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Polyploidy and hybridization in *Spiraea* (Rosaceae): Cytogenetic insights from the hybrid *Spiraea hypericifolia* × *S. media* and its parental species in Tuvinian populations

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***Keywords**:* allopolyploidy, autopolyploidy, chromosome numbers, genome size, hybrids, *Spiraea hypericifolia, Spiraea hypericifolia* × *Spiraea media*, *Spiraea media,* Tuva.

Summary. The polyploidy and hybridization in *Spiraea* evolution make species identification a complex process. The cytogenetic characteristics of hybrid individuals of *Spiraea hypericifolia* × *S. media*, along with those of their parental species, *S. hypericifolia* L. and *S. media* Fr. Schmidt, have been the subject of study. The study presents findings of the newly discovered locations of the hybrid *S. hypericifolia* × *S. media* in the Republic of Tyva. This is the first study to examine the relative DNA content of *S. hypericifolia* × *S. media* individuals. Genomic analysis revealed considerable variability in total genome size, ploidy level and chromosome number among *Spiraea* from three mixed Tuvinian populations. The results of the cytotyping by chromosome counting and genome size determination by flow cytometry were found to be consistent. *Spiraea hypericifolia* was found to exhibit a strictly diploid level ($2n = 2x = 18$). In *S. media*, three euploid levels were identified for the: diploid ($2n = 2x = 18$), autotriploid ($2n = 3x = 27$), autotetraploid ($2n = 4x = 36$), as well as an aneuploid ($2n = 24$). Additionally, mixoploidy is occasionally observed in the *S. media* species. Among the hybrid individuals of *S. hypericifolia* × *S. media*, the expected allodiploids (Tselinnoye) and allotetraploids (Turan, Tapsa) were identified. Hypotheses regarding the appearance of autotetraploid of *S. media* and allodiploid and allotetraploid hybrids of *S. hypericifolia* × *S. media* have been proposed. The most probable mechanism of origin of polyploid and hybrid *Spiraea* species is the disruption of meiosis with the formation of $2n$ gametes and/or somatic endomitosis. Our findings contribute to understanding of microevolutionary processes within the genus *Spiraea*. The hybrid *S. hypericifolia* × *S. media* has significant potential for use in landscape design and breeding and thus requires comprehensive study.

Полиплоидия и гибридизация в роде *Spiraea* (Rosaceae): цитогенетические данные по гибриду *Spiraea hypericifolia* × *S. media* и его родительским видам в популяциях из Тувы

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***Ключевые слова*:** аллополиплоидия, аутополиплоидия, гибриды, размер генома, Тува, число хромосом, *Spiraea hypericifolia, Spiraea hypericifolia* × *Spiraea media, Spiraea media*.

Аннотация. Полиплоидия и гибридизация в роде *Spiraea* усложняют идентификацию видов. Приводятся цитогенетические характеристики гибридных особей *Spiraea hypericifolia* × *S. media*, а также его родительских видов – *S. hypericifolia* L. и *S. media* Fr. Schmidt. Представлены новые местонахождения гибрида *S. hypericifolia* × *S. media* в Республике Тыва. Это первое исследование по изучению относительного содержания ДНК у особей *S. hypericifolia* × *S. media*. Цитогенетический анализ выявил значительную вариабельность как общего размера генома, так и уровня плоидности и числа хромосом у *Spiraea* из трёх смешанных тувинских популяций. Результаты цитотипирования путём подсчёта хромосом и определения размера генома методом проточной цитометрии согласуются между собой. У *S. hypericifolia* обнаружен только диплоидный уровень ($2n = 2x = 18$). У *S. media* выявлено три эуплоидных уровня: диплоидный ($2n = 2x = 18$), триплоидный ($2n = 3x = 27$), тетраплоидный ($2n = 4x = 36$), а также анеуплоидный ($2n = 24$). Кроме того, у *S. media* изредка наблюдается миксоплоидия. Среди гибридных особей *S. hypericifolia* × *S. media* были выявлены предположительно аллодиплоиды (Целинное) и аллотетраплоиды (Туран, Тапса). Предложены гипотезы возникновения автотетраплоидов среди *S. media* и аллодиплоидных и аллотетраплоидных гибридов *S. hypericifolia* × *S. media*. Наиболее вероятным механизмом происхождения полиплоидных и гибридных особей *Spiraea* является нарушение мейоза с образованием $2n$ гамет и/или соматический эндомитоз. Полученные нами результаты способствуют пониманию микроэволюционных процессов в роде *Spiraea*. Гибрид *S. hypericifolia* × *S. media* имеет значительный потенциал для использования в ландшафтном дизайне и селекции, поэтому требует всестороннего изучения.

Introduction

Spiraea L., a member of the tribe *Spiraeeae* DC., of the subfamily *Amygdaloideae* Arn. (Rosaceae Juss.), comprises about 90 species according to "Plants of the World Online" (POWO. URL: https://powo.science.kew.org/) and is widespread in the temperate and subtropical zones of the northern hemisphere (Choi et al., 2019; Zhang et al., 2023). The genus *Spiraea*, like other numerous Rosaceae species, is of great horticultural significance (Laczkó et al., 2024).

The process of interspecific hybridization plays a significant role in the evolutionary history of plants. On the one hand, it favours the emergence of new genetic combinations, improves adaptation and, in some cases, promotes stable natural populations (Soltis P. S., Soltis D. E., 2000; Rieseberg, Willis, 2007); on the other hand, it can lead to outbreeding depression (Lynch, 1991; Whitlock et al., 2013).

One of the principal factors contributing to the success of the Rosaceae is the occurrence of agamospermy, hybridization, polyploidy, and vegetative reproduction (DeVore, Pigg, 2007). A genome sequence analysis of Rosaceae members suggests that this family may have experienced at least one or a few whole-genome duplications (WGDs) (Dickinson et al., 2007); however, a recent phylogenomic analysis of Rosaceae yielded strong evidence for numerous WGDs (Xiang et al., 2017; Laczkó et al., 2024). As for *Spiraea*, genome duplications occurred both within the genus and at the tribe level, between genera (Vamosi, Dickinson, 2006; Dickinson et al., 2007; Xiang et al., 2017). Duplications of the whole genome are associated with ancient hybridization in *Amygdaloideae* s. l., which complicates the understanding of evolutionary history of the tribe *Spiraeeae* (Morgan et al., 1994; Potter et al., 2007; Laczkó et al., 2024). Hybridizations, incomplete lineage sorting and other possible factors might contribute to the uncertainty of the phylogeny of the tribe (Xiang et al., 2017). The presence of polyploidy and hybridization also has a considerable impact on the systematics of *Spiraea*, which in turn makes the process of species identification a challenging polyploidy (Poliakova, 2022). It is not uncommon for *Spiraea* species to be engaged in interspecific and intraspecific hybridization in their natural habitats. However, this phenomenon is still documented mostly on the basis of phenotypic traits (Poliakova et al., 2022). An exception to this is the triploid natural hybrid *S.* × *hitchcockii* W. Hess et N. Stoynoff that was described in 1999 from Oregon (Hess, Stoynoff, 1999), confirmed on the basis of morphology, chromosome count ($2n = 3x = 27$) and determination of pollen viability (low, 2–14 %).

