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RELATIONSHIP BETWEEN CYTOGENETIC CHARACTERISTICS AND MOLECULAR-GENETIC DIFFERENCES IN SPECIES OF THE GENUS *Rhododendron* L. WHEN INTRODUCED

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Abstract

Currently, woody plants attract special attention given the prospects of their involving in bio- and genomic technologies to address challenges of sustainable environment, biodiversity, food security and production of raw materials. Thence, studies of cytogenetic characteristics of woody plants are increasingly relevant. The change in a number of cytogenetic characteristics, in particular, mitotic activity, which may increase and decrease depending on the intensity of stress loads, an increase in the pathology of mitosis, etc., has been shown. However, attempts to identify the similarities and differences in cytogenetic characteristics in woody plants on the basis of the results of molecular genetic comparison weren't conducted yet. Sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal NA were used to generate a phylogenetic hypothesis for disjuncting of wood species of the genus *Aralia* (J. Wen, 2000), to specify of *Rhododendron* systematic state (T.V. Baranova et al., 2014) and other genera of the family *Ericaceae* (O. Schwery et al., 2015). Cluster analysis of nucleotide sequences and construction of the dendrogram were carried out using the ML (Maximum Likelihood, Nearest-Neighbor-Interchange) method in the MEGA software. Germination of *Rhododendron* seeds was carried out in Petri dishes at room temperature. Roots were stained with acetohematoxylin, rinsed with distilled water, and suppressed micro-preparations were prepared using Goyer's fluid. Nucleotide sequences of the ITS1-ITS2 spacer of the parent plants and cytogenetic parameters (mitotic activity, level and spectrum of pathological mitoses, number of cells with residual nucleoli in the metaphase-telophase mitosis stage) we obtained from seed progeny in four *Rhododendron* species introduced into the conditions of the Central Black Earth region of Russia. The identity of the nucleotide sequence of the spacer ITS1-ITS2 in species of the genus *Rhododendron* leads to their greater similarity in the aggregate of cytogenetic indices. However, there is no complete analogy of cytogenetic characteristics in the species studied that have the identical sequence ITS1-ITS2. On the basis of this comparison, it can be assumed that genetic similarity in the studied *Rhododendron* species causes the similarity of cytogenetic indices. According to mitotic activity in the root meristem of the seedlings, two groups can be distinguished among the seed progeny, i.e. with a high value of mitotic activity, namely *Rhododendron dauricum* (7.6±0.3 %) and *Rh. mucronulatum* (7.7±0.7 %), and with low value, namely *Rh. sichotense* (5.6±0.7 %) and *Rh. ledebourii* (6.1±0.6%). The greatest cytogenetic instability is noted in *Rh. ledebourii* (5.2±1.1 %, the level of pathologies of mitosis in this species is maximal), in three other species it was lower (from 3.5±0.5 % for *Rh. sichotense* to 1.6±0.4 % for *Rh. dauricum* mitosis pathologies). A higher level of cells with a residual nucleolus at the stage of metaphase—telophase mitosis indicates a greater intensity of synthetic processes associated with adaptation in conditions of introduction. For this indicator, we can distinguish two groups: i) *Rh. sichotense* (13.3±1.2 %) with a high level of cells with a residual nucleolus at the stage of metaphase—telophase of mitosis, and ii) *Rh. mucronulatum* (9.1±1.1 %), *Rh. dauricum* (10.2±1.0 %) and *Rh. ledebourii* (10.9±1.3 %) with low values. Despite the difference in cytogenetic

parameters in the seed offspring of the studied species, a cluster analysis of the totality of the characteristics of the course of mitosis and nucleolar activity made it possible to distinguish two groups: 1) *Rh. mucronulatum* and *Rh. dauricum*; 2) *Rh. ledebourii* and *Rh. sichotense*. The cytogenetic characteristics of the seed offspring of the species studied are species-specific.

