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Intragenomic polymorphism of the 35S rDNA internal transcribed spacers (ITS) in three species and three interspecific hybrids of *Pulsatilla* (Ranunculaceae)

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Summary. Morphological criteria do not always allow to determine the hybrid origin of samples. The study of intragenomic polymorphism of either protein-coding or rRNA-coding genes provides a more accurate assessment. ITS regions of 35S rDNA are often used as one of such markers. In the presented work, we performed a comparative analysis of ITS1 in three hybrids (nothospecies) of the genus *Pulsatilla: P.* × *intermedia, P.* × *spuria* and *P.* × *hackelii* and their parental species *P. patens*, *P. vernalis*, *P. pratensis* from Leningrad Region (NW Russia). The ITS1-5.8S rDNA-ITS2 region was sequenced by Sanger's sequencing method. On the chromatograms of the ITS sequences of the parental species there were on average, 1.7 (*P. pratensis*) to 3.3 (*P. vernalis*) polymorphic sites (PSs) – positions where two different nucleotides were detected. All three studied nothospecies exhibited more PSs – from 4.7 (*P.* × *intermedia*) to 9.5 (*P.* × *hackelii*). Study of intragenomic polymorphism of the transcribed spacer ITS1 by locus-specific NGS confirmed an obviously assumption that polymorphic sites detected by Sanger sequencing actually reflect the presence in the genomes of different rDNA variants (ribotypes) that were received by the plant from its ancestors, if the proportion of the unusual ribotype is about 20 % or more. Rarer ribotype variants can be detected only by NGS. Among the major ribotypes (ZOTU) of the studied nothospecies there were found any specific ZOTU, which were not detected in the parental species. The intragenomic polymorphism of the ITS1 region revealed by either Sanger sequencing or NGS in parental species may indicate earlier events of homoploid hybridization accompanying speciation within the genus.

Внутригеномный полиморфизм внутренних транскрибируемых спейсеров 35S рДНК (ITS) у трёх видов и трёх межвидовых гибридов *Pulsatilla* (Ranunculaceae)

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Аннотация. Морфологические критерии не всегда позволяют установить гибридное происхождение образцов. Изучение внутригеномного полиморфизма генов, кодирующих белки или рРНК, позволяет дать более точную оценку. В качестве одного из таких маркеров часто используют участки ITS-районов 35S рДНК. В представленной работе мы провели сравнительный анализ ITS1 у трёх гибридов (нотовидов) из рода *Pulsatilla*: *P.* × *intermedia*, *P.* × *spuria* и *P.* × *hackelii* и их родительских видов *P. patens*, *P. vernalis*, *P. pratensis,* все из Ленинградской области (Россия). Регион ITS1-5.8S рДНК-ITS2 был секвенирован по Сэнгеру. На хроматограммах последовательностей ITS родительских видов в среднем было выявлено от 1,7 (*P. pratensis*) до 3,3 (*P. vernalis*) полиморфных сайта (PS) – позиции, где были обнаружены два разных нуклеотида. Все три изученных нотовида показали больше PS – от 4,7 (*P.* × *intermedia*) до 9,5 *(P.* × *hackeli*). Изучение внутригеномного полиморфизма транскрибируемого спейсера ITS1 методом локус-специфического NGS подтвердило очевидное предположение о том, что полиморфные сайты, выявляемые при секвенировании по Сэнгеру, на самом деле отражают наличие в геномах различных вариантов рДНК (риботипов), которые были получены растением от своих предков, если доля необычного риботипа составляет около 20 % и более. Более редкие варианты риботипа могут быть обнаружены только методом NGS. Среди основных риботипов (ZOTU) изученных нотовидов не обнаружено специфических ZOTU, которые не были обнаружены у родительских видов. Внутригеномный полиморфизм региона ITS1, выявленный с помощью секвенирования по Сэнгеру или NGS, у родительских видов может указывать на более ранние акты гомоплоидной гибридизации, сопровождающие видообразование внутри рода *Pulsatilla*.

Introduction

New species of flowering plants arise not only through the accumulation of mutations in allopatric populations, but also saltationally, through distant hybridization (Grant, 1981; Soltis, Soltis, 2009; Qiu et al., 2020). Traditional plant systematics reveals hybrids by their intermediacy of morphological characters of parental species and by sterility. Now genetic methods have become used (Andreasen, Baldwin, 2003; Noyes, 2006). Comparative analysis of internal transcribed spacers ITS1 and ITS2 sequences of the nuclear 35S rRNA genes has been widely used. The level of divergence of these regions appears to be optimal for interspecific comparisons (Feliner, Rossello, 2007; Lopez-Alvarez et al., 2012). Despite the high copy number in the genome, these sequences are usually quite homogeneous, since interspecific hybrids very quickly undergo homogenization or loss of rDNA of one of the parents (Kovarik et al., 2008; Kotseruba et al., 2010; Rodionov et al., 2017, 2018). However, there are examples of long-term intragenomic rDNA polymorphisms. This polymorphism can be detected by Sanger DNA sequencing, when double peaks are detected on the chromatograms, indicating the presence in the genome of a different DNA sequence. Well-known example of the ITS sequence intragenomic polymorphism has been shown in wild and cultivated peonies where polymorphic sites (PS) are almost always recorded in hybrids in nucleotide positions by which parent species differ (Sang et al., 1995; Punina et al., 2012, 2017).

New opportunities for studying rDNA polymorphism have emerged with the advent of high-throughput nrDNA sequencing (NGS). NGS allows not only to identify PS but in addition to determine the ratio of major and minor variants of ITS sequences in hybrid genomes. For example, it was shown that hexaploid wild *Avena* species with genomic composition AACCDD and tetraploid AACC retain only 1.5–3 % of the rDNA of their C-genomic ancestors, which is not detected by Sanger sequencing (Rodionov et al., 2020).

The presence of PS as markers of distant hybridization was also noted in the genus *Pulsatilla* Mill. (Sun et al., 2014). Interspecific hybridization in the genus *Pulsatilla* has been repeatedly noted, in particular, between European species *P. vernalis* (L.) Mill., *P. patens* (L.) Mill. and *P. pratensis* (L.) Mill. (Hegi, Weber, 1975). All these species are diploids with $2n = 16$; this number has been established for different loci (Baumberger, 1971; Kartashova et al., 1974; Dawe, Murray, 1979; Sopova, Sekovski, 1982; Punina, Grif, 1984; Krasnikov, 1991; Sramkó et al., 2019; etc.). The nothospecies were described: *P.* × *intermedia* Lasch, 1828, Linnaea, 2: 164 (*P. vernalis* × *P. patens*), *P.* × *spuria* Camus 1898, J. Bot. (Morot), 12: 100 (*P. vernalis* × *P. pratensis*), and *P.* × *hackelii* Pohl 1814, Tentamen Florae Bohemicae. Prag 2: 213 (*P. patens* × *P. pratensis*). It was shown that *P.* × *intermedia* is also a diploid (Punina, Grif, 1984), and the chromosome numbers for *P.* × *spuria* and *P.* × *hackelii* are unknown. It can be assumed that they are also diploids, in particular, the amount of DNA found for *P.* × *hackelii* is intermediate, between the parent species *P. patens* and *P. pratensis* (Szczecińska et al., 2017). Morphological characters in *P.* × *spuria* and *P.* × *hackelii* are intermediate between the characters of corresponding parental species and stable. Howe-

ver, *P.* × *intermedia* does not have stable morphological characters; different specimens of this hybrid demonstrate the entire range of transitions between parental species (Table 1, Fig. 4). This argues in favor of the introgressive nature of *P.* × *intermedia* (Uotila, 1980; Punina, Grif, 1984).