During field research conducted in the forest-steppe regions of Tuva in 2023, we discovered mixed populations of *Spiraea*. In close proximity,

pure species of *S. hypericifolia* L. and *S. media* Fr. Schmidt, as well as individuals with either intermediate morphological characteristics of leaf blades and habit, or looking to one of the parents, were observed. The putative parental species are well differentiated morphologically. The likelihood of interbreeding between these species was previously reported once for the Krasnoyarsk Territory (Polozhii, 1988). Artificial hybrids of *S. hypericifolia* × *S. media* species named *S.* × *micropetala* Zabel were bred by controlled crossing of *S. hypericifolia* and *S. media* by German dendrologist and gardener Hermann Zabel in the 1880s (Zabel, 1884, 1893; Govaerts et al., 2021). Nevertheless, there are doubts that *Spiraea hypericifolia* was taken as one of the parent species (Schneider, 1906). However, no living plants from his collection have survived to this day (personal correspondence with curators of collections in foreign botanical gardens and herbaria). Therefore, in this study, we do not use the hybrid name *S.* × *micropetala*.

The lack of information on the hybrid *S. hypericifolia* × *S. media*, the frequent errors and difficulties in the identification of *Spiraea* spp., especially by herbarium specimens, which are a common occurrence, indicate the necessity for comprehensive cytogenetic and molecular genetic studies of hybrid populations. This is the first part of a study on *S. hypericifolia* and *S. media*, representatives of the evolutionarily young section *Chamaedryon* Ser., and their hybrids, first discovered in Tuva. Both parental species are typically diploid ($2n = 2x = 18$) and exhibit coincident phenological phases, which facilitates cross-pollination and hybridization.

Spiraea hypericifolia is an East European-West Asian forest-steppe species; it is found in the south-east of the European part of Russia, the Caucasus, Siberia, Central Asia, and western Mongolia. In Tatarstan, this species is at the extreme limit of its distribution. *Spiraea hypericifolia* represents an outcrossing species and is propagated exclusively by seeds, which are easily dispersed by the wind. In the westernmost regions of its range, the species can be considered of special conservation interest as a relic of steppe forests (Attila Molnár et al., 2017).

Spiraea media is common in the temperate zone of Eurasia. It grows wild in northeastern Russia, southern Siberia, the Far East and Central Asia. It is also found in Europe, Mongolia, China, Korea and Japan (Polyakova, Muratova, 2015). *Spiraea media*, in addition to sexual reproduction, often reproduces also through self-pollination and propagates vegetatively (Poliakova, 2022). No information on the occurrence of polyploidy in *Spiraea media* is available.

Polyploidy and genomic rearrangements that are appeared during hybridization can be detected through the estimation of genome size and the determination of chromosome number in different populations in individuals, including plant hybrids. This contributes to a better understanding of the evolutionary and speciation mechanisms involved (Bourge et al., 2018). Cytogenetic studies have not yet provided a comprehensive account of the diversity observed in the genus *Spiraea*. The nuclear genome size of the majority of *Spiraea* species remains poorly understood, and the chromosome numbers of many species are still underrepresented. The study of *Spiraea* genome variability is of great importance for the understanding of its evolutionary history, taxonomy and population genetics, as well as for the identification of valuable phenotypes and genotypic groups for breeding programs.

Thus, the aim of this work is to study ploidy levels in hybrid individuals of *S. hypericifolia* × *S. media* and parental species, *S. hypericifolia* and *S. media*, in Tuva natural populations using a set of cytogenetic approaches. The following objectives were set in this study: (1) to determine chromosome numbers, (2) to measure genome sizes by flow cytometry, (3) to identify ploidy levels through integrated cytogenetic data, and (4) to propose hypotheses for origin of polyploidy and hybrid formation in mixed populations.

Materials and methods

Plant material. The plant material used in this study was collected by T. Poliakova during fieldwork between the dates of 2 and 12 July 2023 in the Republic of Tyva (Figs. 1, 2). The populations were located in the Kyzyl and Piy-Khem districts (kojuuns). A total of 139 individual plants from three natural populations were sampled (Table 1). The herbarium was collected and the seeds were selected. The seedlings were grown from seeds of plants collected in Tuva. The samples for analysis by flow cytometry were fresh young leaves of seedlings of *S. hypericifolia*, *S. media* and their hybrids, which exhibited no visible signs of disease or pest infestation. The herbarium specimens (for which the relevant voucher numbers are listed in Table 1) were prepared for long-term storage and are currently being stored in the Laboratory of Population Genetics of the Vavilov Institute of General Genetics,

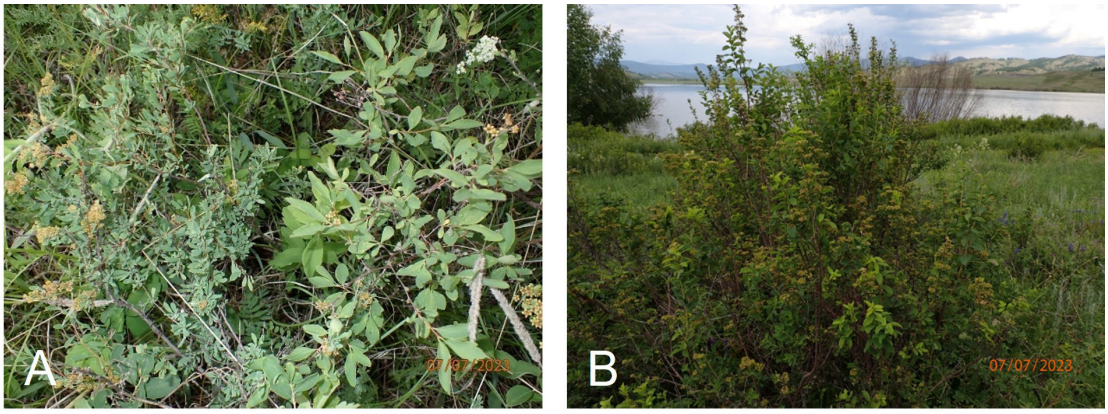

Fig. 1. *Spiraea* shrubs in Turan location (Tuva): A – *Spiraea hypericifolia* (left) and *S. hypericifolia* × *S. media* (right); B – *S. media.*

Fig. 2. The hybrid *S. hypericifolia* × *S. media* from Tapsa location.

