partially resolved taxa. The largest Geinae clade (Figure 4, Clade IV) combined the majority of *Geum* species. This clade is well supported in both our study and the studies of J.E.E. Smedmark and T. Eriksson [30]. In fact, we suggest recognizing this clade as *Geum* s.str. (see below).

In what regards *Waldsteinia*, *Coluria,* and *Tahangia,* in J.E.E. Smedmark and T. Eriksson's study [30], they formed a well-supported clade on the BI tree (PP, 0.99) but showed poor BS support (53) based on joint (ITS + *trnL-trnF*) datasets. Using the expanded sampling of *Waldsteinia* species did not allow us to confirm the relationship between *Waldsteinia* + *C. geoides* and *Tahangia* as sister groups due to the low support (Figure 4). Thus, the position of *T. rupestris* (branch III) within *Geum* s.l./Geinae remains unresolved. The relationship between *Waldsteinia* and *Coluria* is discussed in the next paragraph.

4.2. Relationships between Waldsteinia and Coluria

Previously, *Waldsteinia* and *Coluria* were declared to be closely related genera but separated from the rest of *Geum* [1]. The common feature of *Waldsteinia* and *Coluria* that distinguished them from the other *Geum* s.l. species is a style jointed at the base that is entirely deciduous in fruits [1,3,30]. At the same time, it should be recognized that there were attempts to describe *Waldsteinia* and *Coluria* as *Geum* even before the study of J.E.E. Smedmark [2]. In particular, H. Baillon suggested that floral characteristics and fruit types are features not reliable enough to separate these two groups from *Geum* [76]. The difference between *Waldsteinia* and *Coluria* lies mainly in the leaf shape, with leaves being lobed (*W. geoides, W. idahoensis,* and *W. lobata*) or 3-foliolate/deeply lobed (*W. doniana, W. fragarioides, W. maximowicziana, W. tanzybeica, W. ternata,* and *W. trifolia*) in *Waldsteinia* and pinnate in *Coluria* [1,2]. The differences also manifest in the few carpels, quickly drying stamens, and a cone- or bowl-shaped receptacle in *Waldsteinia,* and the numerous carpels, persistent stamens, and a sacciform receptacle in *Coluria* [6,77–80].

Our analysis based on the joint dataset (ITS + *trnL-trnF*) showed that *C. geoides* is likely to be a sister to *Waldsteinia* (Figure 4; PP, 0.95). At the same time, a dual position of *Coluria* on the plastid DNA tree was demonstrated, i.e., *C. geoides* nested in one clade together with *Waldsteinia*, whereas *C. henryi* formed a single clade with *Taihangia* (Figures 1 and 5). The observation that *Coluria* nested in two different clades on the plastid DNA tree was unexpected because, before the present study, the monophyly of this group was never questioned. Due to the lack of ITS1-ITS2 region sequences for *C. henryi*, the patrilineal lineage for this species and, therefore, the monophyly of *Coluria* currently cannot be established.

The position of *C. geoides* still remains incompletely resolved. In particular, the species was nested in the *Waldsteinia* clade on the plastid DNA tree, and it also tended to form a common clade with North American and Northeast Asian *Waldsteinia* species. At the same time, the nesting of *C. geoides* within *Waldsteinia* could not be confirmed yet based on the nuclear DNA tree (Figure 1, the left side; Figure 2). The *C. geoides* appeared to be a sister to *Waldsteinia*, according to the BI analysis of the joint tree (Figure 4). The inconsistent position of *C. geoides* on the nuclear and plastid DNA trees can be caused either by incomplete lineage sorting with *Waldsteinia* or introgression following a hybridization.

The possible polyphyly of *Coluria* and the incompletely resolved position of *C. geoides* complicate the understanding of the relationships between *Coluria* and other closely related groups. Although our data indicated close affinity between *Waldsteinia* and *Coluria*, the precise phylogenetic relationship between these groups can hardly be deciphered without including other species of *Coluria* and applying additional genetic methods.