Keywords: *Rhododendron* L., rhododendrons, seed progeny, introduced plants, cytogenetic characteristics, mitotic activity, cytogenetic abnormalities, mitotic pathologies, persistent nucleoli, ITS1-ITS2 sequences, cluster analysis

Woody plants attract more and more attention of researchers given the prospects of involving these biological objects in the sphere of application of bio- and genomic technologies for solving environmental problems, preserving biodiversity, food security and production of raw materials. Therefore, studies of the cytological and molecular and genetic bases of inheritance in such plants are relevant.

Currently, cytogenetic characteristics in Russia are widely studied in conifer species [1], especially in the representatives of the *Pinaceae* family [2-4]. Among deciduous plants, aboriginal species are studied, for example, *Betula pendula* [5-7] and *Quercus robur* [8], as well as introduced forms such as *Catalpa, Tilia* [9] and *Rhododendron* [10-12]. In woody plants, there is a change in the range of cytogenetic characteristics (in particular, mitotic activity, which may increase and decrease depending on the intensity of stress loads), as well as an increase in the frequency of pathologies of mitosis, etc. The cytogenetic indicators of seed progeny of woody plants are affected by chemical pollutants [5, 13], radiation contamination [14] and heavy metals [4, 15, 16]. Cytogenetic processes under stress conditions in seed progeny of many woody plants are similar, which raises questions about their species specificity (or non-specificity) and dependence on external factors. At the same time, attempts to reveal the similarity and differences in the cytogenetic features of woody plants by the results of molecular genetic comparison were not undertaken.

Sequences of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA were used to create a phylogenetic hypothesis for distinguishing species of woody plants of the genus *Aralia* [17], refining the systemic position of *Rhododendron* [18], and other genera of the *Ericaceae* family [19]. Assessment of the molecular and genetic similarity of species is carried out not only for the purpose of studying phylogeny (based on comparison of DNA sequences) [20-24] and biogeography [25] but also in the works on biotechnology [26, 27], bioengineering [28], for distinguishing genetic markers [29], confirming the hybrid status and differences of donor parent forms in ornamental hybrids, including *Rhododendron* species [30]. Thus, an ISSR marker was used to separate groups of the same species according to various characteristics, for example, by the change in the activity of enzymes in response to chemical stress [31].

Rhododendron species as an object of research were chosen due to the fact that now these plants are actively studied from the point of view of molecular systematics [20, 22, 31, 32], genetic diversity [34-36] and chemosystematics [37, 38], whereas information on their cytogenetic features is sketchy [39] and is mainly limited to the assessment of ploidy [10, 11]. Detailed studies on the cytology of *Rhododendron canadense* were conducted (*Rhododendron canadense* (L.) Torr.) [12]. In modern classification for the species *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark, the subsection *Rhodorastrum* (Maxim.) Cullen, the section *Rhododendron*, and the subgenus *Rhododendron* [35, 40] are indicated. It is obvious that in order to establish genetic similarity and differences in the species of one subsection close in morphology, comparative studies of several groups of parameters are required to be conducted.

Reducing the number and the genetic diversity of rhododendrons in nat-

ural conditions also leads to a decline in the adaptive potential of plants; moreover, the difficulty in identifying the species of these beautifully flowering bushes by morphological features, in turn, makes it difficult to conserve the biodiversity of these species. Understanding the extent to which the molecular and genetic similarity causes cytogenetic reactions under introduction conditions will allow for a better understanding of the mechanisms of adaptation of plants to the habitat to maintain their biopotential under artificial conditions.

This paper is the first to analyze the ITS1-ITS2 sequences and compare these data and cytogenetic features of the *Dauricum* series of the subsection *Rhodorastrum* (Maxim.) Cullen (section *Rhododendron*, subgenus *Rhododendron*). As a result, species specificity of cytogenetic characteristics is revealed in the four examined rhododendron species when introduced under the conditions of Central Black Earth Region of Russia.

The aim of the research was to study the cytogenetic and molecular genetic features of rhododendron species under conditions, which are unusual for their natural growth.

