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PLANT GENETICS

Phylogenetic Relationships in the Genus *Primula* L. (Primulaceae) Inferred from the ITS Region Sequences of Nuclear rDNA

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Abstract—The nucleotide sequences of the nuclear rDNA ITS regions were determined for 34 species of the genus *Primula* L. and one species of the genus *Cortusa* L., family Primulaceae Vent., and used to infer the phylogenetic relationships among these species. In this analysis species of the Russian flora and the flora of adjacent territories were studied for the first time. The results clarified the taxomic structure of the genus *Primula* and confirmed the entity of some of its sections; but not the subgenera sensu J.Richards. Our data do not support an independent status of the genus *Cortusa*, placing it as one of the terminal lineages of the section *Cortusoides* Balf. f. in the genus *Primula*.

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INTRODUCTION

The genus *Primula* L. (primrose) is the largest in the family Primulaceae Vent., comprising, according to different authors 430–500 species growing in the humid and moderate climate regions of the Northern Hemisphere [1, 2]. Only some *Primula* species occur in the mountains of Africa (Ethiopia), tropical Asia (Java and Sumatra Islands), and South America. Primroses grow in the forest belt, plain meadows, Alpine lawns, and nival and meadow tundras. Several centers of species diversity of the genus *Primula* have been found, namely, the eastern Himalayas and Yunnan Province in China; the western Asian center, comprising the Caucasus, the European mountains from the Pyrenees through Alps to Carpathian Mountains; the mountains of East Asia, and the mountains of the western part of North America [3].

The majority of *Primula* species are perennial short-root herbs, sometimes monocarps, with rosette shoots. Many *Primula* species are ornamental plants and have been used to breed numerous cultivars of garden primroses [4, 5].

The first monograph on the genus *Primula* was published as early as in 1817 [6]; however, it yet lacked any classification. Pax was first to develop a comprehensive classification [7] for the 210 species known at that time and proposed to group them into 21 sections. Later, Smith and Fletcher [8] divided this genus into 30 sections; the system they proposed was partially used in the recently published monograph on the genus *Primula*, which divides over 400 species into 6 subgenera and 37 sections [2]. Some authors classify this genus into several subgenera [9, 10].

The first work on the Russian flora, *Flora Rossica*, by Ledebour [11], described 14 *Primula* species. *The Flora of the USSR* reports 67 species belonging to 2 subgenera, 10 sections, and 19 series [12]. According to our estimates, the Russian flora comprises 41 primrose species. However, foreign researchers do not regard all of them as independent species [2].

The systematics of the genus *Primula* that existed in the 20th century was based on morphological traits and ecological and geographical characteristics: vernation type; shapes and sizes of the leaf blade, leafstalk, bracts, calyx, and petals of corolla; capsule size; and the presence or absence of pubescence or farina on various plant parts [1, 3, 5, 9, 12–15]. The diagnostic traits for sections and some species have been expanded with the data on the structure of primrose seed surface examined with scanning electron microscopy [16, 17]. Nonetheless, the phylogeny of the genus *Primula* still contains disputable issues connected with the size of individual sections, positions of the newly described taxa, and evolution of morphological traits of various taxonomical ranks. It has been demonstrated that the phenotype characterization is not always sufficient to solve the disputable issues in the plant systematics and phylogeny at different taxonomic levels [18–21].

This century brought about the studies of chloroplast DNA, which provided for a more precise systematics of the genus *Primula*. The pioneering study of Trift [22, 23], performed under the guidance of Anderberg and Kallersjo in Stockholm (Sweden), analyzed

the chloroplast gene *rbcL* in 39 primrose accessions belonging to 32 *Primula* species (overall, 90 accessions from the order Primulales Lindl. were analyzed). Then Conti et al. [24] and Mast et al. [25] reconstructed the phylogenetic history of the family Primulaceae based on comparison of the intron sequences of the chloroplast genes *trnL* and *rpl16* for 85 species of the genus *Primula* and 22 genera of the related families. These studies have demonstrated that the largest subspecies in the genus *Primula* is *Aleuritia* (Duby) Wendelbo (containing 15 sections according to the Richards [1]) falls into three rather distant groups. This was considered as an evidence for a polyphyletic origin of the subgenus *Aleuritia*.