4.3. Phylogenetic Structure of Waldsteinia

Waldsteinia geoides, which belongs to the *Waldsteinia* subgenus, significantly differs from the other species in the genus by its morphology. At the same time, according to the plastid DNA phylogenetic tree (Figure 1, the right side) and joint DNA analysis (Figure 4), *W. geoides* clustered together with the Euro-Siberian group of *Comaropsis* (*W. tanzybeica*, *W. ternata*, and *W. trifolia*). Nesting *W. geoides* in the aforementioned group was also confirmed by our previous results based on the *trnH-psbA* intergenic spacer of plastid DNA [81]. In such a way, considering these data, *Comaropsis* does not appear to be a monophyletic group because of *W. geoides* interposition. On the other hand, according to the phylogenetic analysis based on the nuclear DNA, the position of *W. geoides* remained unresolved because of the low support levels, but it was outside the *Comaropsis* group anyway (Figure 1, the left side; Figure 2). No reliable synapomorphies in the ITS sequences that would justify nesting *W. geoides* in the clade of Euro-Siberian species were found. In particular, two sites in the multiple alignment may be claimed as synapomorphies for the two haplogroups of Euro-Siberian species (Figures 1 and 2, nodes K and P′), but none of them was shared with *W. geoides* (Figure 3, sites #78 and #181). Moreover, only one site was found (site #395, dark green) to be a possible synapomorphy for the *Waldsteinia* group as a whole. At the same time, at least nine positions found in the ITS ribotypes of *W. geoides* differed from the sequences of the rest of the *Waldsteinia* species but were shared by *W. geoides* and several *Geum* species.

In such a way, an incongruence was found between *W. geoides* morphology and its position on phylogenetic trees based on ptDNA and ncDNA. The morphological and nuclear DNA data showed that *W. geoides* is a sister to *Comaropsis*. On the other hand, plastid DNA and joint DNA analyses convincingly showed that *W. geoides* nested in one of the well-supported groups within *Comaropsis*, embracing Euro-Siberian species. This fact may suggest a possible introgression following a hybridization event between one of the *Waldsteinia* species from the *Comaropsis* clade and another, yet unknown, paternal ancestor from Colurieae. Our hypothesis mainly builds on the results of other studies, which demonstrated that similar mismatch patterns between nuclear and plastid DNA trees for the species appeared due to hybridization events [82–87]. Another fact that may be related to the hybrid origin of *W. geoides* is the presence of apomictic embryonic sacs in addition to the meiotic ones [17,73]. Most studies devoted to apomixis converge on the recognition

that almost all apomictic plants are polyploids and/or hybrids [88]. Being diploid, the *W. geoides* nuclear genome could have appeared as a result of homoploid hybridization without increasing the ploidy level [89–91] or chromosome number reductions during the 'diploid-tetraploid-dihaploid cycle' following hybridization [92–94]. Phylogenetic analysis based on plastid DNA polymorphism allows us to suggest a candidate for the maternal ancestor of *W. geoides*. It might be some ancestral form of European *Comaropsis*, which is now presented by *W. trifolia* and which has a common plastotype with *W. geoides* (Figure 5).

To clarify the possible intertaxa hybridization events within *Waldsteinia*, with an emphasis on *W. geoides*, and to define the possible paternal ancestors, we have conducted molecular cloning of the ITS region. We expected to get a picture similar to that described for the ITS2 region by M. Zarrei et al. [95]. The study showed nesting of the ITS ribotypes of the *Crataegus* (Rosaceae) species with a hybrid origin in different clades belonging to presumed parents. Our attempts to find any clear patterns of hybridization proved to be unsuccessful. As the result of our study, the identified *W. geoides* ITS ribotypes showed high affinity to each other (Figure 2, node R), but none combined with the ribotypes of other *Geum* s.l. or nested in the *Comaropsis* clade. Only a single position in the alignment (Figure 3, site # 486, dark green) was identical in most *W. geoides* ribotypes and the two minor ribotypes of one *Comaropsis* species and might have possibly been introgressed due to hybridization.