Techniques. We used the seeds of bulk collection of four species of the genus *Rhododendron*, *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark., introduced in the B.M. Kozo-Polyansky Botanical Garden of Voronezh State University (geographical coordinates: 39°22' of northern latitude, 51°40' of eastern longitude, height above sea level of 168.2 m). The age of the analyzed plants is 30-35 years. A sample consisted of 5 plants of each species.

DNA was extracted form plant leaves using a CTAB buffer (1.5 M NaCl, 20 mM Na_3-EDTA, 100 mM HEPES pH 5.3, 25 °C, 1.5% CTAB) using the PrimerDigital Oy protocol (http://primerdigital.com/dna.html). The ITS1-ITS2 site was amplified with primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [41] synthesized in Eurofins MWG, Inc. (Germany) (https://www.eurofinsgenomics.eu/). To amplify the fragments of ITS1-ITS2, a standard protocol for Taq DNA polymerase was used. The reaction was executed in 25 μl reaction mixture containing 25 ng of DNA, 1× DreamTaq buffer (with 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.3 μM of each primer and 1 unit of DreamTaq DNA polymerase (Thermo Fisher Scientific, Inc., USA), in the MasterCycler Gradient amplifier (Eppendorf AG, Germany): initial denaturation at 95 °C for 3 min; 20 cycles — 15 s at 95 °C, 60 s at 60 °C, 30 s at 72 °C; the final elongation is 5 min at 72 °C.

The amplified fragments were separated by electrophoresis in a 1.5% agarose gel (RESolute Wide Range, Biozym Scientific GmbH, Germany); the products were visualized with ethidium bromide. To determine the length of DNA fragments, a molecular weight marker was used (Gene-Ruler DNA Ladder Mix, #SM0331, Thermo Fisher Scientific, Inc., USA).

DNA fragments were extracted from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Inc., USA). Ligation of fragments of PCR products into the plasmid T-vector pGEM-T (Promega, Inc., USA) was performed according to the manufacturer's protocol. *Escherichia coli* cells of the strain JM109 were transformed with plasmid DNA (Promega, Inc., USA). Cells bearing a plasmid with an insert were detected by blue-white selection on medium with ampicillin (at a final concentration of 100 μg/ml), a chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 20 mg/ml) and isopropyl-β-D-1-thio-galacto-pyranoside (IPTG, 200 mg/ml). Colony testing for the presence of an inserted PCR products cloned in the vector was performed by PCR with universal pUC primers (M13, forward and reverse).

Sequencing of amplified DNA fragments was provided by the DNA se-

quencing laboratory of the Institute of Biotechnology (University of Helsinki, http://www.biocenter.helsin-ki.fi/bi/dnagen/sequencing_service.htm). The capillary sequencer 3730xl DNA Analyzer (Thermo Fisher Scientific, Inc., USA) was used. ITS1-ITS2 data were obtained for the species *Rh. mucronulatum* Turcz., *Rh. dauricum* L., *Rh. ledebourii* Pojark. and *Rh. sichotense* Pojark in growing under conditions of the Central Black Earth Region. Cluster analysis of the nucleotide sequences and construction of a dendrogram were performed by ML method (Maximum Likelihood, Nearest-Neighbor-Interchange) with MEGA v.6 software [23, 24].

For cytogenetic studies, rhododendron seeds were germinated in Petri dishes at a room temperature. When the roots were 0.5-1 cm long, they were fixed (at 9 am) in aceto-alcohol (a mixture of 96% ethyl alcohol and glacial acetic acid, 3:1), and stored in the refrigerator at +4 °C. Sprouts rootlets were macerated in 18% HCl solution at 60 °C for 1-2 min, then washed for 15 min in 45% acetic acid, stained with aceto-hematoxylin for 1-1.5 hours, rinsed with distilled water. Hoyer's medium was used for squashed preparations. Slides (20 for each species of rhododendron) were viewed with a LABOVAL-4 microscope (Carl Zeiss, Inc., Switzerland) at a total magnification of 40×1.5×10.