The last system for the genus *Primula*, which was proposed by Richards [2], was constructed based on the analysis and comparison of nucleotide sequences of chloroplast DNA and phenotypic traits of the taxa, The author himself noted that the use of chloroplast DNA imposes certain constraints, as only the maternally inherited genetic material was taken into account; consequently, the resulting phylogenetic constructions could be incomplete. In addition, the species growing on the territory of the Russian Federation, which are the object of this study, either were omitted at all or analyzed only in part. The phylogenetic relationships is now widely assessed based on the internal transcribed spacers (ITS1 and ITS2) in the region of 18S–26S nuclear rDNA [26–29]. Although this is a rather short region (600–700 base pairs) and approximately similar among the angiosperms, it is sufficiently informative for clarifying the phylogenetic relationships at the level of families, genera, end sections, especially when the external similarity between taxa failed to adequately reflect their close relation.

In this paper, we describe the results obtained by comparison of the ITS region nucleotide sequences of the nuclear ribosomal DNA for the *Primula* species growing in Russia and the adjacent territories to establish the phylogenetic relationships within the genus and specify the positions of northern Asian and eastern European *Primula* species.

MATERIALS AND METHODS

In this work, we used 34 species of the genus *Primula* and one of the genus *Cortusa* L. harvested during expeditions to various Russian regions or taken from the collection of the Central Siberian Botanical Garden (Siberian Branch, Russian Academy of Sciences) and the doubled specimens from the herbariums LE, MW, MHA, NS, NSK, and VLA. The oldest herbarium specimens used for successful DNA isolation dated back to 1901 (*P. warshenewskiana* Fedtsch.). The set of analyzed data was supplemented with the corresponding nucleotide sequences of the species belonging to the genus *Primula* and other species of the family Primulaceae (genera *Androsace* L., *Vitaliana* Sesl., and *Douglasia* Lindl.) from the GenBank database (table).

DNA isolation, amplification, and sequencing. Total cellular DNA was isolated and purified with the help of a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. The region of ribosomal operon encoding the 3'-end of 18S rDNA, ITS1, 5.8S rDNA, ITS2, and the 5'-end of 26S rDNA was amplified by polymerase chain reaction (PCR) using the primers N-nc18S10 and C26A. The PCR products were used for a cyclic sequencing using a BigDye v. 3.1 (Applied Biosystems) kit and primers N18L18, N5.8S, ITS2, and ITS4 [29]. The nucleotide sequences of PCR products were determined for both strands in an ABI PRIZM 310 (Applied Biosystems) sequencer. The sequences were assembled using the Staden software package [30].

Sequence alignment and construction of phylogenetic trees. The phylogenetic relationships in the genus *Primula* were analyzed using the data matrix that comprised both the newly acquired sequences and the sequence extracted from the GenBank (table). The genera *Douglasia, Androsace*, and *Vitaliana* (family Primulaceae), as those most closely related to the genus *Primula* [24], were taken as the outgroup. The nucleotide sequences were manually aligned using the SeaView program [31] according to the conserved elements in the ITS primary and secondary structures.

The phylogenetic trees were constructed using maximum likelihood (ML), neighbor joining (NJ), and maximum parsimony (MP) methods with the help of the phylogenetic program PAUP 4.0b10 [32]. The evolutionary models for ML and NJ analyses were selected using Modeltest 3.04 [33]. The distances for NJ analysis were calculated via an ML optimization. A heuristic search for optimal topology was used for ML and MP analyses. The robustness (statistical support) of phylogenetic trees in NJ and MP analyses was assessed by bootstrap [34] using 1000 bootstrap replicates. The bootstrap percentage (BP) below 50% was discarded and is not shown in figure. In the bootstrap analysis of MP trees, ten heuristic searches for optimal topology with a random addition of taxa were performed for each replicate.