Table 1

Comparison of morphological and biological characteristics of the studied *Pulsatilla* species and hybrids

Species and hybrids	Flower shape	Perianth color	Pubescence	Leaf shape	Wintering of leaves and the timing of their development
P. patens	Large, erect at the beginning of flowering, later drooping	Blue or blue-violet (Fig. 2)	Moderately developed, silvery	Palmate-trifid with sharp teeth (Figs 2, 4)	Do not winter, develop after flowering
P. vernalis	Large, erect at the beginning of flowering, later drooping	White inside, pinkish or bluish outside (Fig. 2)	Intensively developed, golden	Pinnate-trifid with blunt teeth, middle part on a short petiole (Fig. 2)	Winter, develop after flowering
P. pratensis	Small, drooping	Various shades from dark purple to pale lilac, also pinkish or greenish inside	Weakly developed, silvery	Pinnate-multifid, with narrow lobes and sharp teeth (Figs 3, 4)	Do not winter, develop during flowering
P. × *intermedia*	Large, erect at the beginning of flowering, later drooping	Various shades from light purple to almost white	Moderately or intensively developed, silvery, pale golden or golden	Various transitional forms between those of parent species (Fig. 2).	A larger or smaller part of the rosette leaves may overwinter, develop after flowering
P. × *spuria*	Medium, erect at the beginning of flowering, later drooping	White inside, pinkish outside	Moderately developed, pale golden	Pinnate-multifid, with rather narrow lobes (Fig. 3)	Partially overwinter, develop after flowering
P. × *hackelii*	Medium, erect or semi-drooping	blue purple	Weakly developed, silvery	Pinnate-trifid or pinnate-five-separate, with rather narrow lobes (Fig. 5)	Do not winter, develop during flowering.

The aim of our work is to check whether the pattern of intragenomic polymorphism in these nothospecies of *Pulsatilla* detected by Sanger sequencing is confirmed by the distribution of sequence variants (ribotypes) detected on the same objects by NGS, and to see whether additional information can be obtained using NGS.

All parental species are rare in Leningrad Region. They have a conservation status, in particular, *P. pratensis* and *P. vernalis* are listed in the Red Book of the Russian Federation (Geltman, 2008), and *P. patens* is in the Red Book of the Leningrad Region (Geltman, 2018). In Finland, which borders the Leningrad Region, *P. vernalis* is also listed in the 2019 red list of Finnish species as vulnerable, and *P. patens* – as endangered (Hyvärinen et al., 2019). Many species of *Pulsatilla* are also protected in other European countries (see, for example, Szczecińska et al., 2016 and others).

Material and methods

The material for the study was collected in 1982–1983 and 2017–2019 in several natural habitats in the Leningrad Region (Table 2, Fig. 1). Due to the protected status of plants, herbarization was not carried out, the plants were photographed, and only a fragment (one leaf) was placed in silica gel (Chase, Hills, 1991). We tested pollen fertility of the species and the nothospecies using an aceto-carmine test. Anthers from the flowering specimens were taken into a paper bag, then pollen completion was assessed using a carmine test. Three or four anthers were placed on a glass slide, 50 μL of 10 % acetocarmine

solution was added, the anthers were covered with a coverslip and slightly heated. After that, the material was distributed under the coverslip by lightly tapping with a wooden stick. Then, under a microscope, the percentage of colored and uncolored pollen grains were counted, taking into account a total of at least 500 pieces (lens 10×). Unstained pollen grains without contents or slightly colored and deformed ones were considered abortive, while uniformly intensely colored and undeformed ones were considered conditionally fertile. The percentage of colored and undeformed pollen grains was calculated.

Table 2

Samples of *Pulsatilla* species and nothospecies from which ITS sequences have been sequenced and where they were collected

Geographic location and coordinates	Examined specimens and sequence numbers		
	Species/nothospecies	GenBank	GenBank (NGS)
Vsevolozhsk district, env. railway st. Lembolovo 60°26'N, 30°18'E	*P. vernalis* 1 *P. patens* Lm1–Lm4 *P.* × *intermedia* 1–6	MN997023 MN997007–MN997010 MN996987–MN996993	SRR12045990 SRR12045988 SRR12045989
Priozersk district, env. settlement Orekhovo 60°29'N, 30°20'E	*P. vernalis* 2, 3 *P.* × *intermedia* 7	MN997024, MN997025 MN996994	
Priozersk district, env. settlement Borisovo 60°37'N, 29°57'E	*P. patens* Bo1, Bo2	MN997011, MN997012	
Priozersk district, env. railway st. Petäjärvi 60°38'N, 30°04'E	*P. patens* Pe1, Pe2	MN997013, MN997014	
Vyborg district, env. settlement Gribnoe 60°35'N, 29°22'E	*P.* × *spuria* 1, 2, *P. pratensis* Gr	MN997021, MN997022 MN997020	SRR12045985 SRR12045986
Luga district, env. railway st. Raz'ezd Generala Omelchenko 58°47'N, 30°52'E	*P. pratensis* Lu1–Lu5 *P. patens* Lu1–Lu12 *P.* × *hackelii* Lu1, Lu2	MN997015–MN997019 MN996995–MN997006 MN997027, MN997028	 SRR12045987 SRR12045984

DNA was isolated using the PlantMini Kit (Qiagen), then the ITS regions were amplified with primers ITS-1p (Ridgway et al., 2003) and ITS-4 (White et al., 1990). Taq polymerase (SibEnzyme) was used for PCR of *P. pratensis* and *P. patens* species, while ITS regions of hybrids and *P. vernalis* could be amplified only with Phire Hot Start II polymerase (Phire Plant Direct PCR Master Mix kit, Thermo scientific). In total, we were able to isolate and amplify DNA for 3 individuals of *P. vernalis*, 20 of *P. patens*, 6 of *P. pratensis*, 7 of *P.* × *intermedia*, 2 of *P.* × *spuria*, and 2 of *P.* × *hackelii* (Table 2). Sanger sequencing of DNA fragments was performed at the Core Facilities Center "Cell and Molecular Technologies in Plant Science" at the Komarov Botanical Institute (St.-Petersburg, Russia) on an AbiPrism 3130 genetic analyzer (Applied Biosystems). The primers ITS-1p, ITS-4, ITS-2 and ITS-3 primers (White et al., 1990) were used for sequencing. The obtained sequence chromatograms were visually checked for the presence of polymorphic sites (PS).

DNA sequence analysis was performed using MEGA X (Kumar et al., 2018), Chromas programs (DNA Sequencing Software. URL: http://technelysium.com.au/wp/chromas/). The degree of similarity of the obtained DNA sequences of the ITS1-5.8S rDNA-ITS2 region of the studied species and hybrids was assessed using the NeighbourNet network built by us in the SplitsTree4 program (Huson, Bryant, 2006).

Library preparation and Illumina MiSeq sequencing were performed at the Core Facilities Center of the All-Russian Research Institute of Agricultural Microbiology. Marker sequences (3'-part of the 28S rRNA gene, complete sequences of ITS1 and 5'-part of the 5.8S rRNA gene) were amplified using primers ITS-1P and ITS-2. Primary sequencing data was processed using FastQC, Trimmomatic, and Fastq-join software tools. Plant ribotypes were sorted by frequency and filtered from fungi and bacteria using BLAST. We aligned the selected ribotypes using the MUSCLE algorithm in MEGA X. Locus-

specific NGS sequencing (Illumina) of the ITS1 region was carried out for 7 plants: *P. vernalis* 1, *P. patens* Lm4, *P. patens* Lu4, *P. pratensis* Gr, *P.* × *intermedia* 3, *P.* × *spuria* 1 and *P.* × *hackelii* Lu2.