In each slide (one slide corresponds to one root and one sprout), about 500-700 cells were examined. A total of about 42500 cells of the studied species of the genus *Rhododendron* were examined. The following cytogenetic parameters were analyzed: mitotic activity (the mitotic index MI is the ratio of dividing cells to the total number of cells counted, %), the percentage of cells across mitosis stages, the frequency of mitotic abnormalities as the percentage of cells with disturbances from the total number of dividing cells, the proportion of cells with residual nucleoli at the metaphase-anaphase mitosis stage as the percentage of cells with residual nucleoli from the total number of cells during the stage). Mitotic abnormalities were classified by I.A. Alov [42].

Data were processed with Stadia v.7.0 software package (http://toptorrent.ws/soft-torrent/4463-camtasia-studio-70.html, TechSmith Corporation, USA). The procedure for data grouping and processing is described by A.P. Kulaichev [43]. To express each cytogenetic index, the mean value was used with an average error (M±SEM). Samples were compared in terms of the frequency of mitotic abnormalities and the proportion of cells with residual nucleoli by the Van der Waerden score criterion, since the analyzed traits do not comply with a normal distribution. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$. Cluster analysis was performed using the metric of the normalized Euclidean distance and the nearest neighbor classification strategy. The following cytogenetic indicators of seed progeny were included in the data matrix for the cluster analysis: MI calculated with regard to the cell number at the prophase stage (%), MI calculated without taking into account the cells at the prophase stage (%), the percentage of cells in the prophase, metaphase, and anatelophase, the frequency of mitotic abnormalities (%) and the proportion of cells with residual nucleoli at metaphase-telophase (%).

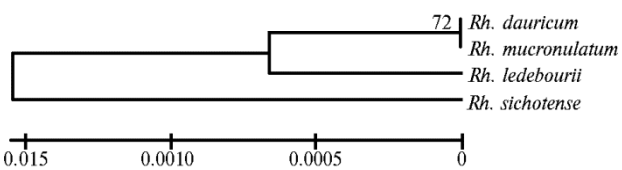

Fig. 1. Nucleotide differences in the ITS1-ITS2 sequence in species of the genus *Rhododendron* L. (Maximum Likelihood method, Nearest-Neighbor-Interchange, MEGA v.6).

Results. The data on analysis of ITS1-ITS2 sequences are presented on a dendrogram (Fig. 1). It is seen that *Rh. mucronulatum* and *Rh. dauricum* have an identical ITS1-ITS2 sequence. *Rh. ledebourii* differs from

Rh. mucronulatum and *Rh. dauricum* in 1 nucleotide of the ITS1-ITS2 sequences, *Rh. sichotense* and *Rh. mucronulatum*, *Rh. sichotense* and *Rh. dauricum* differ in 2 nucleotides. Differences between *Rh. ledebourii* and *Rh. sichotense* for the spacer ITS1-ITS2 is 3 nucleotides.

Tables 1 and 2 show cytogenetic indicators of the studied species of the genus *Rhododendron*.

Cell division is a highly canalized process [8]. In the species of the rhododendrons which were analyzed the parameters of mitosis varied (see Table 1).

1. Cytogenetic characterization of species of genus *Rhododendron* (*M*±SEM)

Species	MI, %		Cells, %		
	with account of prophases	without account of prophases	prophase	metaphase	ana-telophase
Rhododendron dauricum L.	7.6±0.3	3.9±0.2	48.9±2.0	13.3±1.4^{б}	41.7±2.3
Rh. mucronulatum Turcz.	7.7±0.7	4.9±0.7	37.5±1.9^{*в}	10.2±0.9**	51.9±1.4*
Rh. sichotense Pojark.	5.6±0.7^{*a}	3.0±0.4^{*a}	45.8±1.1	14.6±2.2	39.6±3.2^{**б}
Rh. ledebourii Pojark.	6.1±0.6*	3.8±0.4	37.9±1.9^{**г}	18.7±2.1*	43.4±1.7

N o t e. MI — mitotic index.

