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The complete plastid genome and nuclear genome markers provide molecular evidence for the hybrid origin of *Pulsatilla* × *hackelii* Pohl.

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Abstract: This study presents the first molecularly proven data on natural hybridization in the genus *Pulsatilla*, including the complete plastome sequence. A plant with morphological characteristics intermediate between *P. patens* and *P. pratensis* was found near the village of Bocheniec (southern Poland). In this study, several classes of markers based on genotyping (SSR and ISJ) and sequencing (Sanger and NGS) were used to confirm hybridization and its direction, and to determine the prospective parent species. Analysis of ISJ and ISSR genotyping data revealed that all amplified loci were polymorphic. The hybrid species was characterized by only 3 specific bands, and it shared the highest number of bands with *P. pratensis*. A total of 3,204,448 pair-end reads (2 × 250 bp) from *Pulsatilla* × *hackelii* were obtained in a single run of the MiSeq sequencer. The structure of the hybrid plastid genome did not differ from the previously identified genomes of the *Pulsatilla* species. A phylogenetic analysis of plastomes in the *Pulsatilla* species revealed three well-supported clades. The plastome of *Pulsatilla* × *hackelii* was included in the *P. pratensis* clade. *P. hackelii* and *P. pratensis* formed a well-supported clade, and their plastomes differed by 78 substitutions and 74 indels. Most of these differences were found in intergenic noncoding regions (52 substitutions and 67 indels) and introns (11 substitutions and 5 indels). The sequenced plastid genome of the hybrid species was most similar to *Pulsatilla pratensis*, which suggests that the analyzed *P. pratensis* specimen was pollinated by *P. patens*.

Key words: Natural hybrid, nucleolus organizer region, simple sequence repeat, intron-exon splice junction, plastid genome, next-generation sequencing

1. Introduction

Reticulate evolution is one of the key species-forming processes in the plant kingdom (Rieseberg, 1997; Arnold, 2004). Hybridization, the main driving force behind reticulate evolution, contributes to diversity, transfer of adaptive traits between species, and the formation of new hybrid zones (Reiseberg et al., 2003, Whitney et al., 2010, Abbott et al., 2013). Despite the above, hybridization can also decrease diversity (Levin et al., 1996) and lead to the extinction of one or both parental species (Rhymer and Simberloff, 1996) in just several generations (Huxel, 1999). Extinction induced by hybridization is caused by demographic swamping and genetic assimilation of rare species by more common species and their hybrids (Rhymer and Simberloff, 1996; Wolf et al., 2001).

Hybridization is widespread in plants, but estimates of its prevalence can vary considerably between regions and sources (Mallet, 2005; Whitney et al., 2010). Hybrids are found in 40% of plant families with an overall frequency of 0.09 hybrids per nonhybrid species (Whitney et al., 2010). Hybridization is most common among outcrossing species whose reproductive strategies contribute to hybridity, including vegetative reproduction, permanent odd polyploidy, or agamospermy (Ellstrand et al., 1996).

The genus *Pulsatilla* includes 33 species of herbaceous perennials and is characterized by a relatively high hybridization rate (Akeroyd, 1993). Hybridization in this genus has been widely documented within its European and Asian range. In Europe, 9 hybrids have been morphologically described (Hegi and Weber, 1975). Hybridization is most frequently observed between diploid species of the genus *Pulsatilla*. It is far less common between tetraploid species, where it is more frequently observed under laboratory conditions than in natural habitats (Lindell, 1998). Back-hybridization was also reported in the genus *Pulsatilla* (Hegi and Weber, 1975), mostly between species in hybrid zones (Zimmermann, 1964).

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The *Pulsatilla* species are widely distributed in Europe, and the most common hybrid of *P. pratensis* × *P. patens* = *Pulsatilla* × *hackelii* has been frequently noted in natural habitats in Europe. In natural habitats, hybrids of the genus *Pulsatilla* were described mainly based on their morphological characteristics (Hegi and Weber, 1975; Holub, 1978; Lindell, 1998). Morphometric analyses revealed that hybrids had characteristics intermediate between *P. pratensis* and *P. patens*. Detailed morphometric evaluations (Hegi and Weber, 1975; Holub, 1978) demonstrated that *Pulsatilla* × *hackelii* cannot be differentiated based on the color of its flowers, the number of leaf blade segments, or the length of the leaf base. The discussed hybrids are not sterile, but their fertility is decreased. The size of the hybrid genome has been estimated at 2C = 12.63 pg, which corresponds to the average size of its parental forms: *P. patens* at 2C = 11.78 pg and *P. pratensis* at 2C = 13.80 pg.