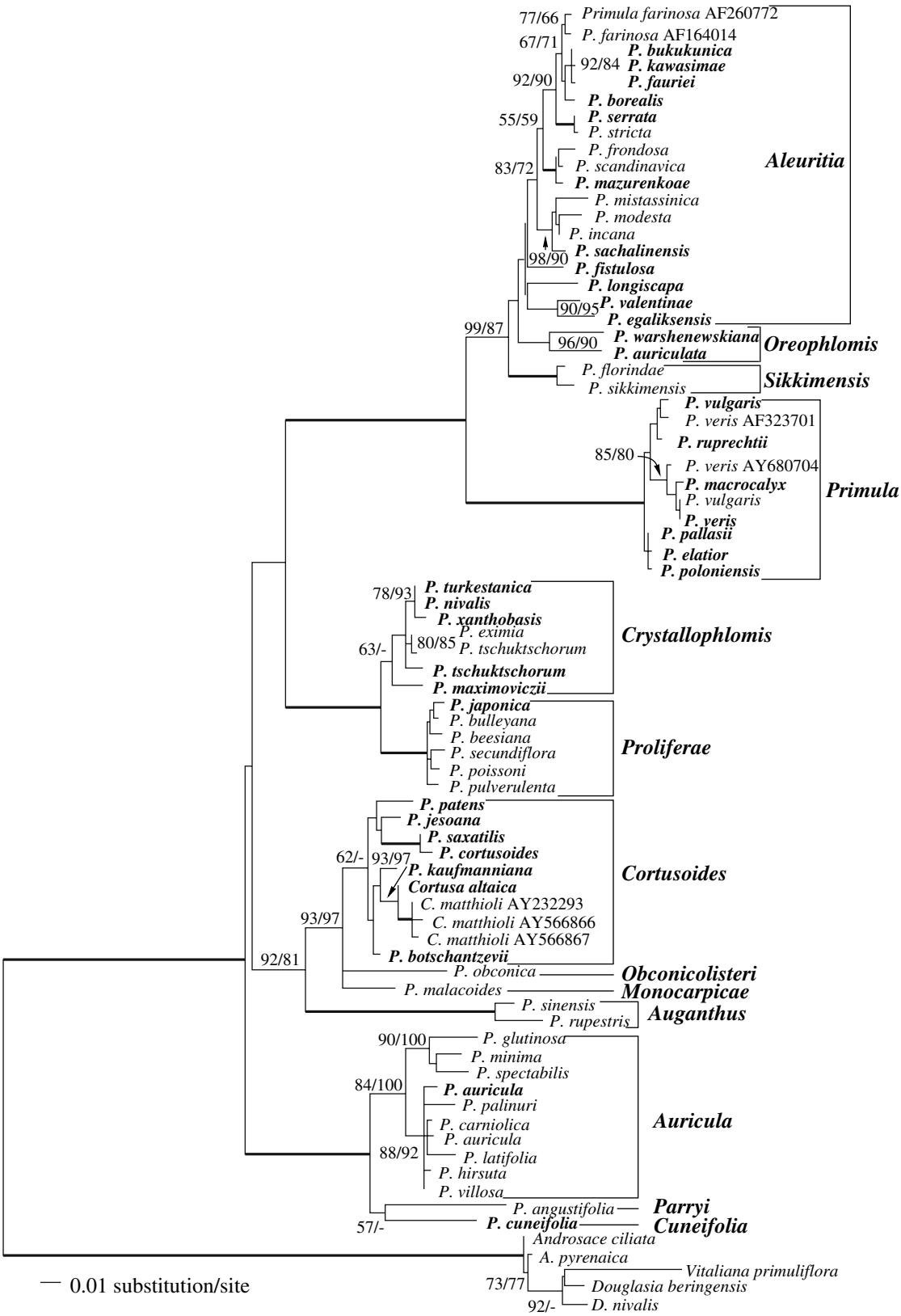
RESULTS

The length of the spacer region (comprising ITS1, 5.8S, and ITS2) in the studied species varied from 618 base pairs (bp) for *Primula jesoana* Miq. to 640 bp in several species with an insignificant increase in the G + C content (53.72 ± 1.3%) The ITS length in the species of the genus *Cortusa* was 621 bp with a similar C + C content. As for the species chosen as the outgroup, the ITS region was shorter (605 ± 1.3 bp) and displayed a higher C + C content (60.7 ± 1.4%). Similar to the majority of angiosperms, the ITS1 in *Primula* species was longer than ITS2 (250.2 ± 3.8 bp versus 215.6 ± 2.8 bp). The SYM model of nucleotide sequence evolution supplemented with the proportion of invariant positions (I) and Gamma parameter (Γ) was selected by Modeltest

Accessions of *Primula* species and related genera examined and GenBank accession numbers of the corresponding rDNA ITS sequences

Species	Accession number	Species	Accession number
P. farinosa L.	AF260772	***P. maximoviczii*** Regel	AM920497
P. farinosa L.	AF164014	***P. japonica*** A. Gray	AM920496
P. bukukunica Kovt.	AM920466	*P. bulleyana* Forrest	AF396690
P. kawasimae Hara	AM920467	*P. beesiana* Forrest	AF396689
P. fauriei Franch.	AM920468	*P. secundiflora* Franch.	AF260759
P. borealis Duby	AM920470	*P. poissoni* Franch.	AF396692
P. serrata Georgi	AM920469	*P. pulverulenta* Duthie	AF323699
P. stricta Hornem.	AF260766	***P. patens*** (Turcz.) E. Busch	AM920485
P. frondosa Janka	AF260765	***P. jesoana*** Mig.	AM920486
P. scandinavica Brunn	AF260769	***P. saxatilis*** Kom.	AM920487
P. mazurenkoae A.P.Khokhr.	AM920471	***P. cortusoides*** L.	AM920488
P. mistassinica Michx	AF260770	***P. kaufmanniana*** Regel	AM920489