Table 1. Habitat and other information on the studied *Spiraea* populations

No.	Location	Latitude, longitude	Coenosis	Population name (number of individuals)	VIGG vouchers
1	Kyzylsky Kojuun, village of Tselinnoye	51°19′37″N, 94°51′14″E	rocks of dwarf hills, shrubby thickets	Tselinnoye (52)	VS11923, VS11723, VS13023
2	Piy-Khemskiy Kojuun, Turan city vicinity, Bilelig River	52°16′14″N, 93°52′43″E	spirea larch, near the river	Turan (34)	VS41923, VS41723, VS43023
3	Kyzylsky Kojuun, Tapsa River	51°54′33″N, 94°39′52″E	larch-birch-aspen forest, shrubby thickets	Tapsa (53)	VS71923, VS71723, VS73023

Russian Academy of Sciences (VIGG, Moscow). The morphological identification of the herbarium specimens was conducted. Samples exhibiting intermediate morphological characteristics were identified as hybrids and subsequently confirmed through molecular genetic analysis (Poliakova et al., in prep.).

Chromosome counting. The method described by Smirnov (1968) was used for chromosome counting. The seeds were germinated on moist filter paper in Petri dishes at room temperature. Because of the small chromosome size and difficulties in obtaining well-distributed metaphases from root meristems, meristems from shoot tips and young true leaves were used for analysis. Before fixation, the germinated material was cold treated for 12–24 h at 0–1 °C. Fixation was carried out in acetoethanol (1 : 3). Before staining, the material was treated with 4 % iron-ammonium alum for 15–20 minutes and then stained with acetohematoxylin. The ground preparations were prepared in 45 % acetic acid by pressing. The slides were examined under the microscope "Biomed-2" (LLC "Biomed-Service", Moscow, Russia) under bright illumination and 1600× magnification.

DNA content by flow cytometry. The DNA content in plant nuclei was determined by flow cytometry (FCM), in accordance with established protocols (Doležel, Bartoš, 2005; Temsch et al., 2022; Fomicheva, Domblides, 2023; Skaptsov et al., 2024). The analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter, USA, cat. no. B53017) equipped with a 488 nm laser. Nuclei were stained with propidium iodide (PI) and fluorescence was measured to quantify DNA content. Leaf plates (0.5 cm^2) were cut from young seedling leaves (Meng, Finn, 2002) and chopped with a sharp razor blade together with an internal standard in 500 µl of ice-cold Galbraith buffer (45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1 % (v/v) Triton X-100, pH = 7.0) with the addition of 50 µg/mL RNase I (Syntol, Russia). *Solanum lycopersicum* 'Stupické', 2C = 2.077 pg or *Arabidopsis thaliana* ecotype Col-0, 2C = 0.366 pg seedling true leaves were used as an internal standard. The corrected standard values were derived from the study that directly compared a number of commonly used cytometry standards (Skaptsov et al., 2024). After chopping the sample was filtered through a 30 µm filter (Miltenyi Biotec, 130-041-407). PI was added to the sample to reach the final concentration 50 µg/ml. After two-minute incubation on ice, the sample was analyzed on a CytoFLEX flow cytometer. Due to the limited amount of material, 100–650 nuclei were recorded for each sample. Deviations between measurements did not exceed 3 %. The absolute DNA content was calculated using the G1 peaks in the sample and the internal reference standard. The following formula was used for calculations: Sample DNA content = Reference DNA content × (Sample average of peak G_0/G_1 / Reference average of peak G_0/G_1) (Doležel, Bartoš, 2005). The ploidy of the sample was determined based on the interpretation of the DNA content of samples with known ploidy.

Statistical analysis. The FCM data were analyzed using the Statistica software (StatSoft, Inc., 1984–2001). The Kruskal-Wallis test, a non-parametric analogue of ANOVA, was employed to assess the differences in relative DNA content between samples. This test was selected due to the non-normal distribution of the data, as confirmed by the Shapiro-Wilk test. The analyses were based on measurements from at least three biological replicates (seedlings)

and one-three technical replicates per plant measured at different days. A total of 8–9 samples were analyzed for each species/population except for *S. hypericifolia* that had 4 samples analyzed in total. All statistical analyses were performed at a significance level of 0.05, and the influence of inter-replicate variability was controlled to ensure that observed differences did not exceed 2 %.

Results

Chromosome counts and ploidy levels

The number of seedlings studied per population ranged from 31 to 60, resulting in a total of 132 seedlings (Table 2). However, not all plants bore viable seeds. In the bushes of *S. hypericifolia* (Tselinnoye, Turan), *S. media* (Turan), and *S. hypericifolia* × *S. media* (Tapsa), the seeds were either absent or failed to germinate. The meristem cells of the shoot apex and leaf meristem of the Tuva populations under study exhibited the presence of diploids, triploids, and tetraploids ($2n = 2x = 18$, $2n = 3x = 27$, $2n = 4x = 36$), as illustrated in Fig. 3. The results of our studies indicate that all seedlings in the Tapsa population of *S. hypericifolia* exhibit a single ploidy level, designated as $2n = 2x = 18$. *Spiraea media* and the hybrid *S. hypericifolia* × *S. media* have been observed to exhibit three distinct ploidy levels, ranging from diploid ($2n = 2x = 18$) to tetraploid ($2n = 4x = 36$). The majority of cell fractions are diploid ($2n = 2x = 18$), representing 79 % of the total. Tetraploid cells represent 11 % of the total. The number of aneuploid cells ($2n = 24$, 1.6 %) is negligible (Table 2).

All cells of the *S. media* species in the Tselinnoye population were observed to be diploid. In the Tapsa *S. media* population, the number of diploid and triploid fractions was approximately equal, while tetraploid and aneuploid cytotypes were also observed.