*, ** Differences with *Rhododendron dauricum* are statistically significant at $p < 0.05$ and $p < 0.01$.

^{a} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.05$; ^{b} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.01$; ^{c} — differences with *Rh. sichotense* are statistically significant at $p < 0.05$; ^{d} — differences with *Rh. sichotense* are statistically significant at $p < 0.01$.

2. Cytogenetic abnormalities in species of genus *Rhododendron* (*M*±SEM)

Species	Abnormal mitoses, %	Cells with residual nucleoli, %
Rhododendron dauricum L.	1.6±0.4	10.2±1.0
Rh. mucronulatum Turcz.	3.4±0.3	9.1±1.1
Rh. sichotense Pojark.	3.5±0.5	13.3±1.2^{a}
Rh. ledebourii Pojark.	5.2±1.1*	10.9±1.3^{в}

* Differences with *Rhododendron dauricum* are statistically significant at $p < 0.05$.

^{a} — differences with *Rh. mucronulatum* are statistically significant at $p < 0.05$; ^{b} — differences with *Rh. sichotense* are statistically significant at $p < 0.05$.

Thus, among the seed progeny, two groups can be distinguished in mitotic activity: the first is *Rhododendron dauricum* and *Rh. mucronulatum* with a high MI value in the root meristem of the seedlings (7.6±0.3% and 7.7±0.7%, respectively), and the second is *Rh. sichotense* and *Rh. ledebourii* with corresponding low indicators (5.6±0.7% and 6.1±0.6%). The analysis of cell distribution through the stages of mitosis showed that the examined species were grouped as follows. The number of cells in the prophase stage in *Rh. dauricum* (48.9±2.0%) and *Rh. sichotense* (45.8±1.1%) was significant, and in *Rh. mucronulatum* (37.5±1.9%) and *Rh. ledebourii* (37.9±1.9%), it was not high. An increased percentage of prophase cells indicates possible irregularities in the mitotic apparatus [44] and the activation of a checkpoint-control system for the integrity of the genetic material [45]. Such cytological responses can be explained by individual (species-specific) features of plants. *Rh. mucronulatum* and *Rh. dauricum*, which do not have differences in the ITS1-ITS2 sequence, are included in the group with high mitotic index values, estimated taking into account cells at the prophase stage. *Rh. ledebourii* and *Rh. sichotense*, differing from each other in the ITS1-ITS2 sequence, belong to the group with a lower mitotic index.

The number of cells in the metaphase of mitosis was maximal in *Rh. ledebourii* (18.7±2.1%) and *Rh. sichotense* (14.6±2.2%); the minimum was in *Rh. mucronulatum* (10.2±0.9%) and *Rh. dauricum* (13.3±1.4%). Delayed cells at the metaphase stage may indicate a violation of the spindle apparatus formation [42]. Grouping of species by the time of passage of the ana-telophase stage turned out to be different. The greatest proportion of cells at this stage is in *Rh. mucronulatum* (51.9±1.4%); in the other species, these values are significantly lower (see Table 1). An increase in the number of cells in the ana-telophase stage of mitosis indicates a violation of the formation of the cell wall [44].

The greatest cytogenetic instability is observed in *Rh. ledebourii* (5.2±1.1%, abnormalities of mitosis is maximal); in three other species, it was lower (from 3.5±0.5% in *Rh. sichotense* to 1.6±0.4% in *Rh. dauricum*) (see Table 2). It can be assumed that under the introduction conditions, the genetic system of *Rh. ledebourii* is the least adapted one. The pattern of irregularities was mainly represented by chromosome lagging in anaphase and metakinesis. Bridges were noted only in *Rh. mucronulatum* (25% of the total number of mitotic abnormalities) and *Rh. sichotense* (12% of the total number of mitotic abnormalities). A higher proportion of cells with a residual nucleolus at metaphase-telophase indicates a greater intensity of the synthetic processes [6] which are associated with the adaptation in the introduction conditions. According to this indicator, two groups were distinguished: the first one was formed by the species *Rh. sichotense* (13.3±1.2%) with a high percentage of cells with residual nucleolus in the metaphase-telophase stage of mitosis, the second one included *Rh. mucronulatum* (9.1±1.1%), *Rh. dauricum* (10.2±1.0%) and *Rh. ledebourii* (10.9±1.3%) with a smaller proportion of such abnormalities