P. modesta Bisset & S.Moore	AF260762	***Cortusa altaica*** Losinsk.	AM920491
P. incana M.E.Jones	AF260771	*C. matthioli* L.	AY232293
P. sachalinensis Nakai	AM920472	*C. matthioli* L.	AY566866
P. fistulosa Turkev.	AM920473	*C. matthioli* L.	AY566867
P. tschuktschorum Kjellm.	AF260767	***P. longiscapa*** Ledeb.	AM920474
P. valentinae Fed.	AM920475	*P. obconica* Hance	AF323698
P. egaliksensis Wormsk.	AM920476	*P. malacoides* Franch.	AF323700
P. warshenewskiana Fedtsch.	AM920477	*P. sinensis* Lour.	AF323695
P. auriculata Lam.	AM920478	*P. rupestris* Balf. f. & Farrer	AF323697
P. florindae Kingdon-Ward	AF396691	*P. glutinosa* Wulfen	AF260755
P. sikkimensis Hookker f.	AF396696	*P. minima* L.	AF260756
P. vulgaris Hudson	AM920479	*P. spectabilis* Tratt.	AJ427794
P. veris L.	AF323701	***P. auricula*** L.	AM920498
P. ruprechtii Kusnez.	AM920480	*P. palinuri* Petagn.	AJ427790
P. veris L.	AY680704	*P. carniolica* Jacq.	AJ427768
P. macrocalyx Bunge	AM920481	*P. auricula* L.	AJ427755
P. vulgaris Hudson	AJ427800	*P. latifolia* Lapeyr.	AJ427783
***P. veris* L.**	AM920484	*P. hirsuta* All.	AJ427777
P. pallasii Lehm.	AM920482	*P. villosa* Wulfen	AJ427797
P. elatior (**L.**) Hill	AM920659	*P. angustifolia* Torr.	AF260754
P. poloninensis (Domin) Fed.	AM920483	***P. cuneifolia*** Ledeb.	AM920499
P. turkestanica Hort.	AM920492	*Androsace ciliata* DC.	AY275034
P. nivalis Pall.	AM920493	*A. pyrenaica* Lam.	AY275035
P. xanthobasis Fed.	AM920494	*Vitaliana primuliflora* Bertol.	AY275050
P. eximia Greene	AF260768	*Douglasia nivalis* Lindl.	AY275026
P. botschantzevii Czukav. et Kovalevsk.	AM920490	*D. beringensis* S. Kelso, Jurtsev et D. F. Murray	AF260773
P. tschuktschorum Kjellm.	AM920495		

