ISSN 1995-4255, Contemporary Problems of Ecology, 2017, Vol. 10, No. 1, pp. 43–51. © Pleiades Publishing, Ltd., 2017.
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Morphological and Genetic Analysis of *Brunnera sibirica* (Boraginaceae) Cenopopulation in the Southern Siberia Mountains

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Received July 9, 2015; in final form, June 10, 2016

Abstract—Fifteen *Brunnera sibirica* cenopopulations have been studied. Morphological and genetic investigations reveal increasing variability and high level of correlation between morphological features (according to decreased sizes of vegetation organs) during the first years after deforestation in fir and aspen forests. High morphological and genetic variability is typical for mountain fir forests, pine–birch, and pine forests.

Keywords: Brunnera sibirica, nemoral relict, morphological and genetic analysis, RAF–PCR, Western Sayan, Northeastern Altai

DOI: 10.1134/S1995425517010127

Brunnera sibirica Stev. is a nemoral relict, endemic to the Altai–Sayan system. This species was included in the Red Data Book of the Soviet Union (*Krasnaya kniga SSSR*..., 1984) and in the Red Data Book of Krasnoyarsk krai (*Krasnaya kniga Krasnoyarskogo kraya*..., 2012). It has narrow and local disjunctive range embracing Altai, Kuznetskii Alatau, Northeastern Tyva, and Western and Eastern Sayans. The small and isolated part of the range is known near the city of Tomsk (Polyntseva et al., 1986). One closely related species is *Brunnera macrophylla* (Adam) Johnst. growing in forests of Caucasus. *Brunnera sibirica,* often dominant in the herbaceous layer, is an element typical for chern forests. In recent years chern forests have been intensively cut down, which is a threat of extinction for this species.

Brunnera sibirica is a plant with one generation of shoots. The main shoot grows monopodially and forms thick, 0.5–1 cm dark brown rhizome partly positioned in soil. The last annual increment of the shoot carries 1–3 radical leaves and 1–2 lateral floriferous shoots. The radical leaves contain long bristled petioles 10–25 cm in length and width. Their plate is cordate–deltoid, with a broad basal cut. Stem leaves are much smaller than radical leaves, sedentary, solid, and lanceolate. Inflorescences are short botryoid bostryces combined at the top into a small tassel. Flowers are pentamerous. Fruit is cenobium, from which one-seed indehiscent erems are separated at maturation. In natural conditions *B. sibirica* reproduces mainly vegetatively by the collapse of rhizomes into separate parts. There are no germs or juvenile plants in the age spectrum of individuals having a vegetative origin. Natural populations of the species are normal and incomplete with a right–hand type of bimodal base spectrum with maxima on young generative and subsenile individuals (Polyntsaeva et al., 1986; Ignatenko, 1995; Amel'chenko, 2010).

Our studies were conducted in the northeastern part of Western Sayan (Krasnoyarskii krai, Ermakovskii district, and the vicinities of settlements Tanzybei and Grigor'evka), in the northeastern part of Altai (shore of Teletskoe Lake, territory of the Altaiskii State Nature Reserve), in vicinities of Tomsk City (vicinities of the village of Anikino). Our studies aimed at analyzing phytocenotic preferences, modification, and genetic variability in natural cenopopulations of *B. sibirica*.

MATERIALS AND METHODS

Materials were collected in the end of May and beginning of July from 1995 to 2011. For studying cenopopulations of *B. sibirica*, we determined projective cover, variability of vegetative and generative characters, seed production, and genetic variability based on RAF–PCR analysis. Upon the study of the influence of forest clearcutting on the condition of cenopopulations of this species, we used cenopopulations growing on relatively undisturbed communities in close proximity to clearcuttings as controls.

The geobotanical description of communities was conducted by the dominance method (Voronov, 1973; etc.). The table shows habitat characteristics of the species. The following gradations in the projective cover of *B. sibirica* were used in determination and