Additionally, several substitutions in one of the ITS ribotypes belonging to *W. geoides* (R5) demonstrated a different ancestry compared to the other ribotypes (R1–R4, R6). Those substitutions may also turn out to be the remains of the former introgression. Nevertheless, we need to admit that the identified single nucleotide substitutions in the ITS region cannot be reliable evidence of the former hybridization and can hardly help in determining the probable ancestors. We are inclined to associate the absence of clear patterns of parental ribotypes in the ITS sequences of *W. geoides* with the concept of concerted evolution, where intra-individual variability in the multicopy DNA units is generally low or absent due to unequal crossing over, high-frequency gene conversion, and large deletions [96]. Thus, if the ribosomal DNA of the hybrid was subsequently homogenized through concerted evolution in the direction of one of the ancestral genomes, the information concerning the other parent would be lost [30,96]. In this case, the present genotype of *W. geoides* may be the result of chromosome rearrangements and DNA recombination, which have led to genetic homogenization [91,97] and the preferential retention of the paternal nuclear genome rather than the maternal one. Although we adhere to the interspecies hybrid hypothesis for *W. geoides* origin with a *Comaropsis* maternal ancestor and a non-*Waldsteinia* paternal ancestor, it cannot be ruled out that the genotype of the studied *W. geoides* specimens could have been formed by complete chloroplast capture from one of the *Comaropsis* species or through other types of introgressions based on backcrossing events with the paternal parent. Nonetheless, stable introgression might be complicated by different ploidy types in *W. geoides* and other modern Geinae (*Geum* s.l.) species, which are mostly polyploid (Figure 4). Furthermore, hybridization events between *W. geoides* and other modern *Geum* s.l. species have never been described, although this does not mean that this is not possible in principle [3]. Moreover, incomplete lineage sorting is a known phenomenon that may lead to the observed mismatches in phylogenies. However, our previous results [81] based on the additional ptDNA marker (*trnH-psbA*) revealed a ptDNA phylogeny that is similar to that presented in this study. In particular, *W. geoides* sharing the common plastotype with *W. trifolia* nested in the clade of Euro-Siberian species. Hence, the question of the probable hybrid origin of *W. geoides* requires further special study, e.g., complete genome sequencing, or targeted sequencing using next-generation technologies.

Nevertheless, based on our data on plastotype diversity, the monophyly of the subgenus *Comaropsis* and the objectivity of dividing *Waldsteinia* into two subgenera [4,13] are becoming controversial. According to our reconstruction, the phylogenetic relationships of *Waldsteinia* taxa are better correlated with geographical patterns than with their morphology. Within *Waldsteinia*, the following geographically separated groups are also

well-supported by genetic data: Euro-Siberian (*W. geoides*, *W. tanzibeica*, *W. ternata*, and *W. trifolia*), East Asian (*W. maximowicziana*), and North American (*W. doniana, W. fragarioides*, and *W. lobata*). The two latter groups could be assembled together on the ML consensus tree, and *W. maximowicziana* appears to be closer to the North American than the Euro-Siberian *Comaropsis* species. Moreover, our data have shown that *W. ternata* s.l. in the commonly accepted sense (i.e., including *W. maximowicziana*, *W. ternata* s.str., and *W. trifolia*) seems to be a paraphyletic or even polyphyletic group considering *W. geoides* nesting in this group and *W. maximowicziana* clustering with the North American species, according to the results of one of the performed analyses (Figure 4). The non-monophyletic status of the aforementioned group was also suggested by our previous results based on the *trnH-psbA* spacer [81]. In such a way, despite the fact that the position of *W. maximowicziana* continues to be unresolved, we support the point of view that *W. maximowicziana* should be separated from the *W. ternata* aggregate and considered a separate species. The appropriate nomenclature combination for *W. maximowicziana* was validly published by N.S. Probatova [98]. Although *W. tanzibeica* has never been considered an infraspecific taxon of *W. ternata*, its close relationship is evident from morphological similarity [10] and genetic analysis [81]. Our present analysis also confirms the high affinity between these two species. This fact was expected because of the distribution of *W. tanzibeica*; its local endemic nature in the Western Sayan Mountains lies within the distribution range of *W. ternata* s.str. [10].

The genetic relationships among North American *Waldsteinia* are not clear. We have not detected any genetic evidence that *W. doniana* and *W. lobata* are more closely related to each other than to *W. fragarioides* [18] or that *W. doniana* is a subordinate taxon of *W. fragarioides* [17]. Our present and previously obtained data [81] indicate that all three species have a common plastotype, i.e., a common matrilineal lineage, but the phylogenetic relationships between these species were not possible to establish from the available datasets. The observed differences between *W. fragarioides* and *W. doniana* in the ITS region are also too minor to suggest species segregation into different taxa. However, we believe that more complex research with additional genetic markers and data from North American populations (including *W. idahoensis*) is needed to make any taxonomic conclusions.