To visualize the intragenomic diversity of ribotypes, Illumina sequences were processed with USEARCH 11.0 (Edgar, 2010). Paired reads were joined. The obtained pool of sequences was adapter trimmed and processed with the aid of Trimmomatic (Bolger et al., 2014) included in Unipro Ugene (Okonechnikov et al., 2012) using the following parameters: PE reads; sliding window trimming with size 4 and quality threshold 12; and minimal read length 130. Then the sequences were sorted by occurrence and clustered using the UPARSE algorithm with a similarity threshold of 97 % (Edgar, 2013). The UNOISE3 algorithm (Edgar, 2016) was used to remove chimeric sequences and sequencing artifacts.

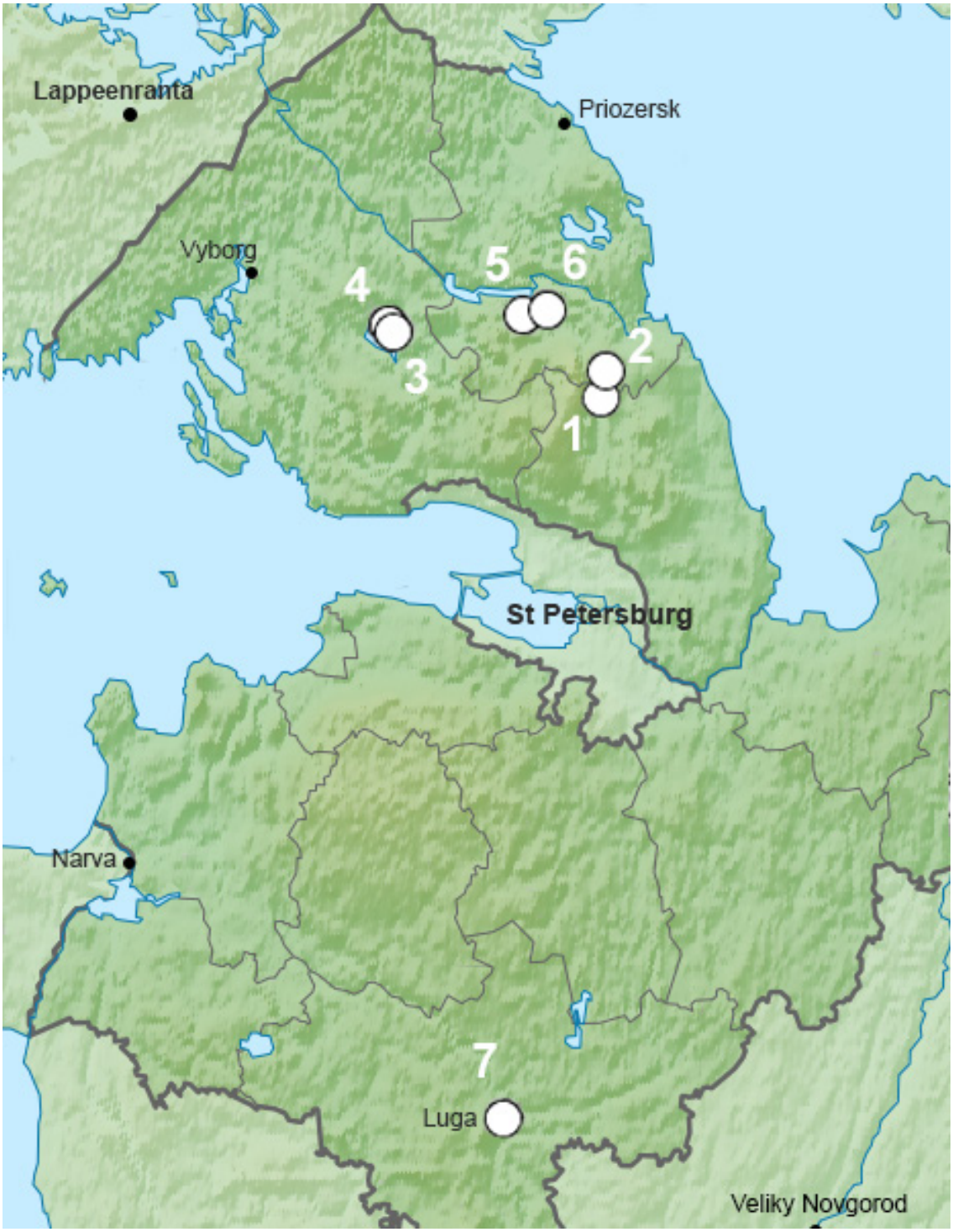

Fig. 1. The map of the locations of collections (see Table 2).

Results

1. Morphology of putative *Pulsatilla* interspecific hybrids

We identified collected plants of *Pulsatilla* based on morphological characters. Characteristic morphological and biological features of the studied *Pulsatilla* species and putative hybrids are given in Table 1 and Figs 2–4. Unfortunately, the previously low number of plants per local population has been declining recent years. Thus, the mixed populations of *P. patens* and *P. vernalis* which we observed near the Lembolovo railway station in 1982–1983, at that time occupied an area of about 0.25 km^2 in a pine forest and consisted of about 50 plants of each species and about of 30 specimens of *P.* × *intermedia*. In May 2017, only 2 flowering and 5 vegetative specimens of *P. vernalis*, 23 flowering specimens of *P. patens,* and 11 of *P.* × *intermedia* were found in this location. In May 2018, in the vicinity of the village Gribnoe, we were able to find only two *P.* × *spuria* and two *P. pratensis* plants, although Dr. A. Yu. Doronina, who first discovered these habitats in 2005, observed 18 specimens of *P. pratensis* and 9 plants of *P.* × *spuria* here (Doronina, 2006). In 2018 and 2019, we were unable to detect the mixed population of *P. vernalis* / *P. pratensis* / *P.* × *spuria* in the vicinity of the village Borisovo; only a few *P. patens* plants have been found in this area in recent years.

2. Study of pollen grains in species and hybrids of *Pulsatilla*

In order to further verify that plants identified by morphology as hybrids are indeed hybrids, we examined the pollen. The study of pollen grains using the carmine test showed that in all parental species, outwardly normal (colored and undeformed) pollen grains range from 64 to 99 % (*P. patens* – 71–93 %, *P. vernalis* – 64–99 %, *P. pratensis* – 96–98 %), while in hybrids this number can be much less and varies greatly in different individuals. Thus, in different individuals of *P.* × *intermedia*, normal pollen grains ranged from 1 to 93 %, in *P.* × *spuria*, from 1 to 18 %, and in *P.* × *hackelii*, 9 % (Table 3, Fig. 5).

Table 3

The proportion of colored and undeformed pollen grains in the studied samples of *Pulsatilla* species and hybrids

Species/hybrids (N)	%% of colored and undeformed pollen grains in different plants
P. patens (10)	71, 76, 81, 85, 86, 90, 91, 92, 93, 98
P. vernalis (7)	64, 81, 85, 86, 95, 96, 99
P. pratensis (3)	96, 96, 98
P. × *intermedia* (19)	1, 15, 16, 29, 32, 45, 51, 53, 55, 59, 61, 67, 68, 73, 74, 78, 80, 82, 93
P. × *spuria* (8)	1, 5, 7, 8, 11, 14, 16, 18
P. × *hackelii* (1)	9

3. ITS1-5.8S rDNA-ITS2 sequence polymorphism in Sanger sequencing and analysis of the ITS1 region in NGS sequencing on the Illumina platform

The length of the aligned Sanger-sequenced ITS1-5.8S rDNA-ITS2 region taken for the analysis was 575 bp, where positions 1–200 bp corresponded to ITS1, positions 201–364 bp corresponded to 5.8S rDNA, and positions 365–575 bp corresponded to ITS2. At the first stage of the analysis, we identified species-specific nucleotide substitutions and PS (Table 4). In all the samples we analyzed, the 5.8S rDNA regions were identical and did not contain substitutions or PS. There were 11 sites with species-specific substitutions (among all three species): *P. patens* and *P. vernalis* differ in 4 substitutions (two C/A transversions in ITS1 and two C/T transitions in ITS2), *P. patens* and *P. pratensis* differ in 10 substitutions (one A/G transition and one C/A transversion in ITS1, one transversion T/A and 7 transitions (two-A/G, 5-C/T) in ITS2); *P. vernalis* and *P. pratensis* – 10 substitutions (three C/A transversions and one G/A transition in ITS1, one T/A transversion and 5 transitions (one A/G, 4 C/T) in ITS2) (Table 5).