Geobotanical characterization of *Brunnera sibirica* habitats

No.	Name of community and location	Composition of tree stands and cover	Dominant species in herb-dwarf shurb layer, projective cover, %
	Subboreal forest of Eastern Sayan, 250–350 m above sea level		
Bs1	Herb-grass-fern pine forest, valley of the River Ui, vicinity of the settlement of Maina	9P1B 0.6	*Pteridium pinetorum* ssp. *sibiricum (50%)* *Carex macroura (20%)* *Brachypodium pinnatum (30%)* *Brunnera sibirica (15%)*
Bs2	Swamp sedge–grass birch forest, vicinity of the settlement of Tanzybei	8B2A 0.1	*Calamagrostis langsdorffii (60%)* *Carex cespitosa (50%)* *Anemone baicalensis (10%)* *Brunnera sibirica (3%)*
Bs3	Tall-grass broad–leaved grass birch forest, Verkhovoi Ridge	8B2A 0.5	*Brunnera sibirica (75%)* *Heracleum dissectum (8%)* *Cacalia hastata (8%)*
	Chern belt of Western Sayan (350–700 m above sea level)		
Bs4	Tall-grass–broad-leaved grass–aspen forest, Verkhovoi Ridge	10A+B, 0.6–0.7	*Anemone baicalensis (80%)* *Brunnera sibirica (60%)* *Heracleum dissectum (15%)*
Bs5*	2-year-old tree clearcutting in the aspen–tall-grass–broad leaf grass forest	no tree stands	*Anemone baicalensis (40%)* *Brunnera sibirica (60%)* *Matteuccia struthiopteris (40%)* *Calamagrostis langsdorffii (15%)*
Bs6*	14-year-old tree clearcutting of the aspen tall–grass–broad leaf grass forest	10A 0.7	*Anemone baicalensis (50%)* *Brunnera sibirica (40%)* *Matteuccia struthiopteris (50%)*
Bs7	Tall–grass–broad leaf grass–fern fir forest, valley of the Vtoraya Belaya River	9F1C 0.6–0.7	*Anemone baicalensis (80%),* *Anemone altaica (50%)* *Brunnera sibirica (15%)* *Athyrium monomachii (15%)*
Bs8*	11-year-old clearcutting of the tall–grass–fern–broad leaf grass forest	no tree stands	*Calamagrostis langsdorffii (80%)* *Brunnera sibirica (50%)* *Athyrium monomachii (15%)* *Anemone baicalensis (10%)*
Bs9*	Willow fern–broad leaf grass, 20-year-old clearcutting, Vtoraya Belaya River floodplain	0.6	*Brunnera sibirica (80%)* *Matteuccia struthiopteris (15%)* *Anemone altaica (15%)*
	Mountain taiga belt of Western Sayan, 700–1050 m above sea level		
Bs10	Fern–broad leaf grass fir forest, valley of the River Chebijek	8F2C 0.6–0.7	*Anemone baicalensis (50%)* *Matteuccia struthiopteris (40%)* *Dryopteris expansa (10%)* *Brunnera sibirica (<1%)*
Bs11*	30-Year-old clearcutting in the fern–broad leaf grass–fir forest, under the power transmission line	no tree stands	*Calamagrostis langsdorffii (50%)* *Anemone baicalensis (15%)* *Brunnera sibirica (5%)*
Bs12	Open fir forest, valley of Chebijek River, upper forest margin	10F+P 0.4	*Anemone baicalensis (85%)* *Anemone altaica (50%)* *Delphinium elatum (5%)* *Brunnera sibirica (5%)*

Table. (Contd.)

No.	Name of community and location	Composition of tree stands and cover	Dominant species in herb-dwarf shurb layer, projective cover, %
	Northeastern Altai, Teletskoe Lake shore, 450–500 m above sea level		
Bs13	Fern–broad leaf grass–birch forest, village of Yailyu, Ok–Porog Site	10B 0.2	*Brunnera sibirica (70%)* *Aegopodium podagraria (60%)* *Athyrium monomachii (20%)*
Bs14	Fern–forb grass pine–birch forest, Chelyushka River floodplain	7B3P 0.4	*Dryopteris expansa (40%)* *Brunnera sibirica (20%)* *Cruciata krylovii (10%)*
	Vicinity of Tomsk city, 150 m above sea level		
Bs15	Fern–glague and birch–aspen forest, vicinity of the village of Anikino	7A3B+P 0.6	*Aegopodium podagraria (40%)* *Pteridium pinetorum* ssp. *sibiricum (10%)* *Calamagrostis arundinacea (10%)* *Brunnera sibirica (6%)*

Here and below the asterisk marks cenopopulations growing on the territory of clearcuttings; B, birch; P, pine; F, fir; C, cedar pine; and A, aspen.

analysis: up to 5% (corresponding to + and 1 score by the scale of Broun–Blanquet, 1964), low projective cover; 5–25% (2 scores), medium; 25–50% (3 scores), high; and more than 50% (4–5 scores), very high. When determining ecological factors limiting this species distribution, we used the ordination system elaborated by Polikarpov et al. (1986) for the mountains of South Siberia. The ordination system shows the altitudinal distribution of forest and nonforest vegetation according to changes in the amount of warmth and moisture expressed as sums of active temperatures and yearly sums of precipitation. If we know the altitudinal distribution of a species and its phytocenotic preference, it is quite easy to determine the ecological amplitude of the species towards the abovementioned factors using this scheme (Yamskikh, 2015).