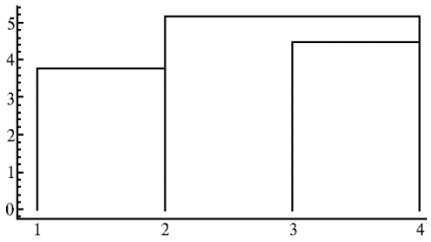

Fig. 2. Dendrogram of cluster distances between species of genus *Rhododendron* L. by cytogenetic characteristics: 1 — *Rh. dauricum*, 2 — *Rh. mucronulatum*, 3 — *Rh. sichotense*, 4 — *Rh. ledebourii* (the metric of the normalized Euclidean distance and the nearest neighbor classification strategy are used, the indicators included in the matrix of data see in the Techniques section).

Despite the difference in the cytogenetic indicators in the seed progeny of the studied species, a cluster analysis of the totality of characteristics of mitosis and nucleolar activity allowed us to distinguish two groups (Fig. 2): the first one is *Rh. mucronulatum* and *Rh. dauricum*, the second one is *Rh. ledebourii* and *Rh. sichotense*. The largest cluster distance is between the cytogenetic index totalities in *Rh. mucronulatum* and *Rh. sichotense*, the smallest ones between the species *Rh. mucronulatum* and *Rh. dauricum*. This agrees with the data we obtained in molecular studies which showed a similar grouping of these species. When introduced into the Central Black Earth Region, the parental plants of the examined species were in the same conditions (B.M. Kozo-Polyansky Botanical Garden of Voronezh State University); therefore, one can speak about the manifestation of the species-specificity of the cytogenetic indicators of their seed progeny. The data of our studies on the similarity of the cytogenetic characteristics of *Rhododendron* species agree with the results of M.B. Belousov et al. [37] on chemotaxonomy, morphology and anatomy, which showed the greatest morphological (secondary) similarity of the same species, explained by similar ecological conditions in the places of growth in the native habitat of species. It should be noted that, according to the molecular and cytogenetic characteristics, the species are grouped in a similar way. The cluster distance, estimated on the basis of studying the cytogenetic properties, between the species *Rh. mucronulatum* and *Rh. dauricum* is the smallest. These species have the same ITS1-ITS2 sequence; *Rh. ledebourii* differs from them by one nucleotide, *Rh. sichotense* by two nucleotides, which corresponds to a greater cluster distance between cytogenetic parameters. The difference between *Rh. ledebourii* and *Rh. sichotense* for the spacers ITS1-ITS2 is three nucleotides. However, the difference in the ITS sequence of 1-3 nucleotides does not play an important role in determining the molecular genetic characteristics. Thus, the similarity of the nucleotide sequence in the species of the genus *Rhododendron* determines their greater similarity according

to the totality of cytogenetic features.

Thus, the identity of the nucleotide sequence of the spacer ITS1-ITS2 in the species of genus *Rhododendron* of the *Dauricum* series leads to their greater similarity in the cumulative set of cytogenetic indicators. However, in the species having the identical ITS1-ITS2 sequence, cytogenetic characteristics do not completely coincide. Based on the comparison we have perforemed it can be assumed that genetic homogeneity in the studied members of the genus *Rhododendron* of the *Dauricum* series causes the similarity of cytogenetic properties. The cytogenetic characteristics of the seed progeny in the examined forms are species-specific.

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