To determine the degree of similarity between the ITS fragments sequenced by the Sanger method in the studied species and hybrids of *Pulsatilla*, we built the NeighborNet network using the SplitsTree4 program (Huson, Bryant, 2006) (Fig. 6).

Sanger sequencing of ITS regions revealed the intragenomic polymorphism in both species and interspecific hybrids of *Pulsatilla* which may indicate the presence of several ribotypes in the each individual genome. In order to estimate the proportions of these ribotypes in each species, we performed sequencing of the ITS1 region on the Illumina platform in 7 samples (*P. vernalis* 1, *P. patens* Lm4, *P. patens* Lu4, *P. pratensis* Gr, *P* × *intermedia* 3, *P.* × *spuria* 1,

P. × *hackelii* 2). The ITS1 region was chosen because it contained a greater number of species-specific transversions (Table 5). We took two accessions of *P. patens* from different locations because they differed in the number of PS (Tables 2, 4). From 7734 to 19838 reads were obtained and analyzed for each sample. Raw sequencing data has been deposited in GenBank under BioProject number PRJNA640136.

Comparison of the Sanger and NGS sequencing results showed that all PS detected by Sanger sequencing indicate the presence in the genome of sequences with different nucleotides at this position. However, in both species and hybrids, the intragenomic polymorphism detected by NGS sequencing turned out to be significantly higher than would be expected based on the number of PS detected by Sanger approach.

To assess the intragenomic polymorphism of the ITS1 *Pulsatilla* species and hybrids we used Illumina high-throughput sequencing. The USERCH algorithm (Edgar, 2012) was employed to process Illumina high-throughput sequencing data to identify the unique variants of the ITS1 sequences as well as to calculate their relative frequency.

Obtained unique sequences, so called ZOTUs (zero-radius OTUs) (Edgar, 2016), or ribotypes (Matyasek et al., 2012). ZOTUs with a read count of 12 or more were used in the further analysis. The hybrids mostly had more ribotypes (22–28) than the parent species *P. pratensis* and *P. patens* (10 and 15, respectively), with the exception of *P. vernalis*, which had 24 ribotypes. In our study, we divided ribotypes into main ones, which accounted for 850 to 28282 reads in the sample, and secondary ones, less than 500 reads. In total, secondary ribotypes accounted for no more than 1–2 % in each sample. Some secondary ribotypes had signs of pseudogenization: deletions of at least 3 bp in length and/or mutations in the GGCRY-(4 to 7 n)-GYGYCAAGGAA motif, which is highly conserved in flowering plants (Liu, Schardl, 1994). Some other secondary variants had numerous (from 20 to 70) SNPs evenly distributed over the sequence. As a result, *P. patens* has 4 main ribotypes (P1–P4), *P. vernalis* has 3 (V1–V3) and *P. pratensis* has 2 (Pr1, Pr2). In the genomes of nothospecies, we observed combinations of parental main ribotypes.

In further analysis, we took into account only the main ribotypes, taking their totality as 100 % for each individual. Each sample of parent species had one significantly predominant ribotype, which we called "major", and the rest were considered as "minor". In Lu4 individual of *P. patens* from the southern population the major ribotype P1 was 66.0 % and its minor ribotypes were 16.76 % (P2), 12.98 % (P3) and 4.26 % (P4). In *P. patens* Lm4 individual from the northern population the major ribotype P1 absolutely prevailed (99.53 %), and the proportions of minor ribotypes were vanishingly small and amounted to 0.12 % (P2), 0.1 % (P3) and 0.25 % (P4). In *P. vernalis* 1, we identified 3 main ribotypes (major V1 – 82.0 %, minor V2 – 13.23 % and V3 – 4.77 %), and in *P. pratensis* – 2 (major Pr1 – 92.71 % and minor Pr2 – 7.29 %) (Fig. 7). Nucleotide substitutions characterizing the listed ribotypes are presented in Table 6.

In hybrids, we found a different ratio of major and minor parental ribotypes (Fig. 7). Thus, in *P.* × *intermedia*, there were two maior ribotypes, P4 and V1, while the proportion of the P1 ribotype, which was major in the parent species *P. patens*, was only 11.33 %, and the P2 ribotype is absent altogether. The ribotypes of the other parental species, *P. vernalis*, prevail in total in *P.* × *intermedia*, and are represented V1 – 41.57 %, V2 – 8.58 %, V3 – 3.10 %. In *P.* × *spuria*, the minor ribotype of the parent species *P. vernalis* V3 becomes the first major ribotype (52.23 %), and the ribotypes V1 and V2 account for 8.84 and 2.25 %, respectively, whereas the other parental species, *P. pratensis*, is represented only by major ribotype Pr1 (36.68 %), thus, in this hybrid, ribotypes of *P. vernalis* also predominate. In *P.* × *hackelii*, the ribotypes of the parent species *P. patens* predominate in total, with P2 becoming major (55.43 %), and P1 and P4 becoming minor (9.15 % and 1.7 %, respectively); the ribotype P3 is completely absent. From the second parental species, *P. pratensis*, this hybrid received only the major ribotype Pr1 (33.72 %).

Discussion

1. Fertility of the studied species and hybrids

Genomic differences between parental species are usually the cause of meiotic disorders, which result in the formation of completely or partially defective pollen grains in hybrids. We found reduced percentage of normal pollen grains in most of presumably hybrid plants comparing to supposed parent species, which confirmed hybrid origin of these plants. Our calculations showed that if in the original species, outwardly normal (colored and undeformed) pollen grains range from 64 to 99 %, the hybrids have much less, and this number varies greatly in different individuals, for example, from 1 to 93 % in 19 studied plants of *P.* × *intermedia* (Table 3). Similar patterns were found by other researchers of *Pulsatilla* hybrids (Krejová, 2014; Szczecińska et al., 2017).