Measurements of vegetative organs parameters and seed productivity characters of the relict for estimating phenotypic variability were conducted on 30 generative shoots. The distance between the plants under study was no less than 10 m. A large part of morphometric characters estimated by us is given as diagnostic characters for species identification in the monographs *Flora of Soviet Union* (Popov, 1953), *Flora of Central Siberia* (Popov, 1959), and *Flora of Siberia* (Nikiforova, 1997). Parameters of seed productivity in the relict were estimated by methods described by Dyuryagina and Ivanova (1985). The following characters were measured in *B. sibirica* shoots: length of the radical leaf petiole (x1), stem length (x2), length (x3) and width (x4) of the stem leaf, shape of the stem leaf x3/x4 (x5), number of stem (x6) and radical leaves (x7), number of floriferous shoots (x8), length (x9) and width (x10) of radical leaf, shape of radical leaf x9/x10 (x11), width (x12) and length (x13) of the cut in the base of radical leaf, ratio x1/x9 (x14), rhizome diameter (x15), length of flourishing part of the stem (x16), number of particular inflorescences on the flower stalk (x17), real (c1) and potential (c2) seed productivities, and seminification coefficient (c3).

Statistica 7.0 software was used for the mathematical treatment of morphological data. Intrapopulation variability of characters was estimated using variation coefficient (C_V), because this parameter allows a comparison of characters having different dimensionalities (Shmidt, 1984). In addition, Mamaev (1972) elaborated the scale of the levels of variability for the variation coefficient. We used one-way ANOVA for determining significant differences between mean population values of similar characters. The difference is considered significant at a level of significance of $p < 0.05$. The interaction of morphological characters was determined using the method of correlation pleiads by Terent'ev (1959) with the subsequent construction of correlation dendrites by the method of maximum correlation path using the algorithm by Vykhandu (1964). The combination of characters into one pleiad was done at correlation coefficients $r \geq 0.6$. The similarity between populations by characters under study was analyzed using cluster analysis. The ward method was used in the treatment of data. Euclidean distance was used as the similarity measure.

Molecular–genetic studies were conducted in laboratories at Altai State University (Barnaul, Russia), and Siberian Federal University (Krasnoyarsk, Russia). The genetic variability of cenopopulations under study was determined using the RAF–PCR (Randomly Amplified DNA Fingerprinting) method. Ten plants, which were not clones, were analyzed from each population. Materials of closely related Siberian species, *B. macrophylla*, were used for comparison in the RAF–PCR analysis. DNA extraction was conducted from 15–25 mg of air-dried tissue using the set AxyPrep Multisource Genomic DNA (AxyGen, United States).

RAF–PCR was conducted in a 12.5-μL mixture (7.4 μL of H_2O, 1 μL DNA, 1.25 μL of 10X buffer, 1.25 μL of 25 mM $MgCl_2$, 1 μL of 10 mM primer, 0.5 μL of 20 mM dNTPs, and 0.1 μL Taq–polymerase) on a MyCycler amplificator. Amplification was conducted by the following program: 94°C, 5 min, 35 cycles: 94°C, 30 sec, 57°C, 1 min, 56°C, 1 min, 55°C, 1 min, 54°C, 1 min, 53°C, 1 min; and concluding stage: 72°C, 10 min, cooling at 4°C. The primer RAF K-02c (5'-GTCTCCGCCT-3') was determined by preliminary experiment from 2 DNA samples from the existent primer set (Waldron et al., 2002). It provided a reproducible polymorphic result. Analysis of amplification products was done using the equipment Experion™ Automated Electrophoresis Station (Bio–Rad, United States).

The statistical treatment of results of the genetic analysis was conducted using TFPGA, version 1.3 (Miller, 1997) (UPGMA analysis, bootstrap test and calculation of genetic distances), and Popgene, version 1.32 (calculation of the percent of polymorphic loci (P), Nei genetic diversity (H_e) software, Shannon index (H_o) and F—statistics for populations (Gst). Genetic distances (D) between cenopopulations were determined by Nei's formula (Nei, 1978).

RESULTS AND DISCUSSION

Geobotanical studies revealed that the phytocenotic range of *B. sibirica* exceeds the range of chern forests on Western Sayan. This species occurs in subboreal pine, birch–pine, and birch forests. The high projective cover of *B. sibirica* takes place in chern aspen forests and middle–mountain fir and cedar pine forests, as well as in birch and willow forests, where it is often a dominant species in the herbaceous layer. Sometimes this species occurs in mountain–taiga fir and cedar pine forests. After a certain interruption, it occurs again on moist subalpine meadows. In the northeastern part of Altai, we noted this species in birch, pine, and mixed forests. Habitats of this species isolated from its main range are known in vicinities of Tomsk. The species grows there under the cover of birch–aspen and aspen forests with an admixture of pine. Depending on growth conditions, the species may play in communities the role of dominant, codominant, or assectator. Its ecological range embraces an area with annual precipitation from 500 to 100 mm and a sum of active temperatures from 1050 to 2000°C. The altitudinal distribution of *B. sibirica* is limited to 200–1400 m above sea level.