Analyses of morphological characteristics are the first step in the identification of hybrid plants in their natural habitats, but this approach has limitations, mainly in cases of introgression or in polyploid species complexes. Various molecular methods are used to detect hybridization. In recent years, molecular approaches (allozymes, DNA analyses) have delivered important insights into the role of hybridization and plant speciation (Reiseberg et al., 2000; Abbott et al. 2013). They include isoenzyme markers (Soltis et al., 1995); dominant markers based on AFLP, RAPD, and ISSR; and codominant specific SSR markers (Reiseberg et al., 1996). The popularization of sequencing methods and the knowledge about cytoplasmic heredity in plants (Baumel et al., 2002) facilitated the identification of hybridization and its direction. At present, hybridization research generally relies on analyses of selected regions of the chloroplast genome, which is useful for discriminating between the potential parental species and the nuclear genome, which is represented by the highly polymorphic ITS region and the codominant SSR markers. Next-generation sequencing (NGS) supports analyses of introgression and hybridization at the genome-wide level, including in nonmodel organisms. The introduction of high-throughput sequencers and the falling cost of sequencing a genome support the application of innovative genomic tools in hybridization analyses of nearly all organisms (Hohenlohe et al., 2011).

In this study, several classes of markers based on genotyping (SSR and ISJ) and sequencing (Sanger and NGS) were used to confirm hybridization and its direction, and to identify the prospective parent species of hybrids in the genus *Pulsatilla* with intermediate morphological characteristics, found growing in the same locality with *P. patens*, *P. pratensis*, and *P. vernalis*. In hybrids, analyses of the nuclear DNA sequence generally involve cloning of the resulting amplicons and analyses of multiple clones. NGS methods do not require this laborious process and speed up the acquisition of results, including sequences of nuclear, chloroplast, and mitochondrial genomes. These methods can also be used to determine the degree of introgression when significant differences in the number of marker sequences are noted for the identified parents (Twyford and Ennos, 2012).

2. Materials and methods

2.1. Materials

A hybrid species was found at the western foot of Bocheniec Mountain (the final hill of the Chęciny Range [Pasmo Chęcińskie], in the Świętorzyskie Mountains), approximately 1.3 km north of the village of Bocheniec near Małogoszcz (ATPOL grid system EE82). The hybrid species was localized on the edge of a fresh mixed coniferous forest (*Querco roboris-Pinetum*), in an area free of trees and shrubs, in the vicinity of a forest road. The hybrid species and other vascular plant species (mainly *Agrostis capillaris*, *Ranunculus serpens* subsp. *nemorosus*, *Clinopodium vulgare*, *Anemone sylvestris*, *Viola riviniana*, *Vaccinium vitis-idaea*, *Rubus saxatilis*, and *Campanula persicifolia*) formed a plant community in the herbal layer.

Pulsatilla pratensis and *Pulsatilla patens* subsp. *patens* (the parent species) were found in the vicinity of the described locality. *Pulsatilla pratensis* is quite common in this region. It occurs in numerous localities along the forest road where the hybrid species was found (Wnuk, 1986). Several tufts of *Pulsatilla pratensis* were observed at a distance of approximately 20 m from the locality of the hybrid. *Pulsatilla patens* subsp. *patens* is far less common in the analyzed region (Wnuk, 1986; Bróż and Przemyski, 1987; Bróż, 1990). It occurs in solitary localities with very few specimens. The previously reported localities of *Pulsatilla patens* subsp. *patens* in the surveyed region were not confirmed. In 2012, the species was observed at a distance of approximately 150 m from the locality of the hybrid specimen.

One leaf was sampled from 2 plants of each species in the evaluated region for genotyping. In the studied locality, *P. pratensis* and *P. vernalis* were represented by individual plants, whereas the *P. patens* population comprised several plants.

2.2. Morphological analysis

The morphological characteristics of *P. patens* and *P. vernalis* should be relatively easy to identify during the growing season. The species are well-identified morphological units that differ in the shape of the leaf blade and the depth of leaf indentation. *P. vernalis* has leaves with 3–5 deep segments, whereas *P. patens* has palmate leaves with three 2–3-pinnatisect segments. In early spring, the two species are highly similar, and they produce one large flower at the

top of a hairy stem. In *P. patens*, flowers are light blue to dark purple, whereas *P. vernalis* generally produces flowers that are light purple on the outside and white on the inside.