Genome size variability

This is the first study to examine the relative DNA content of *S. hypericifolia* × *S. media* individuals. *Spiraea* and internal standard isolated nuclei were isolated, stained with PI and analyzed using the flow cytometer. *Spiraea media, S. hypericifolia* and *S. hypericifolia* × *S. media* (Tselinnoye, Turan) were compared to internal standard *Solanum lycopersicum* (2C = 2.077 pg) (Skaptsov et al., 2024) (Fig. 4A–D). These data showed that *S. hypericifolia* × *S. media* (Tselinnoye) has genome size similar to *S. media* and *S. hypericifolia,* while *S. hypericifolia* × *S. media* (Turan) has two times larger genome. However, the *Solanum lycopersicum* genome is four times bigger than the genome of *S. media, S. hypericifolia* and *S. hypericifolia* × *S. media* (Tselinnoye), but the recommended genome size difference for the standard and the sample is no more than three times (Sliwinska et al., 2021; Skaptsov et al., 2024). Therefore, *Arabidopsis thaliana* (2C = 0,366 pg) (Skaptsov et al., 2024) was used to validate the results obtained for those species. Similar 2C results were obtained with *A. thaliana* standard as with *S. lycopersicum* (Fig. 4E–G). *A. thaliana* was used for further studies to collect the statistical data for three diploid samples, while *S. lycopersicum* was used for the tetraploid hybrid *S. hypericifolia* × *S. media*

Table 2. Variation of chromosome numbers in the examined cells of root and leaf meristem in *Spiraea hypericifolia, S. media, S. hypericifolia* × *S. media*

Sample origin	Of those with the number of chromosomes (%)			
	$2n = 2x = 18$	$2n = 3x = 27$	$2n = 4x = 36$	$2n = 24$ (aneuploidy)
S. media, Tselinnoye	20 (100)	0	0	0
S. media, Tapsa	7 (44)	6 (37)	2 (13)	1 (6)
S. hypericifolia, Tapsa	25 (100)	0	0	0
S. hypericifolia × *S. media*, Tselinnoye	39 (98)	0	0	1 (2)
S. hypericifolia × *S. media*, Turan	13 (42)	6 (19)	12 (39)	0
Total cells (%)	104 (79)	12 (9)	14 (10.5)	2 (1.5)

Note: 0 – no such cytotype was found in this population.

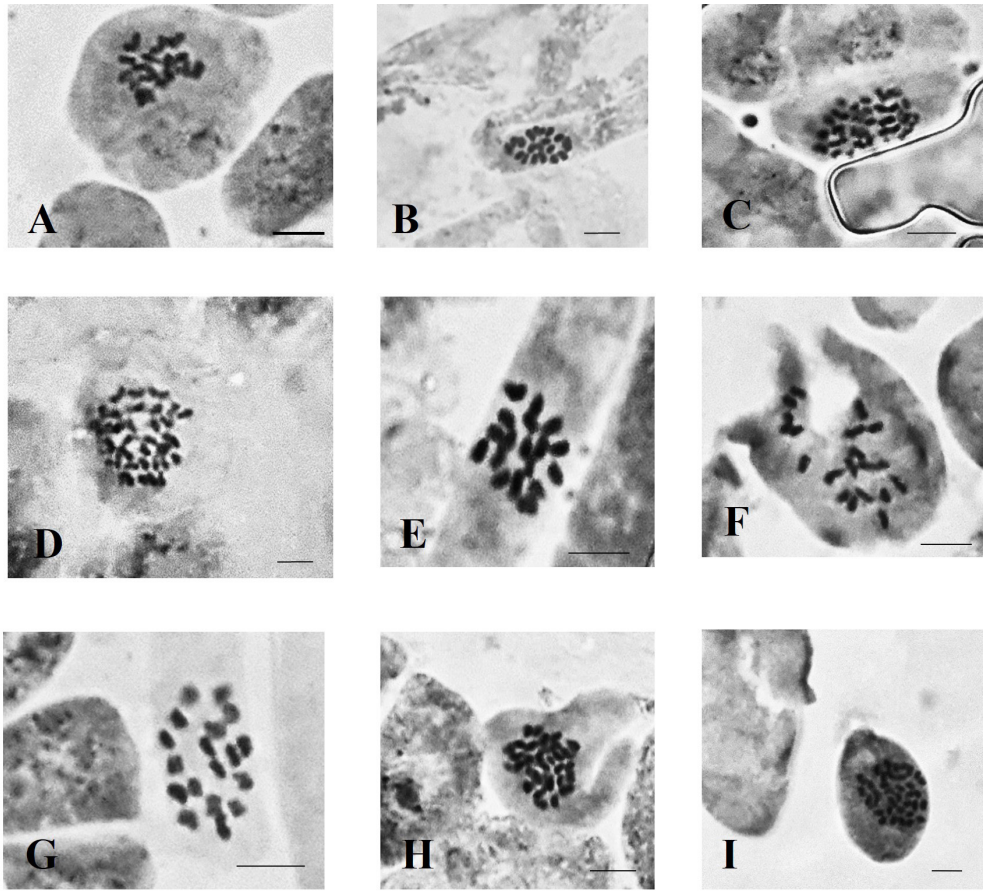

Fig. 3. Somatic chromosomes in the metaphase of *Spiraea hypericifolia*, *S. media*, *S. hypericifolia* × *S. media* at different ploidy levels: A – *S. hypericifolia* (2*n* = 2x = 18), Tapsa; B – *S. media* (2*n* = 2x = 18), Tapsa; C – *S. media* (2*n* = 3x = 27), Tapsa; D – *S. media* (2*n* = 4x = 36), Tapsa; E – *S. media* (2*n* = 2x = 18), Tselinnoye; F – *S. hypericifolia* × *S. media* (2*n* = 2x = 18), Tselinnoye; G – *S. hypericifolia* × *S. media* (2*n* = 2x = 18), Turan; H – *S. hypericifolia* × *S. media* (2*n* = 3x = 27), Turan; I – *S. hypericifolia* × *S. media* (2*n* = 4x = 36), Turan. The scale bar is 10 μm.

(Turan) DNA content calculations (Table 3). The 2C DNA content equals 0.513 ± 0.006 pg in *S. media* and 0.521 ± 0.005 pg in *S. hypericifolia*. Two levels of ploidy were shown in hybrid populations of *S. hypericifolia* × *S. media*: diploid, with 0.516 ± 0.006 pg 2C DNA content in the Tselinnoye population, and tetraploid, with 1.049 ± 0.019 pg 2C DNA content in the Turan population (Table 3). The average calculated Cx values are as follows: 251 Mb in *S. media,* 255 Mb in *S. hypericifolia,* 252 Mb in *S. hypericifolia* × *S. media* (Tselinnoye), 256.5 Mb in *S. hypericifolia* × *S. media* (Turan).