Table 4

Species-specific nucleotide substitutions and polymorphic sites in the region of ITS1 and ITS2 sequenced in Sanger in 3 species and 3 interspecific hybrids of *Pulsatilla*

Region	**ITS1**													**ITS2**								
Position	**2**	**44**	**66**	**67**	**91**	**93**	**101**	**104**	**111**	**112**	**118**	**122**	**135**	**378**	**381**	**418**	**420**	**484**	**529**	**553**	**558**	**568**
Consensus	C	C	A	C	C	G	C	C	A	C	A	C	C	A	G	C	C	T	T	T	C	T
P. × *intermedia* 1							C/A							A/G							C/T	
P. × *intermedia* 2		C/A		C/T			C/A							A/G					C			
P. × *intermedia* 3		C/A		C/T			C/A	C/T	C/A										C		C/T	
P. × *intermedia* 4		C/A		C/T			C/A		C/A					A/G							C/T	
P. × *intermedia* 5				C/T			C/A		C/A										C/T		C/T	
P. × *intermedia* 6							C/A		C/A					G					C/T		C/T	
P. × *intermedia* 7							C/A	C/T	C/A					A/G					C/T			
P. patens Lu1					C/T									A/G	G/T							
P. patens Lu2																			C/T			
P. patens Lu3, Lu4					C/T					C/A	C/A			A/G	G/T							
P. patens Lu5					T						C/A			A/G	G/T							
P. patens Lu6											C/A		C/T	A/G	G/T							
P. patens Lu7														A/G								
P. patens Lu8					T									A/G	G/T							
P. patens Lu9													C/T	A/G								
P. patens Lu10														A/G					C/T			
P. patens Lu11				C/T	C/T					C/A	C/A			A/G	G/T				C/T			
P. patens Lu12				C/T										A/G					C/T			
P. patens Lm1, Lm2, Lm4														G					C			
P. patens Lm3, Bo1														A/G					C			
P. patens Pe2													C/T	A/G					C			
P. patens Bo2, Pe1														A/G					C/T			
P. × *hackelii* 1			A/G	C/A	C/T										A/G	C/T	C/T	A/T	C		C/T	C/T
P. × *hackelii* 2			A/G	C/A						C/A	C/A				A/G	C/T	C/T	A/T	C		C/T	C/T
P. pratensis Lu1, Lu3, Lu5			G	A											A/G	T	C/T	A	C		T	C
P. pratensis Lu4,			G	A											A	T	C/T	A	C		T	C
P. pratensis Lu2			G	A											A	T	T	A	C		T	C
P. pratensis Gr			G	A		G/A						C/T			A	T	C/T	A	C		T	C
P. × *spuria* 1	C/G	C/A	A/G	C/A				C/T								T	T	A/T	C		T	C
P. × *spuria* 2	C/G	C/A	A/G	C/A				C/T								C/T	T		C		T	C/T
P. vernalis 1		A					A	C/T	C					A/T					C	C	T	
P. vernalis 2, 3		A					A	C/T	C/A					A/T			C/T		C	C	T	

Fig. 2. Appearance of plants of *Pulsatilla patens* (a), *P. vernalis* (b), and *P. pratensis* (c).

Fig. 3. Appearance of hybrid plants of *Pulsatilla* × *intermedia* 6 (a), *P.* × *hackelii* 1 (b), and *P.* × *spuria* 1 (c). These individuals were taken in analysis.

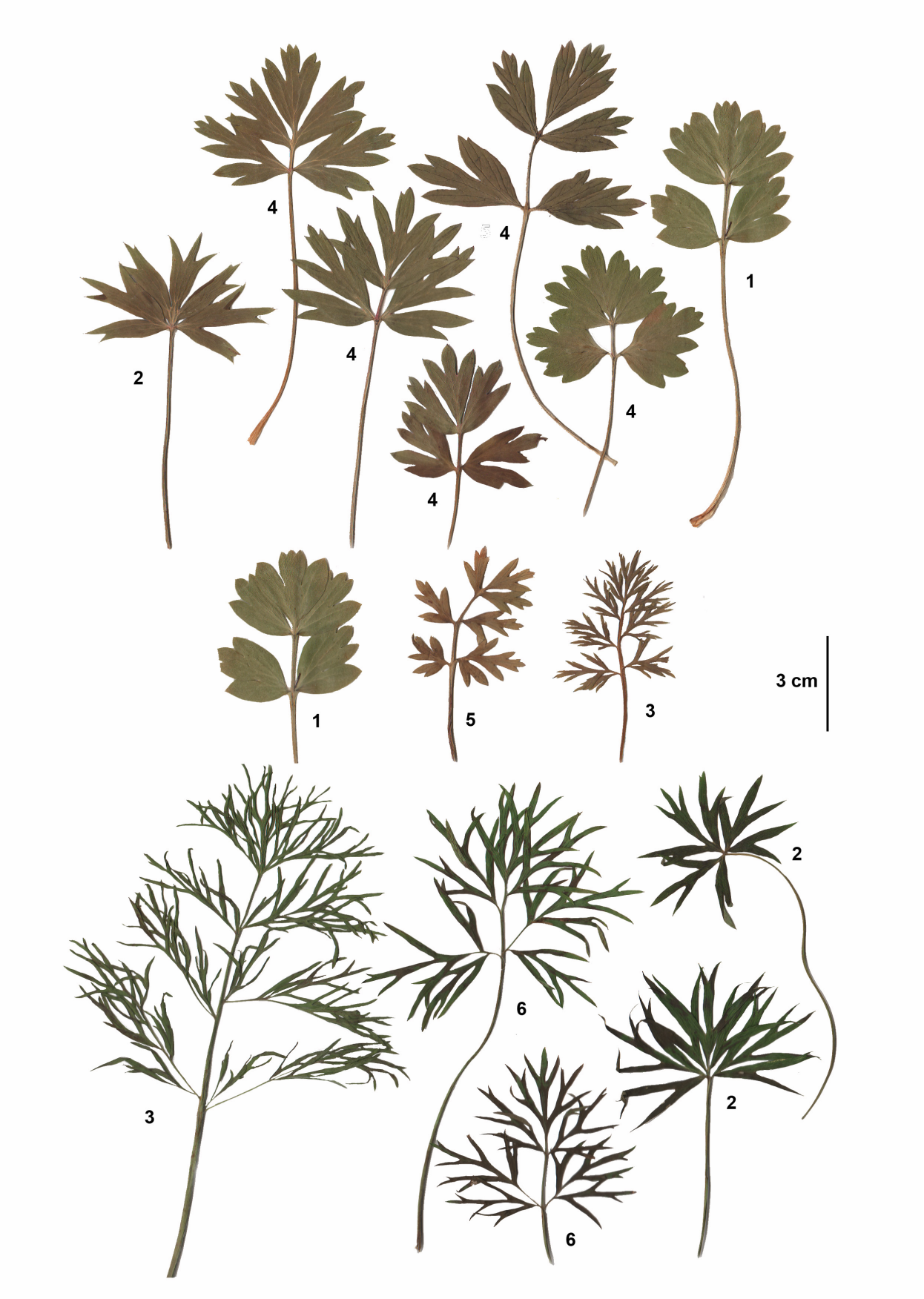

Fig. 4. Leaves of *Pulsatilla vernalis* (1), *P. patens* (2), *P. pratensis* (3), *P.* × *intermedia* (4), *P.* × *spuria* (5), and *P.* × *hackelii* (6).