A very high projective cover of this relict was recorded in communities where edificators are birch and aspen (Table 1), as well as in willow grooves: in the birch forest (cenopopulation Bs3), aspen forest (Bs4), and in fern–wide-grass forest (Bs9*). Maximum density was noted in the willow forest (Bs9*), 58 shoots per 1 m^2. The minimal projective cover of the species under study was recorded in pine forests and mountain taiga fir forests.

On the 2-year-old clearcutting in a aspen forest (cenopopulation Bs5*), the projective cover of *B. sibirica* was almost unchanged when compared to the control cenopopulation Bs4 (table). On the 14-year-old clearcutting of the same forest type (Bs6*), where aspen recovery is fairly active and where tree cover is closed, high cover by *B. sibirica* was also found. On the 11-year-old cutting of the ostrich fern–broad-leaved-grass forest (Bs8*), an increase in the projective cover from 15 to 50% was found when compared with control cenopopulation Bs7, which, probably, is connected with decreased competition from shade-preferring plants like large ferns. On the 20-years-old cutting in the same forest type, representing groves of *Salix viminalis*, projective cover of *B. sibirica* (Bs9*) was very high (80%) and the plants were large. On the 30-year-old cutting in fern–broad-leaved grass–fir forest (Bs11*), intensive growth of individuals of this species was also noted, from less than 1 to 5%. There, *B. sibirica* occurs under the cover of bushes, whereas on open areas *Calamagrostis langsdorffii* dominates.

A comparison of mean population values of *B. sibirica* characters revealed that plants of this species reach maximum size of the vegetative organs in the Western Sayanian broad–leaved grass–fern–spruce forest (Bs7) and in Altaian fern–forb–grass pine–birch forest (Bs14) (Fig. 1). The maximum number of fruits is formed in plants growing in tall-grass–broad-leaved grass–birch forest (Bs3, 27.8 specimens) and on clear-cuttings in tall-grass–broad-leaved grass–aspen forest (Bs5*, Bs6*, by 26–27 specimens). Potential seminal productivity reaches maximum values in plants of the cenopopulations Bs5* and Bs7 (510.4 and 403.6 specimens, respectively), and high values of the insemination coefficient are typical for Bs6* and Bs10 (8.65 and 9.43%) (Fig. 2).

The plants of *B. sibirica,* growing in the West Sayanian subboreal pine forest (Bs1) and open fir forest (Bs12) growing at the upper and the lower margins of altitudinal distribution of species are characterized by minimal sizes and parameters of seminal productivity (Fig. 1). The high level of variability in morphological characters was recorded for these cenopopulations (Fig. 3). The Tomsk population (Bs15), located separately, is characterized by the complete absence of fructification.

There is a general tendency towards an increase in the level of intrapopulation variability and decrease in sizes of reproductive organs of *B. sibirica* on areas with the total cutting of aspen and fir forests in Western

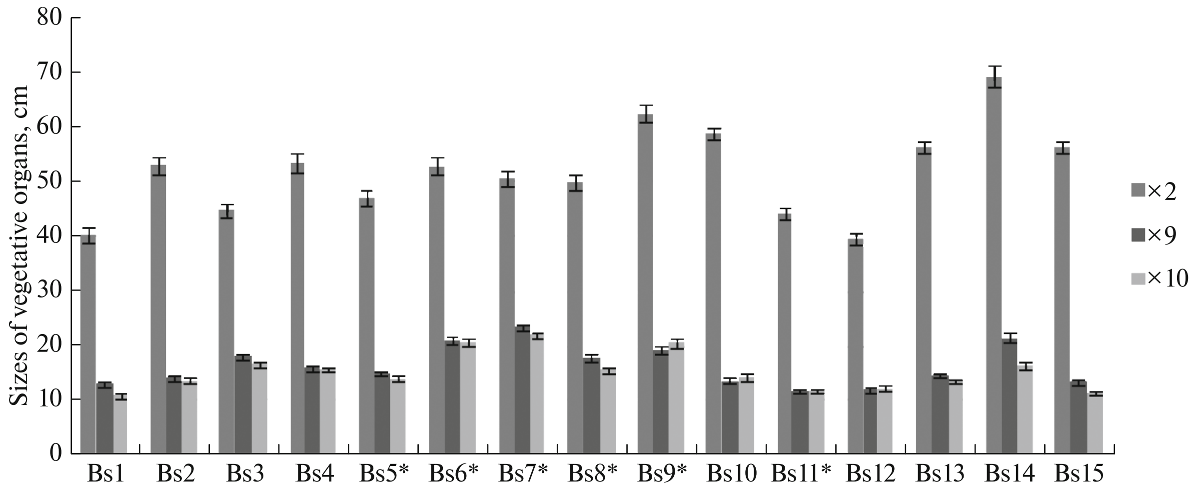

Fig. 1. Mean–population values of morphological parameters in *Brunnera sibirica:* ×2, stem length; ×9, breadth of radical leaf; and ×10, length of radical leaf. Here and below and on Fig. 2, the error of the representation of the mean is indicated as confident interval.