The phenotype of *Pulsatilla* × *hackeli* is morphologically intermediate between the parent species; however, it is morphologically variable (Čelakovský, 1865). In our study, morphological analysis was performed based on the following features: stem length, petiole length, morphology of the basal leaf, number of lateral segments in the leaf, length of the segments in the peak part of the leaf blade, width of the segments in the peak part of the leaf blade, and length of the perianth segments.

2.3. DNA extraction

Total genomic DNA was extracted from 3-month-old herbarium specimens. A small fragment of the leaf was homogenized in the Mini-Beadbeater-1 cell disrupter for 50 s, processed with the use of the Plant MiniSpin DNA kit (A&A Biotechnology) according to the manufacturer's protocol, and stored at –20 °C.

2.4. SSR and ISJ genotyping

Dominant intron-exon splice junction (ISJ) markers were amplified with ISJ4, ISJ5, and ISJ1 primers. Primer sequences and amplification conditions were identical to those described by Sawicki and Szczecińska (2011).

Codominant *Pulsatilla*-specific simple sequence repeat (SSR) markers were isolated in a previous study (Szczecińska et al., 2013). All primer pairs were tested for amplifications in *P. pratensis* and *P. vernalis*. Three primer pairs (loci) capable of amplifying SSR loci in all 3 species were selected for the experiment (Table 1). Amplification conditions and applied reagents were described in previous studies (Szczecińska et al., 2013, 2016).

The amplicons were separated in the QIAxcel capillary electrophoresis system (QIAGEN) using the QIAxcel High Resolution Kit with 15–2500 bp (ISJ) and 15–500 bp (SSR) alignment markers (QIAGEN). The applied size markers were 25–1000 bp (ISJ) and *Hae*III-puc (SSR) DNA size markers (QIAGEN). Standard OM500 and OM700 settings were used in the electrophoresis program for ISJ and SSR markers, respectively.

The reproducibility of the SSR and ISJ markers was checked by extracting and amplifying all samples twice. However, since there were no differences between repeated genotyping experiments, the error rate was not calculated.

2.5. NGS library preparation and sequencing

The quantity of DNA was estimated with the use of the Qubit fluorometer system (Invitrogen) using the Quant-IT ds-DNA BR Assay kit (Invitrogen). The quality of DNA was checked in 1% agarose gel. One nanogram of DNA was used to prepare the genomic library using the Nextera XT Kit (Illumina). The library validation procedure was described in a previous study (Szczecińska et al., 2014). The obtained libraries were sequenced with the MiSeq Reagent V2 Kit (Illumina) in a 500-cycle format. The resulting reads were assembled using the Velvet de novo assembler implemented in the Illumina Base Cloud service.

2.6. Data analysis

The obtained contigs were mapped in *Pulsatilla patens* (GenBank KQ134910), and the longest contigs of 7–11 kb were used to reconstruct the *Pulsatilla* hybrid plastome using a flow chart developed in a previous study (Szczecińska et al., 2014). The obtained plastid genome was annotated in Geneious 6.0.1 using the previously identified *Pulsatilla* genomes (Szczecińska and Sawicki, 2015) as a reference.

Table 1. Number of amplified alleles in the hybrid specimen and the analyzed *Pulsatilla* species.

Locus	Repeat motif	Primer sequence	Hybrid	*P. patens*	*P. patens*	*P. pratensis*	*P. pratensis*	*P. vernalis*	*P. vernalis*
*Pul*01	$(GCT)_4$	F: CACCTTGTCCACGGTTCTG R: ACCAGGTCAGAGAGCTCAAC	168	168	168	168	168	168	168
*Pul*04	$(CT)_6$	F: ACCGTTACTGTCCAACGGG R: CCTGTATGAATGCAACTTGACG	223	227	227	223	Null allele	227	227
*Pul*05	$(CT)_8$	F: GATTAATGGCGGGCGACAG R: TGGGTGTCGCTAATCGAGG	244	244	244	244	244	244	242
*Pul*06	$(ATT)_4$	F: TGGCATTCCTAGTTGAGGATGG R: GCTAGACAAACAAGAATCCCTGC	164	164	164	164	164	164	164
*Pul*07	$(AG)_6$	F: ATCCCGAGGGAGAATGCAC R: AAGCATGAGGTGTCTTGGC	332	332	331	Null allele	Null allele	332	331
*Pul*11	$(CTT)_5$	F: TCAATCAACCGCATGTAGAGC R: CACGTGTATTCGGCAGTCAG	302	302	302	302	302	302	302

The IGS regions were mapped on the previously identified 18-its1-5.8S-its2-26S (Szczecińska and Sawicki, 2015) regions using medium sensitivity settings in Geneious 6.0.1 with 100 iterations.