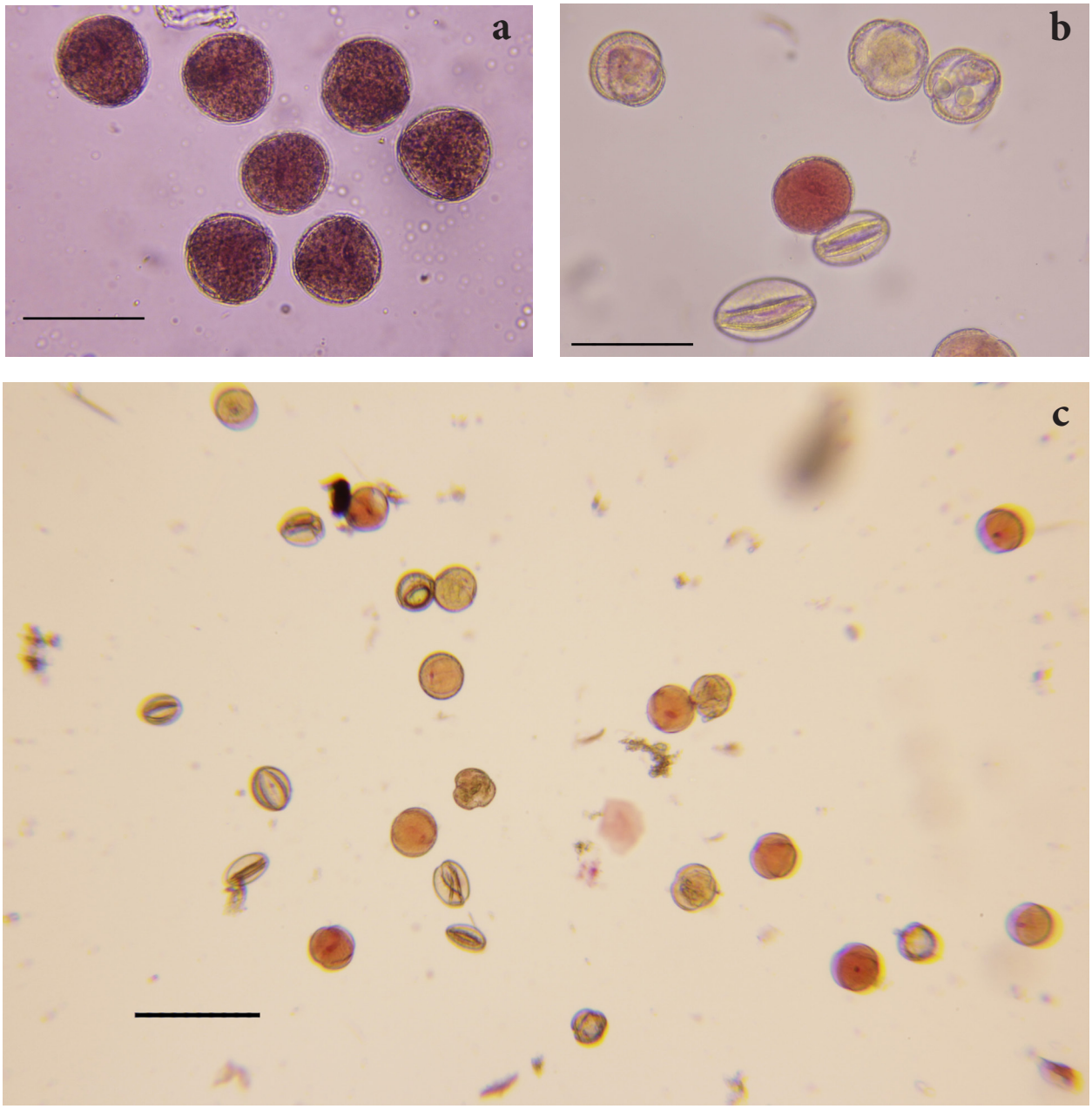

Fig. 5. Pollen grains after aceto-carmine staining: *Pulsatilla pratensis* (a) and *P.* × *hackelii* (b, c). Bar = 10 μm (a, b) and 100 μm (c).

Table 5

Species-specific nucleotide substitutions in the studied species of *Pulsatilla* detected by Sanger sequencing

Region	ITS1				ITS2						
Position	44	66	67	101	381	418	420	484	553	558	568
P. patens	C	A	C	C	G	C	C	T	T	C	T
P. vernalis	A	A	C	A	G	C	C	T	C	T	T
P. pratensis	C	G	A	C	A	T	T	A	T	T	C

Table 6

Nucleotide substitutions in ITS1 region characterizing the intragenomic diversity of the main ribotypes detected by NGS sequencing and the corresponding PS detected by Sanger sequencing

Position	2	44	66	67	69	91	93	101	104	111	112	118	122	135	171
patens P1	C	C	A	C	C	C	G	C	C	A	C	A	C	C	C
patens P2	.	.		.	.	T	.	.	.	.	A	C	.	.	.
patens P3	.	.		.	.	.	.	.	.	.	.	.	.	T	.
patens P4	.	.		T	.	.	.	.	.	.	.	.	.	.	.
vernalis V1	.	A		.	.	.	.	A	.	C	.	.	.	.	.
vernalis V2	G	A		.	.	.	.	A	.	C	.	.	.	.	.
vernalis V3	G	A		.	.	.	.	.	T	.	.	.	-	.	T
pratensis Pr1	.	.	G	A	.	.	.	.	.	.	.	.	.	.	.
pratensis Pr2	.	.	G	A	G	.	A	.	.	.	.	.	T	.	.
P. patens Lu4	.					C/T					C/A	C/A			
P. patens Lm4	.														
P. vernalis 1	.	A							C/T						
P. pratensis Gr	.						A/G						C/T		
P. × *intermedia* 3	.	C/A		C/T				C/A	C/T	A/C					
P. × *hackelii* 2	.	.	A/G	C/A		C/T					A/C	A/C			
P. × *spuria* 1	C/G	C/A	A/G	C/A					C/T						

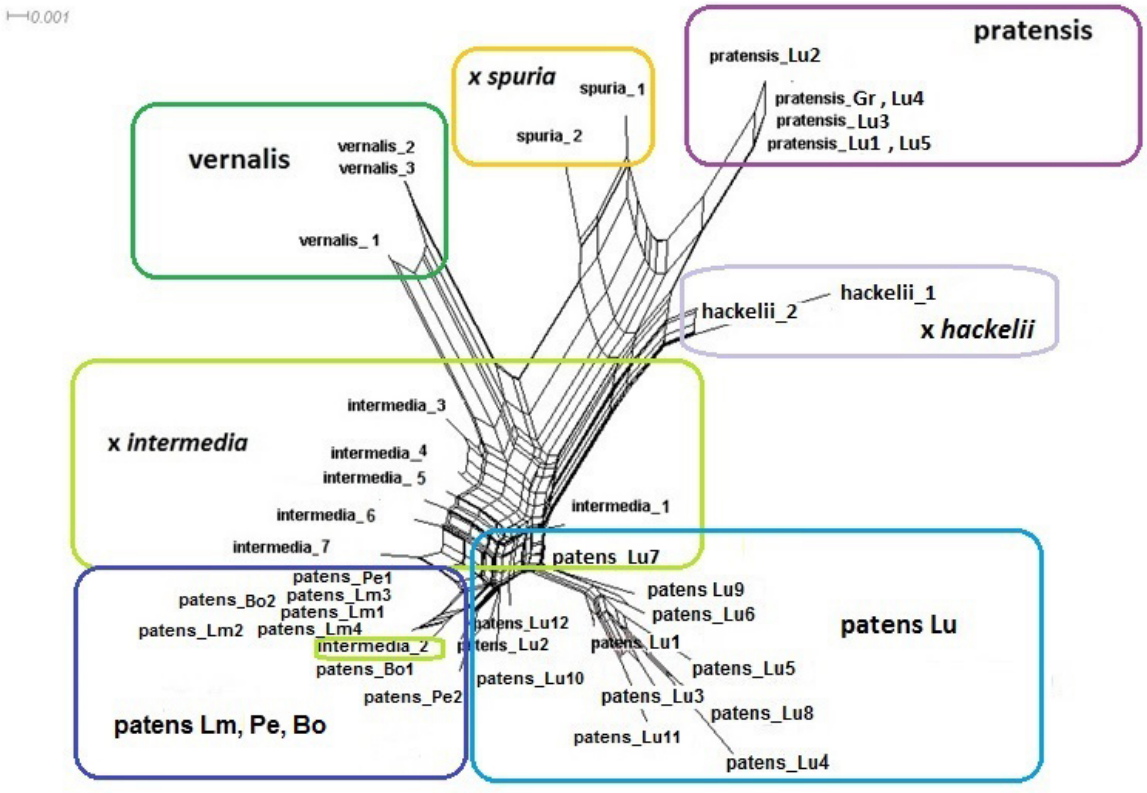

Fig. 6. NeighborNet network showing the degree of similarity between the ITS1 + ITS2 sequences (Sanger method) of *Pulsatilla* species and hybrids.