4.4. Possible Scenarios of Waldsteinia's History

The level of genetic distance based on the ITS phylogenetic tree indicated that the ribotype of *W. maximowicziana* (R1) was the nearest to the common ancestor of the *Comaropsis* group (Figure 2). According to the plastid DNA (Figure 5a,b, and [81]), *W. maximowicziana* carried the maternal ancestral plastotype for *C. geoides* and the entire *Waldsteinia*. Considering the current distribution range of *W. maximowicziana* (Table 1), we suggest an East Asian origin for the genus *Waldsteinia* and its subsequent speciation and distribution toward Europe and North America (Figure 5c).

Considering the estimated age of Colurieae, 38–49.6 million years old [39], and the tertiary age of some *Waldsteinia* species [4,10,20–22], the probable time of *Waldsteinia's* origin as a genus can be suggested as the Late Oligocene or Miocene, when the climate, even at high latitudes, was warm and wet and mixed mesophytic forests were more or less continuously distributed throughout the Northern Hemisphere [99,100]. These conditions should be favorable for warm- and moisture-dependent *Waldsteinia* [10,20,101]. During that time, intercontinental migrations were possible through the Bering Land Bridge, which connected northeastern Asia and western North America throughout the late Cretaceous and the Neogene [99,102,103]. The scenario of intercontinental dispersal events from the Old World to the New World, in particular, was assessed by Xiang and Soltis [104] and appeared to be the most common among temperate species with intercontinental disjunctions in the Northern Hemisphere [105–109].

The significant climate cooling approximately 15 Ma and the disappearance of the Bering Land Bridge about 5.5–5.3 Ma [110,111] simulated the divergence of Asian and American *Waldsteinia* populations and their distribution through the continents.

In North America, further progression of climatic cooling and aridification culminated in the Pleistocene and led to phytogeographic barriers around the arid center of the continent [112]. As a result, elements of temperate and subtropical mesophytic biotas survived in refugial regions of the south-eastern (Atlantic) and western (Pacific) parts of North America [111]. Evidently, climate change was the reason for the split in the distribution range of *Waldsteinia*. Currently, *W. idahoensis* is endemic to Idaho and Montana and is the only representative of the genus in the Pacific refugium, whereas the Atlantic complex is represented by three species, *W. fragarioides, W. doniana,* and *W. lobata*, distributed from north to south from the Great Lakes to the southern piedmont of the Appalachian Mountains [19].

Concurrently, Eurasian *Waldsteinia* was spreading from East Asia to the west. The expansion of ancestral *Waldsteinia* to Europe might occur through continental Siberia following the outlines of the trans-Palearctic broad-leaved zone [20,21,101,113]. Climate change and the increase in continentality were the reasons for the fragmentation of the broad-leaved forest zone into European and East Asian parts and the formation of a gap in continental Eastern Siberia and Central Asia in the Quaternary [101]. After all, those events led to the disjunction of *Waldsteinia* into European (*W. trifolia*), South Siberian (*W. tanzibeica, W. ternata*), and East Asian (*W. maximowicziana*) fragments. Since then, the distant populations have developed independently, and their genotypes have accumulated nucleotide substitutions and DNA rearrangements at different rates.

The mountains of southern Siberia are of great importance because of the surviving mesophylous remnants of broad-leaved forests during the Pleistocene [101]. In the case of *Waldsteinia*, such refugia are located on the piedmonts of the Khamar-Daban Ridge, the Eastern Sayan Mountains, and the Western Sayan Mountains, which are inhabited by *W. ternata* and *W. tanzibeica*, which are treated as tertiary (Neogene) nemoral relicts [9,10,20,21,114]. A status of tertiary relict was also suggested for European *W. trifolia,* characterized by a highly fragmented range in the southeastern Alps and the Carpathian Arc [4,22]. Considering that *W. trifolia* occurs mostly in the territory of modern Romania, the southern part of the Carpathians could be considered the main Pleistocene refugium for this species. Herwig Teppner [4] also defined some possible refugia in the southeastern Alps near Carinthia.

Although the European plastotype was evidently more ancient than the Siberian one, there was no other way to reach Europe than through continental Siberia. The youngest plastotype was found in the South Siberian populations, which may indicate the highest value of genome plasticity among *Waldsteinia* species or genetic drift. In particular, a dramatic reduction in the population size of *Waldsteinia* both in Europe and in southern Siberia during the Pleistocene cooling and glaciation could lead to accidental haplotype extinction or fixation resulting from the bottleneck and/or founder effects. By that, we may explain the fact that European species retained the plastotype that is ancestral to the Siberian species.