Discussion

Chromosome counts and ploidy levels

Chromosome number and nuclear genome size are among the most important markers for the study of polyploid plants (Bennett, Leitch, 2005). At present, the chromosome numbers of approximately 30 taxa within the genus *Spiraea* are known according to "Plant Chromosome Number Index database" (URL: https://www.tropicos.org/Project/IPCN). *Spiraea* is distinguished by a higher level of ploidy (Sun et al., 1997) with multiple polyploid forms described in a number of species (Polyakova, Muratova, 2015). A majority of *Spiraea* species are diploid or tetraploid, as indicated by cytological analyses (Singhal et al., 1990; Poliakova, Shatokhina, 2021; Shatokhina et al., 2022). Some species of *Spiraea* demonstrate intraspecific variability in chromosome number. For example, different populations of *S. media* are represented by diploid, polyploid and aneuploid cytotypes (Polyakova, Muratova, 2015; Shatokhina et al., 2022).

S. hypericifolia has been less extensively studied from a karyological perspective than *S. media*. The present study corroborates previous findings that the number of somatic chromosomes in *S. hypericifolia* is consistently $2n = 2x = 18$ in specimens from Kazakhstan, Daghestan (Poliakova, Shatokhina, 2021) and Tuva (present study).

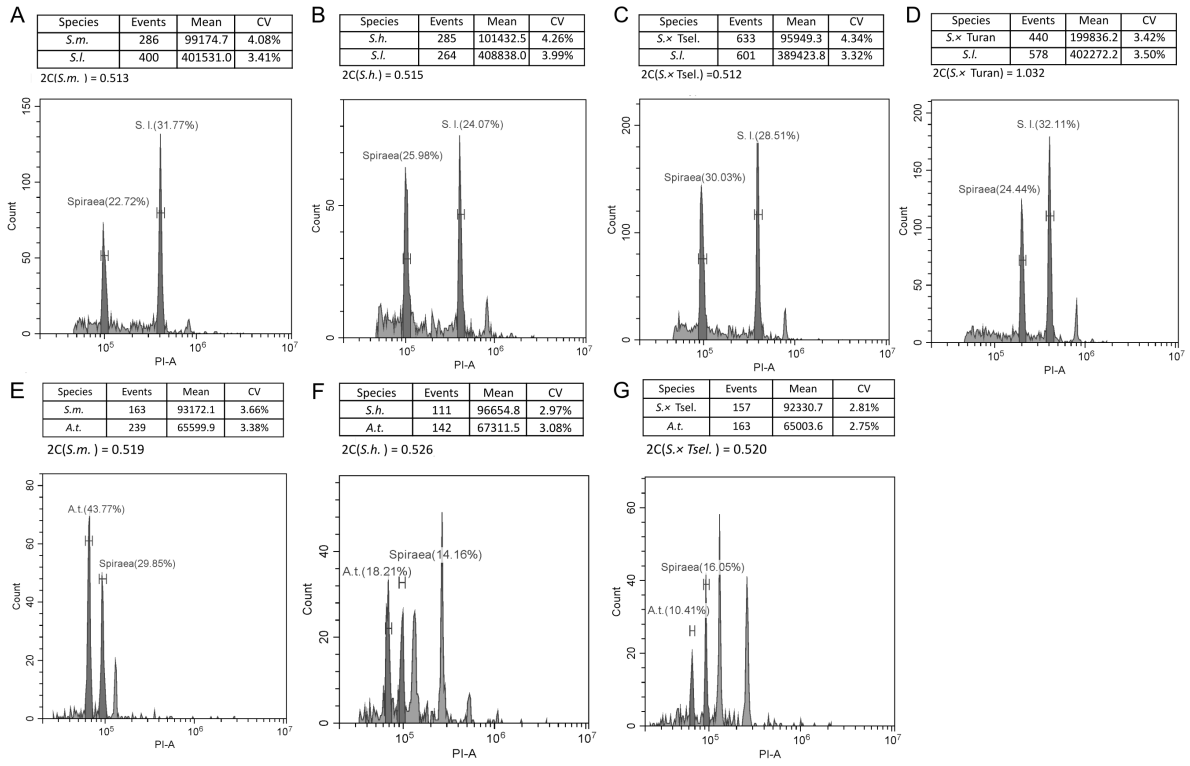

Fig. 4. Histograms of PI fluorescence intensity (logarithmic scale) in the nuclei of young leaves of *Spiraea* from Tuva population samples and internal standard. *Solanum lycopersicum* 'Stupické' (*S. l.*), 2C = 2.077 pg or *Arabidopsis thaliana* ecotype Col-0 (*A. t.*), 2C = 0.366 pg were used in the study. The tables show the number of nuclear events, Mean and CV for the sample and the standard, as well as calculated 2C for the demonstrated sample. A – *S. media* (*S. m.*), Tselinnoye with *S. l.* standard; B – *S. hypericifolia* (*S. h.*), Tapsa with *S. l.* standard; C – *S. hypericifolia* × *S. media* (*S.*×), Tselinnoye with *S. l.* standard; D – *S. hypericifolia* × *S. media*, Turan with *S. l.* standard; E – *S. media*, Tselinnoye with *A. t.* standard; F – *S. hypericifolia*, Tapsa with *A. t.* standard; G – *S. hypericifolia* × *S. media*, Tselinnoye with *A. t.* standard. Light grey peaks in E–G are from dividing and endopolyploid cells in *Arabidopsis thaliana* seedlings.

Table 3. The nuclear DNA content in *Spiraea hypericifolia*, *S. media, S. hypericifolia* × *S. media* populations

Species, origin	Average 2C DNA content ± SD*, pg	1C**, average, Mb	1Cx, Mb	Number of analyzed samples***
S. media, Tselinnoye	0.513 ± 0.006	251	251	9
S. hypericifolia, Tapsa	0.521 ± 0.005	255	255	4
S. hypericifolia × *S. media,* Tselinnoye	0.516 ± 0.006	252	252	8
S. hypericifolia × *S. media,* Turan	1.049 ± 0.019	513	256.5	8

Note: * SD – standard deviation; ** 1 pg of DNA = 978 Mb according to Doležel et al. (2003), *** at least 3 biological repeats (different seedlings from the same parent), 2–3 measurements at different days for *S. media* and hybrids. Three biological repeats were used for *S. hypericifolia*; one plant was measured twice at different days.

The widespread species *S. media* being the subject of more extensive research (Polyakova, Muratova, 2015) was shown to be a heteroploid species, that is, a species comprised forms with different ploidy level. *Spiraea media* is distinguished by a notable diversity of cytotypes, encompassing diploid, triploid, and tetraploid variants, as well as aneuploid cytotypes. This observation corresponds to findings of previous studies in this field (Oginuma et al., 2004; Polyakova, Muratova, 2015). In this species, along with the most common diploid, $2n$ = 18 (Novosibirsk Region, Irkutsk Region, Zabaikalye Territory, Amur Region, Yakutia, Sakhalin) chromosome number, tetraploid, $2n = 36$ (Siberia), triploid, $2n = 27$ (Amur Region) and aneuploid, $2n$ = 24, fractions (Tuva) are also present in meristematic cells. In addition, mixoploidy was also detected in some cells of *S. media* (Poliakova, Shatokhina, 2021; Shatokhina et al., 2022, 2024).