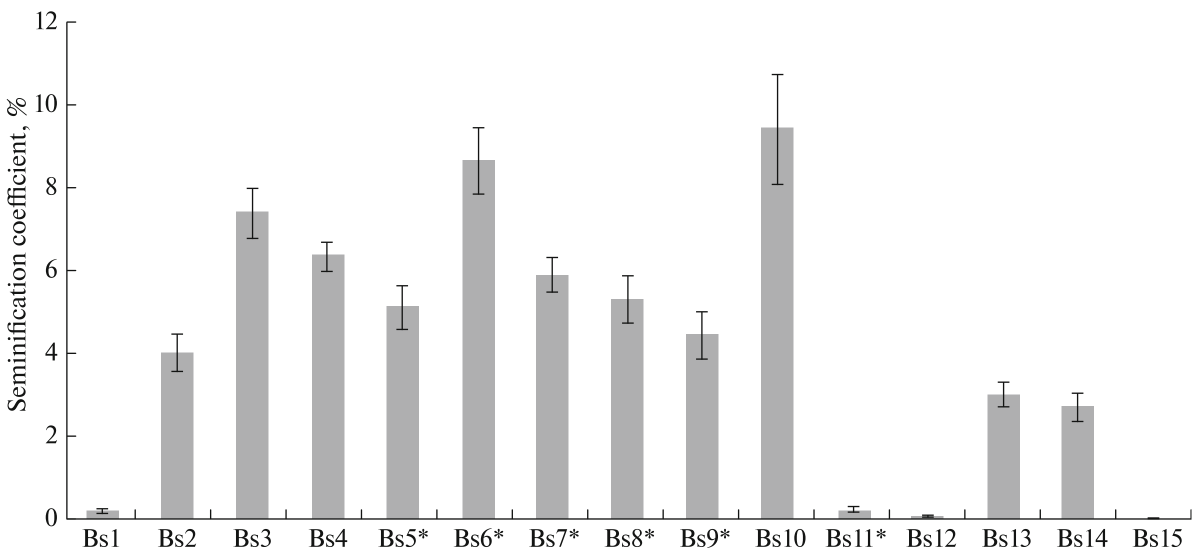

Fig. 2. Mean population values of the coefficient of semnification of *Brunnera sibirica*.

Sayan (Figs. 1 and 3). Cenopopulation Bs8* growing on the 11-year-old clearcutting in the chern fir forest is an example. Compared with the control (Bs7), these plants display a decrease in leaf length and breadth from 23.2 ± 0.5 to 17.7 ± 0.7 and from 21.8 ± 0.5 to 15.5 ± 0.6 m, respectively. Cenopopulations growing on clearcuttings with forming tree stands (Bs6*) or with a well-developed bush layer (Bs9*) are less different from the control by parameters of variability.

In addition to the study of external features of plants, lately it is considered very important also to study the interactions of characters, i.e., reveal the correlation structure of plant populations (Zlobin, 1989; Rostova, 2000; Tikhonova, 2005; Yamskikh, 2007, 2008). Upon the influence of unfavorable factors in different plant species, the number of significant correlative relations between characters increases sharply, and the structure of their dendrites is changing. In *B. sibirica,* the similar structure of correlative dendrites and position of pleiads revealed cenopopulations growing in pristine aspen, spruce, and pine forests, as well as in secondary birch forests floodplain willows in Western Sayan, Altai, and the vicinities of Tomsk. In such habitats, the number of significant correlative relations ($r \geq 0.36$) varies from 38 to 53 and the number of strong ones ($r \geq 0.7$) does not exceed 8. On the correlative dendrite of such cenopopulations (dendrite of the cenopopulation Bs4 as an example),