Despite the fact that populations of *Comaropsis* species have been isolated from each other since at least the Early Pleistocene, the modern plants morphologically [1,3,4,8,14,17,78] and genetically [81,115] are still very similar. The morphological similarity fits well with the fact that tertiary relict floras on separate continents have a shared history, leading to a slow morphological evolution ('stasis') in many taxonomic groups as compared to their genetic differentiation [112]. However, further investigation is needed to understand whether a low genetic variance among *Comaropsis* species is due to a prevalence of vegetative propagation or apomixis over sexual one [4]. We supposed that the observed moderate resolution in *Comaropsis* phylogeny (e.g., the unresolved position of *W. maximowicziana*) together with the clustering of ribotypes (Figure 2) or plastotypes [81] from a single species in non-monophyletic groups may be due to the progenitor/derivative situation where the ancestor (*W. maximowicziana* in our case) and in the short term, the derivative species do not show clearly monophyletic patterns. Moreover, ancestral populations may often show a lack of autapomorphies, which makes it problematic to designate them as separate species. Monophyly may be obtained over time, however, via the sorting and extinction of lineages [116].

Considering the incongruence in the position of *W. geoides* in plastid and nuclear DNA phylogenetic trees, we speculated that the species may have a hybrid origin with a matrilineal ancestor belonging to a European representative of the subgenus *Comaropsis* and an unknown patrilineal ancestor from Colurieae (see Section 4.3). European *Comaropsis* is currently represented only by *W. trifolia*, which has the same plastotype as *W. geoides* (Figure 5). The modern distribution ranges of both species overlap in the Carpathian Mountains, which could be the center of *W. geoides* origin. The age of *W. geoides* is hard to estimate based on available data; however, it had a chance to arise when *Comaropsis* relatives reached Europe. This assumption is also relevant because *W. geoides* has never been considered a tertiary relict in contrast to the European *W. trifolia* [4,22]. These facts allow us to consider *W. geoides* as the youngest *Waldsteinia* species of the late Cenozoic age.

Thus, we suggest the scenario of a one-directional dispersal of *Waldsteinia* to the New World that was accompanied by a one-directional dispersal through Asia towards Europe (Figure 5c). This obvious dispersal pattern is known for many other East Asian angiosperms [104,108]. The hypothesis is also confirmed by the distribution patterns of races with different ploidy levels.

Diploids in most cases represent the ancestral chromosome race in species with variable ploidy levels. Thus, the presence of diploids in *W. maximowicziana* fits well into the concept of the East Asian origin of the genus. At the same time, diploids frequently occur in all fragments of the disjunct *Waldsteinia* range. Furthermore, taking into account that polyploidization in plants happens much more often than the polyploid-diploid transition, we assume that the intra- and transcontinental dispersal of the group occurred predominantly with diploids (Figure 5c). In particular, we suppose that the transcontinental (trans-Pacific) dissemination of the ancient *Waldsteinia* from East Asia to North America may have been driven by diploids, with their subsequent and independent polyploidization and probable hybridization within continents. The appearance of the modern polyploid races *in loco* may also be indicated by the different dominant ploidy levels in the East Asian and North American populations. Specifically, the 4x race dominates in East Asia, while the 3x race dominates in North America. The same chromosome races, which are rarely found on either one (4*x* in North America) or both (6*x*) continents, could arise independently of each other. The possibility of such independent convergent evolution of different ploidy levels within the genus was first suggested by H. Teppner et al. [17].