***Spiraea* genome sizes and ploidy levels**

As an effective method of establishing genome size and ploidy FCM is helpful in studying species taxonomy, evolution and ecology, as well as being invaluable for breeding polyploid and hybrid varieties (Sliwinska, 2018). Published genome size data for *Spiraea* species are limited, as evidenced by the major plant genome size databases: according to "FLOWer, a plant DNA flow cytometry database" (FLOWer. URL: https://botany.natur.cuni.cz/flower/) (Loureiro et al., 2008), and "Kew Plant DNA C content database" (URL: https://data.kew.org/cvalues) (Bennet, Leitch, 2011; Leitch et al., 2019). The C-values of genera in the subfamily *Spiraeoideae* are among the smallest observed among flowering plants (Dickson et al., 1992). In comparison to the genomes of other angiosperms, which exhibit a range of sizes from 1C = 0.0648 pg in *Genlisea margaretae* to 1C = 152.23 pg in *Paris japonica* (Bennet, Leitch, 2005; De Storme, Mason, 2014), the genomes of *Spiraea* species are relatively small. It has been previously documented that the genome sizes of *S. chinensis* and *S. sargentiana* span a range from the smallest 2 C-value of 0.42 pg to the largest 1.84 pg, respectively (Dickson et al., 1992; Bennett et al., 2011; Kostikova et al., 2018). It is assumed that this fourfold difference corresponds to the transition from diploid to octoploid ploidy levels.

The number of chromosomes, as well as the size of the genome, is a characteristic of the species (Bennett, Leitch, 2005). This is the inaugural experimental confirmation of the correlation between relative DNA content and ploidy level in *Spiraea*, based on direct chromosome counts and flow cytometry measurements conducted on the same samples. Similarly, this correlation has been demonstrated to be applicable to other plant species. For instance, the fluorescence of *Rubus* genotypes whose ploidy ranged from diploid to dodecaploid was observed to increase with increasing chromosome number (Meng, Finn, 2002).

The range of ploidy levels identified in *S. hypericifolia* × *S. media* indicates that this hybrid exists at a minimum of two distinct ploidy levels. Furthermore, the results demonstrate that the tetraploid populations of *S. hypericifolia* × *S. media* (Turan, Tapsa) exhibit a relative DNA content (2C) that is twice that of the diploid populations (0.52 pg) (Tselinnoye) (Table 3).

A twofold difference in genome size has been documented for the *S. media* species. The genome size of *S. media* has been observed to range from 0.45 pg (in the Amur Region, Mogot Village) to 0.98 to 1.01 pg in Siberian populations (Kostikova et al., 2019). It is evident that such discrepancy in genome size within a single species is attributable to disparate ploidy levels. Nevertheless, the authors did not undertake a chromosome count of the samples under study.

Hypotheses for the origin of polyploids and hybrids of *Spiraea media* and *S. hypericifolia* × *S. media*

Polyploidy, or whole-genome duplication, is a pervasive phenomenon, occurring with high frequency and recurrence within plant species (Tamayo-Ordonez et al., 2016; Van de Peer et al., 2017; Clo et al., 2022). It is an active and ongoing process that has been identified as a significant factor in the diversification of plant species (Bennett, Leitch, 2005; Alix et al., 2017; Pelé et al., 2018; Doyle, Coate, 2019). Its impact extends to plant evolution and ecology (Van de Peer et al., 2017). On the other hand, according to the fundamental hypothesis of Susumu Ohno (1970), with paralogues arising from both tandem duplications and polyploidization, evolution is free to experiment, and this is the basis for progressive evolution.

Polyploidy is a typical phenomenon for many species and genera of the family Rosaceae (Vamosi, Dickinson, 2006; Dickinson et al., 2007). Polyploidy represents a pivotal mechanism influencing the evolutionary trajectory and adaptive capacity of the genus *Spiraea* (Sax, 1936; Byung-Yun et al., 1997; Poliakova, 2022). The karyotype of the tribe *Spiraeeae* represents a group of angiosperms of hybrid origin (Morgan et al., 1994; Evans, Dickinson,

1999; Potter et al., 2007). It plays a significant role in the formation of genetic diversity, adaptation to different environmental conditions and evolutionary success of representatives of the genus *Spiraea*.

The findings of autotetraploid specimens of *S. media*, as well as allodiploid and allotetraploid hybrids of *S. hypericifolia* × *S. media* in mixed natural populations of Tuva provide an opportunity to discuss the probable genetic mechanisms of polyploidy in the genus *Spiraea*.

Polyploids are formed through a wide range of mechanisms, including the formation of unreduced gametes or doubled somatic cells (Doyle, Coate, 2019). These mechanisms are important for accelerating the adaptive evolution of the plant genome and speciation (De Storme, Mason, 2014). Three principal cytological mechanisms are recognized as responsible for ploidy change. These are: (1) meiotic non-reduction and the formation of 2*n* gametes; (2) somatic genome duplication; and (3) minor karyotype changes resulting from aneuploidy and/or dysploidy (De Storme, Mason, 2014; Sattler et al., 2016).

Diploid plants, including hybrids, produce unreduced gametes at relatively high rates, which contribute to the formation of numerous polyploid lineages (Alix et al., 2017). In instances of bilateral meiotic non-reduction, the fusion of two diploid gametes results in the formation of a tetraploid individual. Depending on the prevailing selection conditions, this can lead to the *de novo* establishment of a stable tetraploid lineage (De Storme, Mason, 2014). In instances of unilateral sexual polyploidization, a single diploid gamete fuses with a normal haploid gamete, resulting in the formation of a triploid embryo (Ramsey, Schemske, 1998). Although triploid seeds are often non-viable due to imbalances in the dosage of the parental genome, this triploid block is sometimes incomplete or absent, thereby allowing triploid plants to form (Kohler et al., 2010; De Storme, Mason, 2014). The meiotic cell division of these triploids is typically highly unbalanced, resulting in the formation of aneuploid gametes. Nevertheless, through random segregation, triploids also produce some euploid gametes, both haploid and diploid, which can contribute to the establishment of stable polyploid populations over time (De Storme, Mason, 2014). This process is commonly referred to as the triploid "bridge" hypothesis. The discovery of triploid cytotypes within the Turan population provides support to this hypothesis.