Note: The species for which the sequences were determined in this work are boldfaced.



Phylogenetic tree of the genus *Primula* constructed according to comparison of 77 ITS rDNA sequence by maximum likelihood method; robustness of the branches for NJ/MP methods calculated by bootstrap is shown; heavy lines denote the branches with 100% BP for both methods; and the species for which the sequences were determined in this work are boldfaced.

as the most adequate for description of the used data. The results of ML analysis of 77 taxa are shown in the figure. The members of the genus *Primula* in this tree formed four clusters.

Cluster I with a high probability (100% BP) includes the members of four sections of the studied genus. This particular cluster contains the majority of the sequences that we determined. Characteristic of the clade with a high BP (100%) of the type section *Primula* (seven species and ten sequences) is a long branch; this clade is a sister for the large clade (22 species and 23 sequences) displaying a 87–99% BP and comprising the members of three sections—*Aleuritia* Duby, *Oreophlomis* (Ruprecht) Fedorov, and *Sikkimensis* Balf. f. The last two sections are represented in our analysis by two species each; their monophyletic character is supported by high BP values (90–100%; figure). Several small significant subclades were determined within the species-rich section *Aleuritia*; however, the overall section has no statistical support.

Cluster II (100% BP) includes the clades approximately similar in size formed by the species belonging to the section *Crystallophlomis* (Ruprecht) Fedorov and *Proliferae* Pax. The clade *Proliferae* is robust, whereas the support for the clade *Crystallophlomis* is low (63% BP only for NJ). This clade comprises two geographically isolated groups of species—south Siberian species *P. turkestanica* Hort., *P. nivalis* Pall., and *P. xanthobasis* Fed. (78–93% BP), very similar phenotypically, and the Beringian species *P. eximia* Greene and *P. tschuktschorum* Kjellm. (80–85% BP).

Cluster III includes (92/81 BP) the *Primula* species from the sections *Auganthus* (Link) Pax ex Balf. f., *Cortusoides* Balf. f., *Obconicolisteri* Balf. f., and *Monocarpicae* Flanchet ex Pax and the genus *Cortusa*. This cluster is well structured, and its branching order is statistically significant. The section *Auganthus* (100% BP) occupies a basal position in this cluster and is followed by the sections *Obconicolisteri* and *Monocarpicae*, and the clade of the section *Cortusoides* (62% BP) occupies the crown position in this cluster. Representatives of the genus *Cortusa* form one of the crown clades (93–97% BP) in the section *Cortusoides*.

Robust (100% BP) cluster IV contains members of the sections *Auricula* Duby, *Parryi* W.W. Smith ex Wendelbo, and *Cuneifolia* Balf. f. The clade *Auricula* has a high bootstrap support (84–100% BP) and contains several robust subclades. The clade containing *Parryi* and *Cuneifolia* (57% BP only in NJ) is a sister group relative to *Auricula*.

We failed to significantly determine the branching order of clusters in the tree. Topologically, cluster IV occupies a basal position followed by cluster III, while clusters II and I form a sister pair.