The western (trans-Eurasian) pathway of *Waldsteinia* dispersal was also most likely related to diploids. The distribution by only one race is also confirmed by matrilineal plastotype evolution (Figure 5a,c), which definitely indicated that both tetraploid *W. ternata* and diploid *W. tanzybeica* had the same 'South Siberian' plastotype that arose from the common East Asian ancestry. However, the parallel distribution of the tetraploid race cannot be excluded (Figure 5c). Tetraploids are dominant in South Siberia (*W. ternata*) and codominant in Europe (*W. trifolia*), whereas diploids are very rare in Siberia (locally endemic *W. tanzibeica*) and represented in Europe by a single species, *W. geoides*, from a monotypic subgenus. If the homoploid origin of *W. geoides* took place, feasibly, the ancient diploid *Comaropsis* species could have disappeared after it was involved in hybridization. For another European species, *W. trifolia*, diploids were not known, and its present chromosome races (4*x*, 5*x*, and 6*x*) could have originated either from the common diploid ancestor with *W. geoides* or from the tetraploid *Comaropsis* ancestor that arrived from South Siberia. Noteworthy, hexaploids occur rarely in species from different geographical regions [4,13,53,54] and cannot be reliable criteria for relationship establishment between species and populations.

The relationships between chromosome races of the same species are unclear because of poor morphological differences [54]. The distribution pattern of chromosome races was revealed only for *W. trifolia* [4,54]. To obtain a complete picture of the phylogenetic relationships between *Waldsteinia* species, the study of DNA differences in clones with the same and different ploidy levels from matching and distant regions needs to be conducted.

4.5. Geum Sensu Lato vs. Geum Sensu Stricto

Despite the fact that the phylogenetic reconstruction of Colurieae proposed by J.E.E. Smedmark et al. [28,30] was well justified, in our view, the taxonomic decision to combine all herbaceous species into *Geum* [2,26] was too generalized. The challenges in *Geum* s.l. taxonomy are mainly associated with the complex interaction of processes such as hybridization, polyploidization, and gametophytic apomixis, which are widespread in the Rosaceae as a whole [84,117–119]. Some works have suggested a broad generic concept as the most appropriate solution to the convoluted nomenclature of groups with such kinds of relationships [29]. Debates between 'lumpers' and 'splitters' are as old as taxonomy itself, and, apparently, it should be recognized that the taxonomic level of a particular taxon can be arbitrary and seems to be less important than the actual establishment of its monophyly. It should also be considered that clear monophyly patterns, at least at the species level, maybe occasionally obtained only with time via the sorting and extinction of lineages [116]. The loss of generic status by small groups cannot always be justified since their unique 'identity' is ignored in these cases. It looks especially lamentably in the case of groups with well-defined morphology and evolutionary history, e.g., *Waldsteinia*. And although we generally support the allopolyploidy monophyly arguments in favor of Geinae (or *Geum* according to the new concept), we still believe that the suprageneric status of this clade better reflects the present genetic structure and evolutionary perspectives of internal groups, such as their divergence [116]. In addition, gene flow between extant species has been limited for a long time. For example, the experimental attempts to perform intergeneric crosses between *Waldsteinia*, *Coluria,* and different *Geum* species were unsuccessful [1,3]. Although the importance of reticular evolution and hybridization events throughout the early evolution of Geinae can hardly be ignored, we believe that the present relationships between the existing taxa are more important for making taxonomic decisions.

Despite the question of whether to consider Geinae as a suprageneric clade or as a genus *Geum* s.l. with a subgeneric structure being only a taxonomic issue and looking secondary, we would still suggest considering the genus *Geum* in a partly restricted sense combining most of the herbaceous species starting with *G. reptans* and other taxa that share more recent common ancestors with it than with *Waldsteinia*, *Coluria,* and *Taihangia* on the joint phylogenetic tree (Figure 4; clade IV). The designated clade was also well supported on the plastid DNA phylogenetic tree, both in our study (Figure 1, the right side, node G, BS, 92; PP, 1.00) and in the study of J.E.E. Smedmark and T. Eriksson [30] (BS, 99; PP, 1.00). The high affinity of species in the proposed sense of *Geum* was also shown by the phylogenetic reconstruction based on the nuclear low-copy granule-bound starch synthase (GBSSI) gene sequences [28]. Thus, no evidence exists that at present any species belonging to the clade *Geum* s.str. have a part of the genome that is closer to *Waldsteinia*, *Coluria*, or *Taihangia* than to the other species of the mentioned clade.

If we follow the 'split' conception, New Zealand *Oncostylus* and probably several American *Geum* species (Figure 4, clade V, BS, 98, PP, 1.00) linked by the common origin should be separated from *Geum* s.str. The distinct status of this group is even more evident because of its sister position to the clade embracing the other Geinae (*Geum* s.l.) (Figure 4, clade IV, BS, 73; PP, 1.00). However, the confusing merging of *Geum schofieldii* and *G. andicola* (on the plastid DNA tree) with *Oncostylus* needs further investigation. In this view, *G. andicola* could probably be an intergeneric hybrid [30].