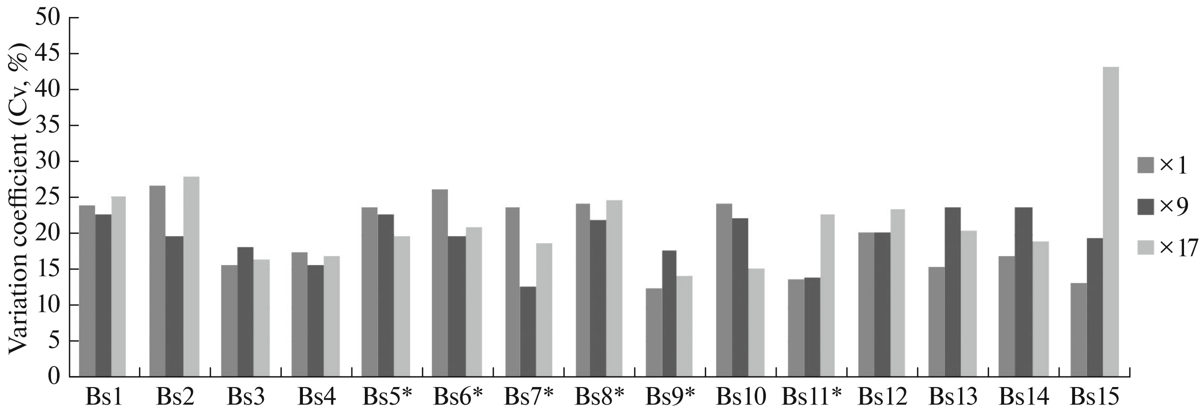

Fig. 3. Intrapopulational variability of morphological characters of x1, petiole length pf the radical leaf; x9, breadth of radical leaf; and x17, number of particular inflorescences on the flower stalk.

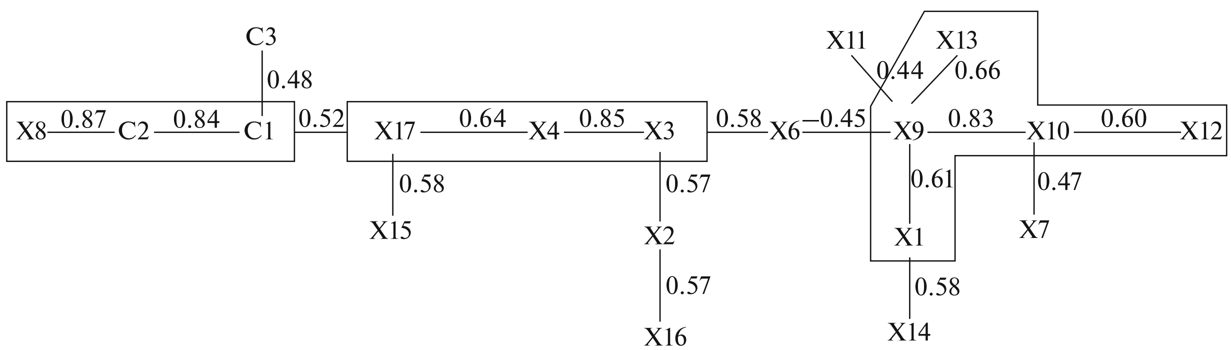

Fig. 4. Correlational dendrite of *Brunnera sibirica* morphological characters in population Bs4, tall-grass–broad-leaved grass–aspen forest. Numerals indicate correlation coefficients.

most often pleiads of the sizes of radical and stem leaves are formed, as well as parameters of seminal productivity (Fig. 4). Cenopopulations growing in conditions untypical for *B. sibirica*—swampy birch forest (Bs2) and rarefied fir forest (Bs12)—are characterized by a high level of conjugacy of parameters: the number of significant relations is 71–77. Their correlation structure is characterized by the formation of two pleiads combining the majority of characters. On total clearcuttings of the first years (Bs5*), an increase in significant relations is observed, from 53 to 89, while on the dendrite, a combination of different characters into one pleiad is visible (Fig. 5). On older clearcuttings, a gradual decrease takes place in the level of interrelation between *B. sibirica* parameters, whereas, on the dendrite, the collapse of a singular pleiad into several smaller ones is observed. We observed the complete recovery of the correlation structure in this species on the 30-year-old fir clearcutting.

On a similar dendrogram built using cluster analysis, splitting of the set of individuals by geographic principle is visible (Fig. 6). A separate cluster is formed by Altai populations Bs13 and Bs14, for which we noted more elongated shape of the stem and radical leaves. Close morphological similarity is typical also for the Tomsk Bs15 and Western Sayan subboreal forest cenopopulations Bs1 growing in similar phytocenotic conditions. Western Sayan cenopopulations growing in natural and disturbed habitats differ little from each other by the complex of parameters under study.

Genetic polymorphism in *B. sibirica* was studied on cenopopulations growing in the forests of Western Sayan (Bs1, Bs4, Bs10, and Bs11*), Northeastern Altai (Bs14), and in the vicinity of Tomsk (Bs15). For comparative analysis, we used genetic material from a closely related species, *B. macrophylla* (Bm). In the course of analysis, we identified 61 DNA fragments. The sum percent of polymorphism was 93.44. The level of identified intrapopulation genetic diversity was rather high, 80.33–88.52%. Parameters of genetic polymorphism were maximum in the Altai cenopopulation Bs14 ($P = 88.52\%$, $H_e = 0.347$, and $H_o = 0.508$). Values of the genetic diversity and Shannon index are also typical for Western Sayan Bs1 (subboreal pine forest) and Bs11* (30-year-old clearcutting of spruce forest). Minimal values were noted for cenopopulation in