DISCUSSION

In this work, we for the first time have analyzed the nucleotide sequences of rDNA ITS regions of 34 species belonging to eight sections of the genus *Primula* representing the flora of Russia and adjacent territories, which have not been included in earlier studies, and the relevant data deposited with the GenBank. Overall, 77 accessions were analyzed; of them, 68 accessions of 63 *Primula* species from 13 sections. The genera *Cortusa* (four accessions from two species), *Androsace* (two species), *Douglasia* (two species), and *Vitaliana* (one species) from the tribe Primuleae, family Primulaceae, were taken as an outgroup. We have determined four large groups within the genus *Primuleae* that correspond to clusters I–IV.

Basal cluster IV corresponds to the subgenus *Auriculastrum* (Link) Wendelbo of the genus *Primula* [2]. It contains three sections—*Cuneifolia* ($x = 11$), *Parryi* ($x = 22$), and *Auricula* ($x = 33$). Note that the base chromosome numbers $x = 12$, 11, 10, 9, and 8 were found in this genus; $x = 11$ is the most frequent variant in both the tribe Primuleae and genus *Primula.* This suggested that $x = 11$ is an ancestral trait characteristic of the most primitive *Primula* species [2]. A trend of ploidy ($x = 11$, 22, 33) is evident within the subgenus *Auriculastrum*, which comprises the species displaying a specific leaf type—involute vernation of the dense leathery leaves. As for the other *Primula* species, they display a revolute vernation of membranous (rarer, dense) leaves. Only for some species of the section *Crystallophlomis*, the leaf vernation in the ontogenesis is first involute and then changes for revolute [12]. Considering the ontogenesis as a brief form of phylogenesis, the involute leaf vernation, characteristic of the subgenus *Auriculastrum*, should be regarded as an ancestral trait and the subgenus *Auriculastrum*, as a more ancient in the genus *Primula*.

Cluster III (figure) includes the sections *Auganthus* ($x = 12$), *Monocarpicae* ($x = 9$), *Obconicolisteri* ($x = 12$), and *Cortusoides* ($x = 11, 12$) and the *Cortusa* species. This cluster corresponds to the subgenus *Auganthus* Wendelbo according to Richards [2] except for the genus *Cortusa*. The primroses belonging to this subgenus have soft membranous leaves, frequently rugose and covered with multicellular hairs.

Species of the genus *Cortusa* (three European accessions of *C. matthioli* L. and Russian accession, *C. altaica* Losinsk.) belong to clade III and are significantly close to the species of section *Cortusoides*, which is confirmed by a high bootstrap value (93–97% BP). Similar results were obtained in the earlier studies [23, 25]. The genus *Cortusa* ($x = 12$), which is considered by several researchers to be monotypic with the only species *C. matthioli*, has a wide Eurasian distribution and was spited several small species in the eastern part of its area; phenotypically, these species are very close to primroses of the section Cortusoides (*P. kaufmanniana* Regel, *P. jesoana* Mig., and *P. geranifolia* Hook). The

Cortusa species differ from them only by the flower structure: the filaments of all *Cortusa* species are adnate at the base, whereas they are free in the primroses. This trait demonstrates a close relation of the genus *Cortusa* to the oligotypic genus *Kaufmannia* Regel, two Central Asian species of which display adnate filaments forming a thick ring. An erroneous attribution of the herbarium specimens to either *Cortusa* or *Cortusoides* primroses still takes place. Our studies do not favor an independent status of the genus *Cortusa*; presumably, it should be regarded as an individual section within the subgenus *Auganthus.*

Cluster II comprises the sections *Proliferae* ($x = 11$) and *Crystallophlomis* ($x = 11$). The section *Crystallophlomis* contains three significantly separate species with similar morphological characteristics—*P. nivalis, P. xanthobasis*, and *P. turkestanica* (78 and 93% BP).