In perennial taxa, both auto- and allopolyploids are formed preferentially through triploid bridging (Ramsey, Schemske, 1998), which is also likely to be the case for the observed triploid and tetraploid cytotypes of *S. media*. The triploid "bridge" hypothesis postulates that hybrids with intermediate ploidy levels facilitate gene flow, particularly at the interface between diploid and autotetraploid populations. In populations comprising admixtures of plants with different ploidy levels, interploidy hybridization results in the formation of triploids. Although triploid hybrids are rarely formed, they can interbreed with the parental cytotypes, resulting in viable introgressed offsprings, and these backcrosses are often euploid. The probability of autotriploid formation is higher when unreduced gametes of 2*n* ovules are involved than when 2*n* pollen (Ramsey, Schemske, 1998). The formation of triploids plays a significant role in the rate of autopolyploid formation, irrespective of the mating system, and in the formation of allopolyploids in taxa with outcrossing (Ramsey, Schemske, 1998). Bidirectional introgression has been observed, with a stronger tendency towards the tetraploid cytotype (Bartolić et al., 2024). It is possible that autotriploids of *S. media* may act as a triploid "bridge", facilitating the formation of allotetraploid individuals of *S. hypericifolia* × *S. media*.

Hybridization may also occur in the absence of corresponding genome duplication, that is, without polyploidisation. This phenomenon may also play an important role in speciation (Mallet et al., 2007; Abbott et al., 2013). It is probable that allodiploid formation occurs in 2x × 2x crosses, as evidenced by the processes in the Tselinnoe population, in which hybrid diploids are formed as a result of crossing between two diploid species, *S. hypericifolia* and *S. media*. The formation of allotriploids is most probable in 4x × 2x crosses (Ramsey, Schemske, 1998). We assume that triploid cytotypes of *S. hypericifolia* × *S. media* hybrids are formed as a result of fertilization by autotetraploid maternal pollen of *S. media* and diploid paternal pollen of *S. hypericifolia*. Previously, it was assumed that triploids were completely sterile. However, recent research suggests that they are, in fact, semi-fertile (Ramsey, Schemske, 2002), thereby contributing to the formation of tetraploids.

The process of meiotic restitution is also a significant factor in the formation of allopolyploid hybrids, as evidenced by the findings by De Storme and Mason (2014). The formation of allopolyploid F1 hybrids is significantly influenced by meiotic non-reduction and sexual polyploidization (Ramsey, Schemske, 1998). F1 plants derived from extensive hybridization events typically

produce non-viable gametes as a consequence of instability in meiotic chromosome segregation and gametophytic aneuploidy (De Storme, Mason, 2014). Notwithstanding the meiosis-based gametophytic sterility, F1 hybrids typically produce a modest or occasionally considerable number of seeds, which in the majority of cases exhibit a duplicated chromosome number (De Storme, Mason, 2014).

Allopolyploids are prevalent among F1 hybrids due to the increased effective frequency of 2*n* gametes in hybrid systems. Given that the average fecundity of allotetraploids is typically lower than that of auto- and allotriploids (Ramsey, Schemske, 1998), it can be supposed that allotetraploids may not play a significant role, or at least a marginal one, in the formation of higher-level polyploids. It is anticipated that allopolyploidy will result in a greater adaptive potential than autopolyploidy (Van de Peer et al., 2017).

Whole genome duplication in plants depends not only on meiotic cell cycle changes, but can also be due to somatic ploidy instability (Ramsey, Schemske, 1998), which largely depends on the mode of reproduction (De Storme, Mason, 2014). Somatic polyploidization in plants can occur through two different mechanisms: endoreduplication and endomitosis (De Storme, Mason, 2014), i. e., doubling of the chromosome set in somatic cells without subsequent cell division. Endoreduplication is common in plants but is rarely reported in reproductive tissues (De Storme, Mason, 2014). Endomitosis and endoreduplication can occur either in the zygotic cell or in apical meristematic tissues, leading to the formation of mixoploid or even fully polyploid organisms (Sattler et al., 2015). Somatic polyploidy via endomitosis is also not a common plant biological process (De Storme, Mason, 2014), but somatic ploidy change may act as a stress-induced mechanism, providing adaptive chromosomal change or polyploidization to overcome stress conditions (De Storme, Mason, 2014).

The consequences of endomitosis can be consolidated in offspring through vegetative reproduction. Vegetative reproduction is a significant factor in the establishment of polyploids, along with an outcrossing mating system that allows for hybridization (between species, subspecies, races, populations, etc.) in the formation of the polyploid (Soltis P.S., Soltis D.E., 2000). In plants that reproduce vegetatively, genome duplication in tissues that are required for sexless reproduction (e. g., rhizomes) can result in the establishment of stable polyploid lines (De Storme, Mason, 2014). Polyploids exhibit reduced inbreeding depression compared to their diploid progenitors, enabling them to tolerate elevated levels of selfing (Soltis P.S., Soltis D.E., 2000). In some *Spiraea* species, particularly *S. media*, selfing and vegetative reproduction are the primary reproductive strategies (Poliakova, 2022). This may also be associated with the observed variations within a species of several cytotypes, including diploid, tetraploid, triploid, and aneuploid, as well as mixoploidy, which is likely also the result of endomitosis.

Furthermore, the impact of environmental stressors on the extent of polyploidization in plants is well documented (Tossi et al., 2022). Polyploidy, whether autotetraploid, allotetraploid, or endopolyploid, is associated with enhanced tolerance to a wide range of stresses (Doyle, Coate, 2019). An adaptive mechanism such as polyploidization may provide enhanced tolerance to unfavourable environmental conditions by increasing the frequency of mutations and whole genome duplications. It has been demonstrated that the prevalence of polyploids is greater in humid habitats. For example, the distribution of diploids and allotetraploids in *Brachypodium distachyon* exhibits a geographic structure throughout its range in the Iberian Peninsula, with patterns of aridity influencing the distribution of these cytotypes. Tetraploid cytotypes may demonstrate enhanced tolerance to wet conditions (Manzaneda et al., 2012). The microclimatic conditions in Tuva may be extreme and stressful for a mesophytic species, with autotetraploid *S. media* and allotetraploid hybrids demonstrating the capacity to master new environmental conditions.