2.7. Phylogenetic relationships

The obtained plastid genome was aligned with all known Ranunculaceae plastomes using the Mauve genome aligner (Darling et al., 2010), excluding the second IR repeat. The chloroplast genome of *Nicotiana tabacum* was chosen for an outgroup. The *Pulsatilla* hybrid plastome was added to the previously published datasets and analyzed according to the method described by Szczecińska and Sawicki (2015).

3. Results

3.1. Morphological analysis

The analyzed specimen was a caespitose perennial herb with morphological characteristics intermediate between the parent species. Stem length ranged from 20 to 25 cm (during flowering), and the stems were entirely pubescent. Basal leaves were petiolate (sparsely haired petioles had the length of 6 cm). Leaf blades were ovate to oblong in outline, 4–6 cm × 3.5–5 cm in length, adaxially glabrous, with white abaxial hairs. Leaf blades were pinnately divided into 5 segments. Lateral segments were 2–3-pinnatisect, and the middle segment was 3-palmatisect. Less divided, irregularly shaped palmate basal leaves were rarely observed. Leaf segments were divided into narrow (2–4 mm), linear, and pointed lobes ranging from 30 to 45 mm, with a tuft of hairs on each apex. Cauline leaves were sessile and united on basal pubescent leaves. They were divided into 17–20 lobes with the width of 1.9–3.1 mm. Flowers were solitary, campanulate, 3–4 cm in diameter, and slightly nodding. Six perianth segments were purple-violet, oblong-ovate (25–27 × 11–13 mm), glabrous inside, sparsely hairy outside, and narrowly recurved at the apex.

3.2. Sequencing and mapping the hybrid genome

A total of 3,204,448 pair-end reads (2 × 250 bp) from *Pulsatilla* × *hackelii* were obtained in a single run of the MiSeq sequencer. Raw reads mapped on the plastid genome of the *Pulsatilla* hybrid resulted in 55× coverage. The structure of the hybrid plastid genome did not differ from the previously identified genomes of the *Pulsatilla* species (Figure 1).

A phylogenetic analysis of plastomes in the *Pulsatilla* species revealed three well-supported clades (Figure 2). The analyzed *Pulsatilla* species formed a distinct, well-supported clade. *P. vernalis* was resolved as a sister to *P. patens*, whereas *P. pratensis* was resolved as a sister to the *P. patens*/*P. vernalis* clade. The plastome of *Pulsatilla* × *hackelii* was included in the *P. pratensis* clade.

P. hackelii and *P. pratensis* formed a well-supported clade, and their plastomes differed by 78 substitutions and 74 indels. Most of these differences were found in intergenic noncoding regions (52 substitutions and 67 indels) and introns (11 substitutions and 5 indels). Only 12 SNPs were identified in protein-encoding genes. In most of these genes, only single synonymous mutations (*psb*B, *psb*D, *rps*11, *ycf*1) or nonsynonymous mutations (*psb*A, *rps*2, *rpo*B, *rpo*C2, *ycf*4) were found. Two SNPs were found in *rps*4 (synonymous) and *pet*B (one synonymous and one nonsynonymous). In the group of protein-encoding genes, the greatest differences were observed in the *ndh*F gene that contained 2 SNPs (one synonymous and one nonsynonymous) and a 15-bp-long indel.

The absence of differences in nuclear rRNA genes between *P. patens* and *P. vernalis* and minimal variations in ITS1 and ITS2 sequences made it practically impossible to identify the hybrid's origin. Species-specific SNPs, including 16 in the 5' direction and 10 in the 3' direction, were identified only when the IGS fragment was included in the 18SrRNA-ITS1-5SrRNA-ITS-26SrRNA region. The differences between *P. patens* and *P. vernalis* were used to determine the hybrid's origin and to identify the pollinator species as *P. patens* (Figure 3). The nucleolus organizer region (NOR) was covered more than 280 times. Around 120 copies were identical to the NOR sequence of *P. pratensis* and around 160 copies were identical to the ITS sequence of *P. patens*. The sequenced genome library did not contain sequences complementary to the analyzed regions of the chloroplast and nuclear DNA of *P. vernalis*.