Clusters II and I together without the section *Primula* correspond to the subgenus *Aleuritia* in the Richards system [2]. Our studies provide another confirmation for heterogeneity and artificial character of the subgenus *Aleuritia.*

In cluster I, the clade *Primula* ($x = 11$) with diploid species ($2n = 22$) corresponds to the type section and subgenus *Primula* according to Richards [2]. In the section *Primula*, we have analyzed the nucleotide sequences of the vicarious species *P. macrocalyx* Bunge and *P. veris* L. (BP = 85/80) and three closely relates species—*P. pallasii* Lehm., *P. elatior* (L.) Hill., and *P. poloniensis* (Domin) Fed. The monotypic section *Sredinskya* Stein with the only species *P. grandis* Trautv. ($2n = 22$) from Abkhazia and Svanetia (Georgia) also belongs to this subgenus. Characteristic of this subgenus are the hairy plants without farina and very rugose leaves.

Cluster I also contains also three clades that correspond to the sections *Sikkimensis* ($x = 11$, $2n = 22$), *Oreophlomis* ($x = 11$, $2n = 22, 44$), and *Aleuritia* ($x = 9$). These three sections from cluster I and cluster II belong to the subgenus Aleuritia in the Richards system [2], constructed based on the phenotypic traits and analysis of chloroplast DNA.

The section *Aleuritia*, which is the most distant from the tree root, is represented by the most polymorphic North Asian and North American primrose species. The base chromosome number of these species, $x = 9$, is regarded as a more progressive trait [2]. The majority of the species in this section are diploids with $2n = 18$ (*P. farinosa* L., *P. longiscapa* Ledeb., *P. modesta* Bisset & S. Moore, *P. frondosa* Janka, and *P. mistassinica* Michx.). However, the section also contains tetraploids with $2n = 36$ (*P. borealis* Duby and *P. serrata* Georgi), hexaploids with $2n = 54$ (*P. scotica* Hook. and *P. incana* M.E. Jones), and octaploids with $2n = 72$ (*P. scandinavica* Brunn and *P. magellanica* Lehm.) and 2n = 126 (*P. stricta* Hornem.).

Similarly to other relevant studies, our work does not provide a statistical support for a monophyletic origin of the section *Aleuritia.* A relatively high sequence divergence within this section can be a reason of its polymorphic pattern.

Of the section *Aleuritia*, we plan to describe the species *P. bukukunica* Kovtonyuk in shet., which we collected in the Sokhondo National Park and introduced at the Central Siberian Botanical Garden. Analysis of the nucleotide sequences of the rDNA ITS region suggests that *P. bukukunica* is close to *P. kawasimae* Hara and *P. fauriei* Franch. (92–84% BP); these species differ in the shape of leaf blade and have nonoverlapping areas.

The endemic species *P. sachalinensis* Nakai, belonging to the same section (collected on the Sakhalin Island, the mud volcano Maguntan, near the railroad station Pugachevo), displayed a statistically significant similarity (BP = 98/90) to the species *P. mistassinica, P. modesta*, and *P. incana* from Alaska and Northern Canada rather than with *P. farinosa*, with which it is frequently united. Our studies are another confirmation for an independent of the endemic species *P. sachalinensis.*

Earlier, RAPD assay, a more sensitive method at an interspecific level, confirmed a genetic relation between the Baikal endemic *P. pinnata* M. Pop. et Fed. and the South Siberian species *P. serrata* Georgi [35–38].

The performed comparison of the nucleotide sequences of rDNA ITS region allowed us to determine more precisely the phylogenetic relationships between the taxa within the genus *Primula* as well as the phylogenetic relationships among the genera within the corresponding family. This method provides for determining the clades that correspond to the sections of the genus *Primula.* Our results to a considerable degree comply with the sectional divisions of this genus and the earlier data of other researchers based on different data sets and markers [23–25]. On the other hand, the determined clusters not always agree with the division of this genus into subgenera proposed by Richards [2]. Presumably, the division of the genus *Primula* into two subgenera according to the vernation type and several sections according to other traits looks more logical. The studies do not support an independent status of the genus *Cortusa*, suggesting that it could be reasonable to regard it as an individual section of the subgenus *Auganthus*.

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