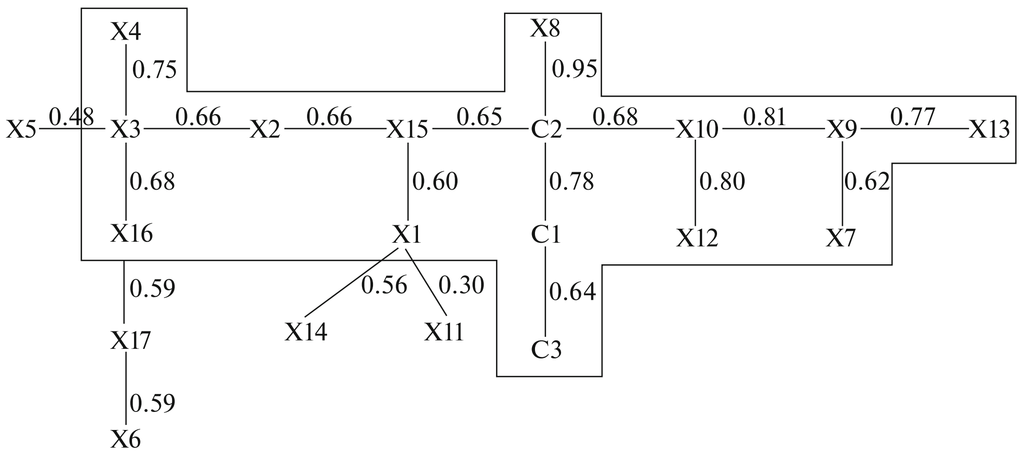

Fig. 5. Correlation dendrite of *Brunnera sibirica* morphological characters from cenopopulation Bs5* (2-year-old clearcutting of the tall-grass–broad-leaved grass–aspen forest).

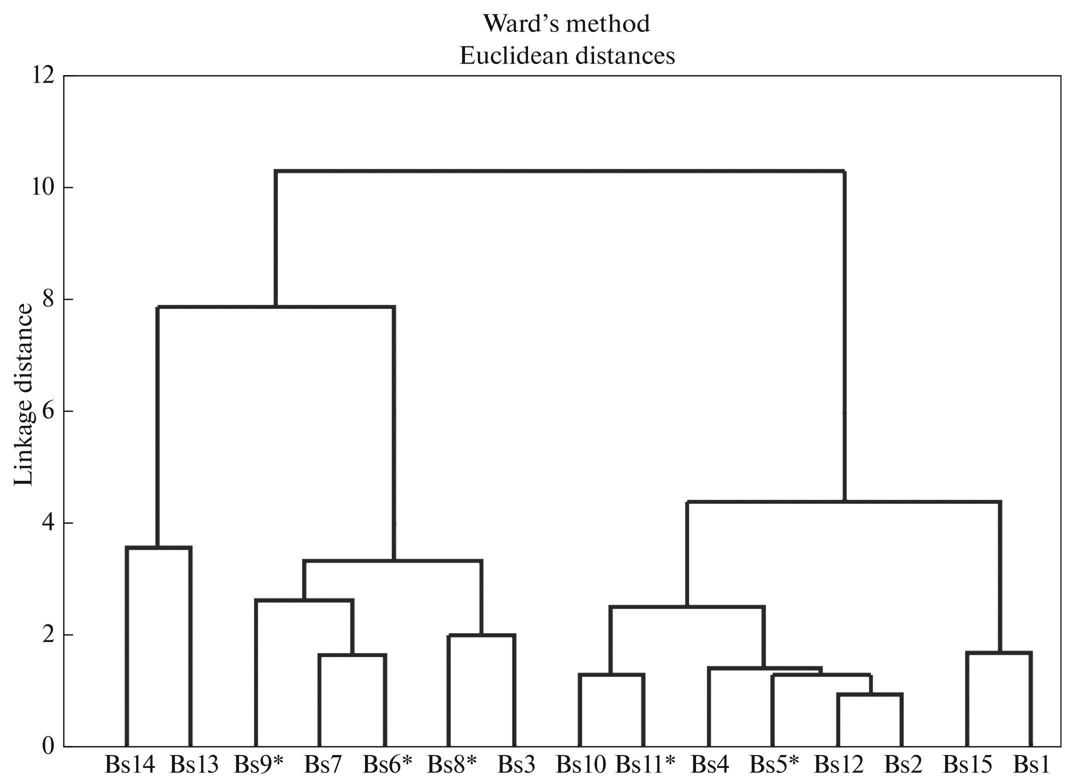

Fig. 6. Dendrogram of similarity of *Brunnera sibirica* cenopopulations by morphological characters.

the vicinity of Tomsk Bs15 ($P = 80.33\%$, $H_e = 0.282$, and $H_o = 0.423$).