3.3. ISJ and SSR genotyping

The applied ISJ primers – ISJ4, ISJ5, and ISJ11 – revealed 26, 33, and 21 loci, respectively. All amplified loci were polymorphic. The number of species-specific loci ranged from 1 for the ISJ11 primer of *P. patens* to 9 for the ISJ4 primer of *P. pratensis*. In total, ISJ markers revealed 23 bands specific for *P. pratensis*, 17 bands specific for *P. vernalis*, and 11 bands specific for *P. patens*. The lowest number of species-specific bands (3) was noted in the hybrid specimen (*Pulsatilla* × *hackelii*) (Table 2). *P. patens*, *P. vernalis*, and *P. pratensis* shared 7, 9, and 15 bands with the hybrid specimen, respectively (Table 3). Six out of the 12 identified SSRs for *P. patens* successfully amplified products in the hybrid specimen and the remaining *Pulsatilla* species. Three of the analyzed loci were monomorphic (*Pul*01, *Pul*06, and *Pul*11), whereas the *Pul*05 locus revealed only an intraspecific polymorphism in *P. vernalis* (Table 1). Hybrid heterozygosity was not observed in any of the remaining loci. The *Pul*04 locus in the hybrid specimen contained an identical allele to that found in *P. pratensis*, whereas the *Pul*07 locus harbored an allele that was also found in one *P. patens* plant and one *P. vernalis* plant.