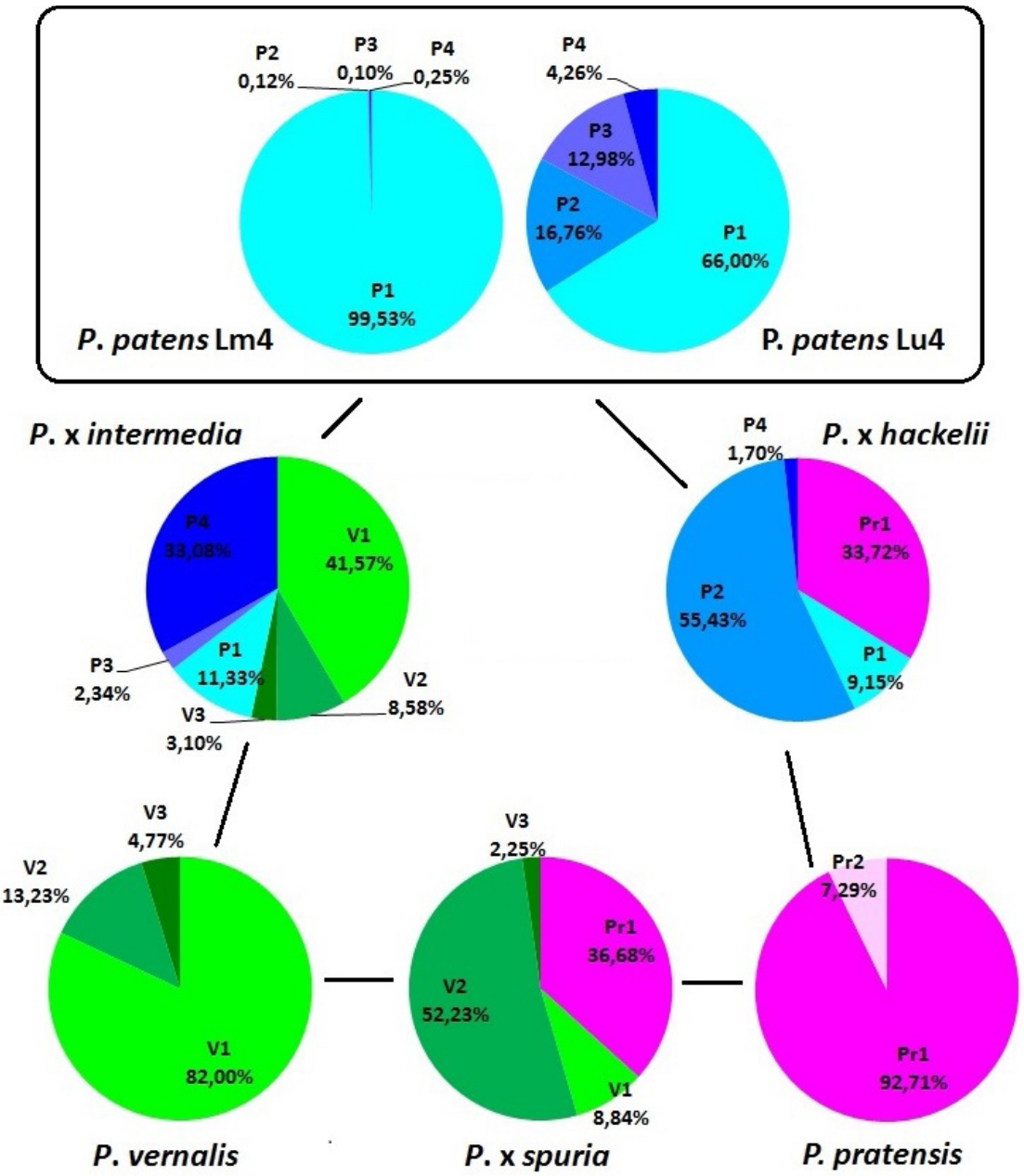

Fig. 7. Proportions of the main ribotypes identified by NGS (Illumina) sequencing of ITS1 region in species and hybrid plants of *Pulsatilla*. Color designations are the same as in Table 6.

Different individuals of *P.* × *intermedia* have varying degrees of fertility; the percentage of outwardly normal pollen grains varies greatly; there are both individuals with almost completely sterile pollen, and with a significant proportion of outwardly full-fledged pollen grains. Our observations in 1981–1983 and in 2017–2019 showed that *P.* × *intermedia* sometimes forms underdeveloped and extremely rarely, full-fledged, germinating seeds, which made it possible to carry out a karyological study of this hybrid and establish its diploid nature (Punina, Grif, 1984). Interestingly, similar karyotypes of the parental species differ in the position of NOR on chromosome 8: in *P. patens*, it is located terminally on the short arm, and in *P. vernalis*, it is subterminally located on the long arm (Punina, Grif, 1984; Sramkó et al., 2019), whereas in the studied seedling from the seed of a hybrid plant, both chromosomes of the 8th pair had NOR as in *P. vernalis* (Punina, Grif, 1984). Together with a wide range of morphological variability and a significant proportion of completed pollen grains in

some plants, this indicates that *P.* × *intermedia* is an introgressive hybrid, which confirms the earlier assumption of P. Uotila (1980).

2. Interspecies differences and intraspecific and intragenomic polymorphism of the ITS1-5.8S rDNA-ITS2 region detected by Sanger sequencing

Comparison of the ITS1 and ITS2 sequences obtained by us with the data of other authors reveals more species-specific nucleotide substitutions in these species. Thus, it was previously shown that the ITS1 regions in *P. patens* and *P. vernalis* are completely identical (Szczecińska, Sawicki, 2015; Sramkó et al., 2019), while in ITS2 these species differ only in one C/T transition (Szczecińska, Sawicki, 2015). In *P. pratensis*, two species-specific substitutions were noted in ITS1 (positions 66 and 67) (Sramkó et al., 2019) and 4 (positions 418, 484, 558, and 568) in ITS2 (Szczecińska, Sawicki, 2015; Sramkó et al., 2019).

Three studied *Pulsatilla* species, *P. vernalis*, *P. patens*, and *P. pratensis*, have a wide distribution area and morphological polymorphism, and many researchers have repeatedly identified forms, subspecies, and even independent species within these species (Aichele, Schwegler, 1957; Tzvelev, 2001; Sramkó et al., 2019; etc.). In particular, *P. vernalis* was divided into 4 varieties characteristic of lowland or high mountain habitats (Aichele, Schwegler, 1957). The study of chloroplast DNA polymorphism showed the presence in *P. vernalis* of 7 haplotypes belonging to two different groups, where one group of haplotypes was distributed over the entire range of the species, and the other was present only in certain narrow local populations (Ronikier et al., 2008). The same authors note that with direct sequencing of ITS in this species, double peaks are revealed in electropherograms. M. Ronikier et al. (2008) interpret this as occurrence of multiple rDNA repeats of different length in the same genome, but, in our opinion, this, first of all, indicates the presence of PS in ITS regions in *P. vernalis*. It is not clear yet whether genetic heterogeneity is accompanied by morphological features of the samples. A checking of the *P. vernalis* herbarium specimens stored in LE and our numerous observations in nature did not reveal any noticeable morphological polymorphism in this species in the territory of the Leningrad Region.