The coefficient of subdivision of cenopopulations (G_{st}) is 0.204. Therefore, the portion of interpopulation diversity should be 20.40% and the cenopopulations studied demonstrate a quite high degree of differentiation (Wright, 1978). Nei genetic distances (Nei, 1978) were minimal between Bs1 and Bs4 ($D = 0.069$) and Bs10 and Bs11* ($D = 0.080$). Maximum distances were recorded between populations of *B. sibirica* and *B. macrophylla*. Two clusters were determined on the UPGMA dendrogram of cenopopulation similarity (Fig. 7). The first cluster is formed by Bs1 and Bs4 grown in the foothill and low-mountain belts of Western Sayan. The second cluster is formed by Western Sayan highland Bs10 and Bs11* and the adjacent Altai population Bs14. A somewhat separated position is occupied by Bs15, for which low parameters of genetic

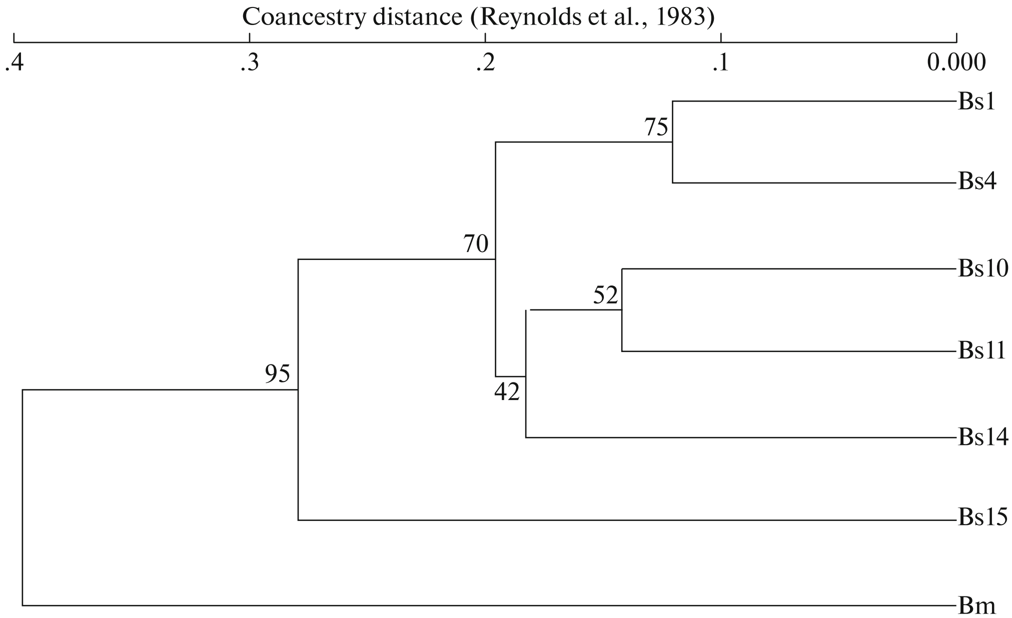

Fig. 7. Dendrogram of similarity of *Brunnera sibirica* and *B. macrophylla* cenopopulations based on data from RAF–PCR analysis. Numerals mean bootstrap values, %.

variability were noted. The manifestation of this cenopopulation in the vicinity of Tomsk is a matter for discussion. Some scientists suppose a natural origin of the Tomsk populations of *B. sibirica*, others tend to think that this species originated in vicinities of Tomsk as a result of introduction. Anyhow, specimens of this cenopopulation display close genetic and morphological similarity with the lowland Western Sayan population rather than with those from Altai.

Therefore, a high level of intrapopulation genetic variability of *B. sibirica* is typical for cenopopulations growing not in typical chern communities, but rather for those from subboreal forests and only for the clearcuttings made for power transmission line.

The studied cenopopulations are in satisfactory condition, according to the scale of Boronnikova (2009). They are capable of self-reproduction.

CONCLUSIONS

The analysis of variability and interactions of characters in *B. sibirica* revealed that, on total clearcuttings of fir and aspen forests of tall-grass and fern–broad-leaved-grass groups in the northeastern part of the Western Sayan, an increase is noted in the projective cover and some parameters of the seed production of species. A decrease in parameters of vegetative organs, an increase in variability in some morphometric characters, and the level of correlation are typical for cenopopulation of the relict growing on the younger clearcuttings. Individuals growing on older clearcuttings, as well as on territories with the active formation of arboreal and bush layers, are less different from plants in pristine forest types and sometimes exceed the latter by the parameters studied.

The plant *B. sibirica* in its type of strategy expresses parameters of the violent in covered communities or, otherwise, those of the phytocenological patient, and on the clearcuttings those of the explerent, due to its capability of sprawling in the first years. This species grows beyond refugia in the nemoral zone. It occurs in subboreal and mountain taiga forests, where it displays a high extent of morphological and genetic diversity, which indicates the possibility of its further dispersal on the territory of southern Siberia.

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Translated by S. Kuzmin