The relationships between the three remaining groups within Geinae (*Coluria, Taihangia,* and *Waldsteinia*) are still not entirely resolved. *Coluria* appears to be a polyphyletic group based on its matrilineal lineage and has common maternal ancestors with both *Waldsteinia* and *Taihangia* (Figure 1, the right side). In this case, we suggest a hybrid origin for some *Coluria* species or incomplete lineage sorting with other groups (see above). *C. geoides* clustering together with *Waldsteinia* on the plastid DNA phylogenetic tree and its predicted position as a sister to *Waldsteinia* according to the results obtained with the joint DNA dataset may mean that these groups may be more closely related to each other than to other groups of *Geum*. *Taihangia rupestris*, a generic endemic of China, still exhibits an unresolved

position (in relation to *Geum* s.str., *Coluria*, and *Waldsteinia*) on the joint phylogenetic tree (see Figure 4). However, the position of *T. rupestris* on the plastid DNA phylogenetic tree suggests a certain affinity to *Coluria* (Figure 1, the right side, node O). Therefore, the discussed issues concerning the origin and the relationships between *Coluria*, *Taihangia*, and *Waldsteinia* could hardly be solved without including other representatives of *Coluria* in the analysis and applying additional genetic methods. We understand that our study has raised more questions than it has answered, and we admit that our present results are still preliminary and do not definitively demonstrate the taxonomic status of the groups included in the analysis.

5. Conclusions

Based on the present phylogenetic structure of *Geum*, *Waldsteinia*, and other closely related taxa, we believe that excluding *Waldsteinia* together with *Coluria*, *Taihangia*, and *Oncostylus* from *Geum* s.l. is more justified than 'lumping' them together into a single polymorphic genus. Even though, in our opinion, our phylogenetic reconstruction has resolved with greater precision the relationships between several groups, in fact, our taxonomic proposals are only based on a different 'chopping pattern' of the tree than the one previously suggested by J.E.E. Smedmark and T. Eriksson [30]. The earlier division of *Waldsteinia* into two subgenera (*Waldsteinia* and *Comaropsis*) is now getting controversial since the subgenera appear to be non-monophyletic, as is indicated by the genetic data, mainly ptDNA. We tend to explain the morphological differences (on which the division into subgenera is based) between *W. geoides* (the only representative of the type subgenus) and *Comaropsis* species by the presumed hybrid origin of the former one. Our 'hybrid' hypothesis is based on the discrepancy observed between *W. geoides* positions on the plastid and nuclear DNA trees, along with a mismatch between the species morphology and its position on the plastid DNA tree. We also suggest that this species may have a matrilineal ancestor belonging to the European representatives of the *Comaropsis* subgenus and an unknown patrilineal ancestor from other Colurieae. Our data convincingly show that the phylogenetic relationships of *Waldsteinia* species are better explained by their geographical distribution than by the morphological differences between them. The Euro-Siberian (*W. geoides*, *W. tanzibeica*, *W. ternata*, and *W. trifolia*), Northeast Asian (*W. maximowicziana*), and North American (*W. doniana*, *W. fragarioides*, and *W. lobata*) phylogeographic groups within *Waldsteinia* were identified, and East Asia was proposed to be considered the place of the genus's origin. Three *Waldsteinia* species from eastern North America (*W. doniana*, *W. fragarioides*, and *W. lobata*) belong to a single maternal lineage, but the observed genetic differences are too small to serve as a convincing argument for the species segregation, so their relationships still remain unresolved. Despite the fact that the position of *W. maximowicziana* continues to be not entirely resolved, we support the point of view that *W. maximowicziana* should be separated from the *W. ternata* aggregate (the one including *W. trifolia*, *W. ternata* s.str., and *W. maximowicziana*) and considered a separate species. Our suggestion is based on the possible paraphyly or even polyphyly of the *W. ternata* aggregate considering *W. geoides* nesting in this group and on *W. maximowicziana* clustering with the North American *Comaropsis* species according to the results of one of the performed analyses. Our data has also shown that *W. tanzibeica* may belong to the aforementioned species aggregate because of its high affinity with *W. ternata* s.str.

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