The formation of polyploids is more prevalent among self-pollinating individuals than cross-pollinating ones (Ramsey, Schemske, 1998). *Spiraea media* is a species that displays plasticity and alters its reproductive strategies in response to environmental conditions (Poliakova, 2022). In more humid habitats, situated beneath the forest canopy, *S. media* individuals determine for vegetative reproduction and self-pollination. The Tapsa population comprises individuals displaying a range of cytotypes ($2n = 18$, 27, 36, 24), which flourish in a larch-birch-aspen forest setting. In the Tselinnoye population, *S. media* individuals are observed to grow on open rocky slopes of dwarf hills and have only a diploid number of chromosomes, indicating that outcrossing occurs in this population. The observed advantage of self-pollination in establishing polyploids demonstrates the importance of the crossbreeding system in

polyploid evolution (Ramsey, Schemske, 1998; Dickinson et al., 2007).

In the case of *Spiraea*, aneuploidy is defined as the loss of entire chromosomes. Aneuploid offspring are frequently the consequence of crosses with triploids or are observed in polyploid systems (Ramsey, Schemske, 1998) and in newly formed hybrids (De Storme, Mason, 2014). Somatic aneuploidy is a phenomenon observed in polyploid plants. It can occur in undifferentiated tissues that subsequently form generative organs, which in turn gives rise to meiotic production of aneuploid offspring. Somatic aneuploidy is frequently observed in plants that are capable of reproducing clonally. Chimeric aneuploid tissues or organs can contribute to the formation of new plants through vegetative propagation, for example, shoots or rhizomes (De Storme, Mason, 2014). It is hypothesized that such processes occur in *S. media* individuals that are more capable of vegetative reproduction in the Tapsa population and in hybrid individuals in the Tselinnoye population.

A considerable number of polyploid speciation events entail the crossing of two or more species that are either closely or distantly related, resulting in the formation of a stable allopolyploid lineage (De Storme, Mason, 2014). The formation of new combinations of alleles resulting from the crossing of two different species can be a factor in hybrid viability or heterosis (i. e., the amplification of traits resulting from the combination of the genetic contributions of both parents), which in turn can lead to the emergence of more extreme phenotypes in the hybrid population (Van de Peer et al., 2017). From a genomic perspective, these polyploid hybrids benefit from both fixed heterozygosity and chromosome redundancy, which provides them with increased genomic flexibility that can be influenced by selection (De Storme, Mason, 2014). New auto- and allopolyploids may possess novel physiological, ecological or phenological characteristics (Tamayo-Ordonez et al., 2016), occupy new ecological niches (Ramsey, Schemske, 1998), and often demonstrate higher adaptability than their progenitors, as evidenced by their enhanced tolerance to abiotic stresses (Pelé et al., 2018). The gradual colonization of a new niche and the emergence of reproductive isolation from their diploid progenitors may ultimately result in the formation of a new species.

Conclusion

The study of cytotypes and genome variability in mixed populations of the genus *Spiraea* (*S. hypericifolia*, *S. media* and hybrid *S. hypericifolia* × *S. media*) in Tuva revealed diploid, triploid and tetraploid plants, with *S. hypericifolia* (Tapsa population) characterized by a single diploid cytotype ($2n$ = 2x = 18), while in *S. media* and *S. hypericifolia* × *S. media* ploidy levels varied from $2x$ to $4x$. Analysis of relative DNA content showed significant variability and confirmed the presence of two major cytotypes, with both diploid (2C = 0.516 ± 0.006 pg) and tetraploid (2C = 1.049 ± 0.019 pg) forms occurring in hybrid populations of *S. hypericifolia* × *S. media* (Tselinnoye and Turan, respectively). Thus, the revealed cytogenetic and genomic heterogeneity indicates the complex character of population formation and emphasizes the need for further studies of ploidy and genome structure in interspecific hybrids of the genus *Spiraea*.

Overall, the results confirmed that the genus *Spiraea* is characterized by a diversity of ploidy levels and corresponding variability in genome size, with direct comparison of FCM data with chromosome counts showing for the first time a reliable correlation between relative DNA content and ploidy for the samples studied by both methods: diploids for *S. media* (Tselinnoye), *S. hypericifolia* (Tapsa) and hybrids (Tselinnoye), and also tetraploids for hybrids (Turan). However, so far, tri- and tetraploid cytotypes for hybrids from Turan, as well as tri-, tetra- and aneuploid cytotypes for *S. media* from Tapsa, have not been confirmed by flow cytometry. Additional studies are planned on a more extensive material from the same populations. The identified tetraploid forms had twice the genome size compared to diploid forms. Nevertheless, these findings complement the known results on C-value variation in the genus and emphasize the important role of FCM in distinguishing taxa and understanding the genetic structure of *Spiraea* populations.

Thus, the cytotypes (diploid, triploid, tetraploid, and aneuploid forms) identified in mixed populations of *S. media* and *S. hypericifolia* × *S. media* reflect a wide range of putative mechanisms leading to the formation of polyploids: from meiotic non-reduction and triploid "bridge" to somatic endomitosis and vegetative reproduction. These processes contribute to the increase of genetic diversity and potential adaptability of *Spiraea* plants – both due to the combination of genomes of different species (allopolyploidy) and due to the duplication of their own set of chromosomes (autopolyploidy). We suggest that stressful environmental conditions, as well as self-pollination

and vegetative (clonal) propagation, may favour the formation of allopolyploid *Spiraea* cytotypes, while providing them with the flexibility to adopt new niches. Thus, polyploidy and hybridization indicate the dynamic nature of *Spiraea* evolution, laying the foundation for the formation of new ecotypes and, potentially, new species.

The study of hybrid populations such as *S. hypericifolia* × *S. media*, allows us to understand the mechanisms of adaptation, speciation and stabilization of hybrids. These findings have both fundamental and applied implications for the biology and conservation of these plants. The potential ecological and agricultural significance of polyploids exhibiting novel phenotypic traits is the subject of ongoing research (Tamayo-Ordonez et al., 2016). Understanding the factors involved in the formation of unreduced gametes and in endomitosis, which represent optimal pathways for the formation of polyploid species, can be used to breed new polyploid varieties with improved ornamental properties (Manzaneda et al., 2012; Tamayo-Ordonez et al., 2016). We believe that the *Spiraea* polyploids under study may have enhanced aesthetic qualities and genome stability. The hybrid *S. hypericifolia* × *S. media* found in Tuva may become valuable for landscape design and breeding not only because of its ornamental features, but also because of its greater ecological stability due to its relatedness to the eurybiotic species *S. media*.

Author contributions

Tatiana Poliakova: concept and design of the experiment, collection of plant material, analysis and interpretation of data, writing of the manuscript. Anna Shatokhina: chromosome counting, data analysis and interpretation, drafting of the manuscript in the karyology part. Maria Fomicheva: flow cytometry, analysis and interpretation of flow cytometry data, drafting of the manuscript in the flow cytometry part. Dmitry Politov: critical revision of the manuscript.

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