Throughout its native range (Central and Northern Europe to Central Asia), *P. pratensis* sensu lato is also morphologically heterogeneous. In particular, the color of tepals and the size of the flower in this species mainly vary. Different authors identified up to 4 such variations, giving them different taxonomic ranks, from forms to independent species. In particular, on the territory of the European Russia, N. N. Tzvelev (2001) recognized the independence of *P. pratensis* (L.) Mill. and *P. bohemica* (Skalický) Tzvel. (= *P. pratensis* subsp. *nigricans* (Störck) Zämelis) in European Russia. In a recent extensive comprehensive study of the genus *Pulsatilla* (Sramkó et al., 2019) based on morphological, cytogenetic, and molecular phylogenetic data, the authors in addition to the type subspecies, recognize three more subspecies of *P. pratensis*: *P. pratensis* subsp. *bohemica* Skalický, *P. pratensis* subsp. *nigricans* (Störck) Zämelis and *P. pratensis* subsp. *hungarica* Soo. In this study, the ITS sequences of the first two subspecies were found to be identical, while the third one differed from them by one G/A substitution at the position 381 in ITS2. In our study the ITS sequences obtained from plants from two geographically distant (> 200 km) populations turned out to be the closest to those of *P. pratensis* subsp. *hungarica* (GenBank MK551006 [Sramkó et al., 2019]), and the species-specific substitutions at positions 67, 381, 418, 484, and 568 are the same. Nevertheless, we attributed all the specimens studied by us to the type subspecies *P. pratensis* subsp. *pratensis* on the base of their morphology. The color of flowers of the individual from location 4 (Fig. 1) was purple, and the color of flowers in five individuals from the location 7 varied from purple to pinkish outside and was greenish inside. Specified for the northern part of the Leningrad Region *P. pratensis* subsp. *nigricans* was not found in 2018, although we examined the locations where we ourselves observed this subspecies in 1982.

Pulsatilla patens is the species with the widest range among these three species (Northern and Central Europe to northern and Central Asia) and a wide spectrum of morphological diversity, which is the reason for its still unclear intraspecific taxonomy. The color of tepals varies in this species throughout the range from blue-violet to yellow, specimens with blue and pale lilac, almost white flowers were also noted. The degree of dissection and the width of the lobes of the leaf blade vary greatly. In some cases, morphological features are geographically confined, which prompted various researchers to single out several subspecies or even species. Thus, N. N. Tzvelev (2001) distinguishes 5 species, and in total 23 names of taxa of species and intraspecific rank are known only for Eurasia (Kricsfalusy, 2015). So there is every reason to think that *P. patens* may be considered as

a very problematic taxon (Kricsfalusy, 2015). In a molecular phylogenetic study of this species, small differences in 1–2 nucleotide substitutions in the ITS1 and ITS2 sequences were found in the group of Eurasian and North American subspecies of *P. patens* (Sramkó et al., 2019). Nevertheless, a detailed study of the relationship of *P. patens* subsp. *patens* and *P. patens* subsp. *flavescens* (Zucc.) Zämelis (= *P. uralensis* (Zämelis) Tzvelev), based on the combination of chloroplast and nuclear sequences rbcL+matK+ITS2, didn't reveal any differences at the molecular level, despite differences in the color of tepals and in the width of leaf lobes (Valuyskikh et al., 2020).

We attributed all the specimens studied by us to the type subspecies *P. patens* subsp. *patens* but drew attention to the fact that the specimens from the Karelian Isthmus always have wide lobes, while the specimens from the vicinity of Luga have both wide and narrower lobes (see Figs 4, 5).

In all the hybrids studied by us, we also detected PS in the ITS1 and ITS2 sequences (Table 4). In many cases, these PS were found precisely in those positions by which the parental species differ; however, this regularity was not absolute. Thus, in *P.* × *intermedia* the number of PS varied from 3 to 6 in seven individuals, and only in four positions these PS corresponded to species-specific nucleotide substitutions in the parent species *P. patens* and *P. vernalis*. Some of these PS coincided with those in one of parental species, and in some positions there was no such correspondence. In *P.* × *hackelii* we found 6 PS, which could be considered as "traces of hybridization", and 1–2 PS which corresponded to those in parental species. In *P.* × *spuria*, there were 3–4 PS resulting from hybridization, two corresponded to PS in *P. vernalis*, and one corresponded to neither the species-specific substitution nor PS in parental species.

To determine the degree of similarity between the ITS sequences of the studied species and hybrids of *Pulsatilla*, we built the NeighborNet network using the SplitsTree4 program (Huson, Bryant, 2006) (Fig. 6). On this network, the pool of ITS sequences was distributed into distinct clusters corresponding to diploid species. The "pratensis" cluster included specimens from both the Karelian Isthmus and Luzhsky district. Representatives of *P. vernalis* formed their own compact cluster. The "patens" cluster turned out to be more "loose", than that of other two species, which demonstrates its lesser genetic homogeneity; two subclusters can be distinguished in it, one including only "southern" specimens from the vicinity of Luga, and the other subcluster with both "southern" and "northern" specimens from the Karelian Isthmus. Hybrids are distributed in the network in an interesting way. *Pulsatilla* × *spuria* occupies an intermediate position on the branch leading to the "pratensis" cluster from the common "trunk", but closer to the "pratensis". Two specimens of *P.* × *hackelii* occupy a position on this branch closer to the "trunk" from which the rest of the clusters diverge, and closer to *P. patens*. Finally, 6 individuals of *P.* × *intermedia* took up a position on the "trunk" from which the rest of the clusters diverge, i. e. an intermediate position between *P. patens* and the rest of the accessions, and *P.* × *intermedia* 2 was in the cluster with *P. patens* of mixed geographical origin. Thus, most hybrids occupy an intermediate position between species clusters. The position of *P.* × *intermedia* 2 hybrid within the *P. patens* species cluster, in our opinion, also can indicate its introgressive nature.

3. Comparison of the results of sequencing of the ITS1 region by Sanger and NGS (Illumina)

Comparison of Sanger sequencing and NGS (Illumina) results showed that all PS detected in ITS by Sanger sequencing are located in positions where the ITS sequences of the parent species differ. However, with Sanger sequencing, only those variants of ancestral sequences that occur in a hybrid with a frequency of at least 15–20 % can be identified as PS. Thus, PS identified by Sanger sequencing of multicopy ITS fragments can serve as a reliable criterion for the presence of intragenomic polymorphism when studying hybridization processes, though do not fully reflect its spectrum.

The ZOTU approach, in which we took into account all reads repeated at least 12 times, revealed 54 intragenomic ribotypes, with ITS1 regions in hybrids having more ribotypes than in parental species, with the exception of *P. vernalis*. Using NGS, we identified 9 main ribotypes in parental species, the combination of which was found in hybrids. Two *P. patens* plants from different geographical locations, differing in the number of PS, had the same set of ribotypes, but the proportions of these ribotypes were significantly different. At the same time, in hybrids involving *P. patens*, the proportion of the main parental ribotype P1 sharply decreased, but the proportion of any of the minor ribotypes increased significantly. In all hybrid plants, only those major ribotypes that were present in the parent species were found; no other hybrid-specific ribotypes were found. The non-additive ratio of

ancestral ribotypes in hybrids can be explained both by backcrossing and yet poorly studied processes of rDNA homogenization in hybridogenic genomes (Winterfeld et al., 2009; Matyasek et al., 2012; Lunerova et al., 2019).

Thus, we have shown that not only interspecific hybrids of *Pulsatilla* but also some species are characterized by intragenomic polymorphism of repeated ITS sequences and the presence of several ribotypes in each genome. It can be assumed that these are "traces" of previous repeated acts of homoploid distant hybridization that accompanied speciation in the genus *Pulsatilla*, in particular, the species *P. patens*, *P. pratensis*, and *P. vernalis*.

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