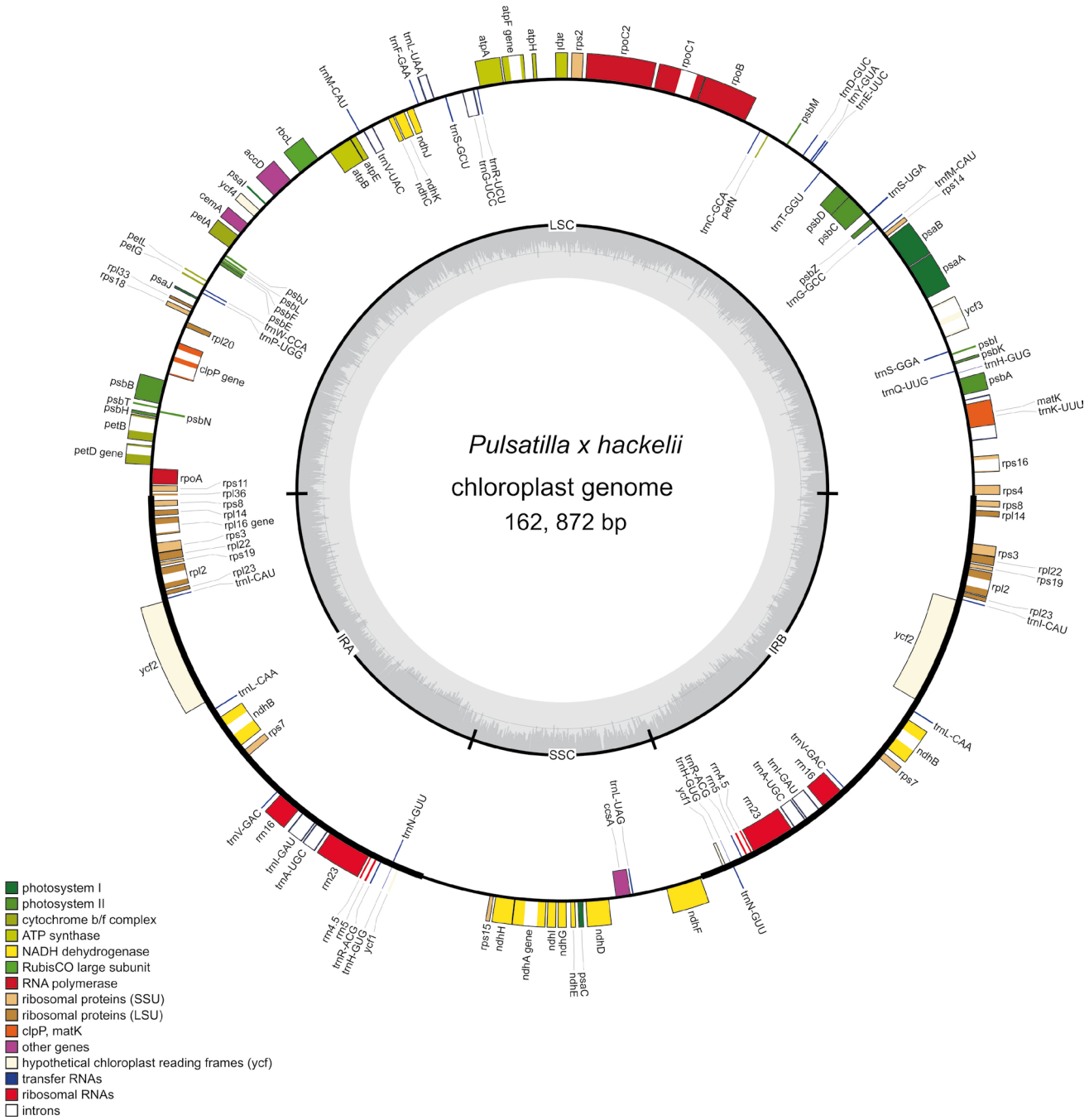

Figure 1. Gene map of the *Pulsatilla* × *hackelii* plastid genome. Genes outside the outer circle are transcribed clockwise and genes inside the outer circle are transcribed counterclockwise. Genes belonging to different functional groups are color-coded.

4. Discussion

Potential interspecies hybrids of the genus *Pulsatilla* were described mainly based on their morphological characteristics (Zimmermann, 1964; Hegi and Weber, 1975; Lindell, 1998), and the hypotheses formulated based on analyses of chloroplast DNA only (Lee et al., 2010) were not confirmed by nuclear genome data (Sun et al., 2014).

The origins of a hybrid specimen and its parents may be difficult to identify due to the presence of several hybridizing species in the vicinity. The identification of parents based on morphological characteristics only is burdened with the risk of error. Genome size analyses can also produce misleading results, in particular when the genomes of hybrid specimens are similar in size. The

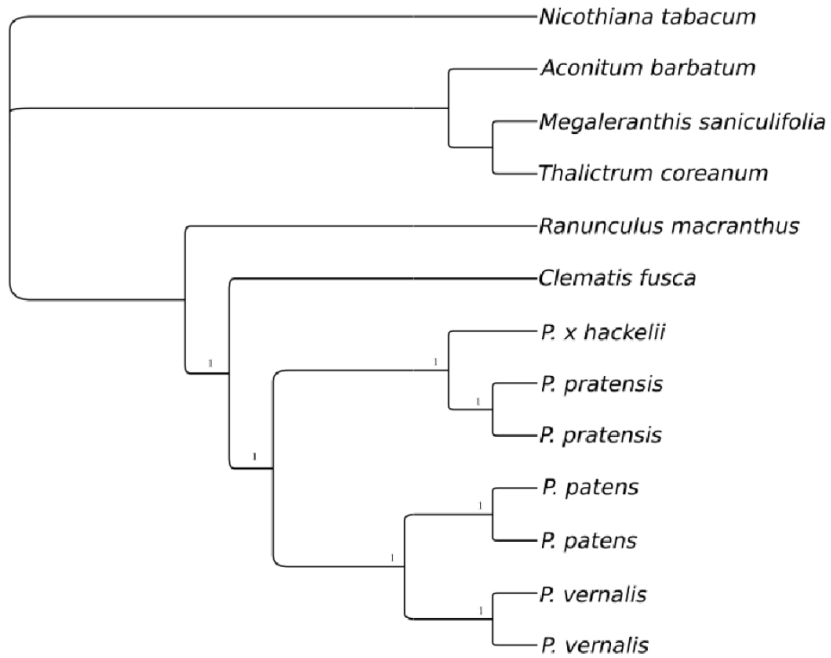

Figure 2. Phylogenetic relationships between the studied species determined in Bayesian analysis.

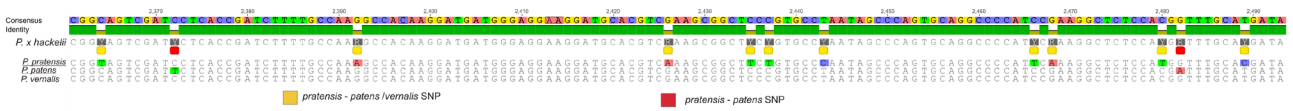

Figure 3. Based on a polymorphism in the NOR-IGS region, the pollinating species was identified as *P. patens*. Yellow marks denote common SNPs for *P. pratensis*-*P. patens*/*vernalis*, and red marks denote common SNPs for *P. pratensis*-*P. patens*.

Table 2. Number of amplified bands for the analyzed *Pulsatilla* species.

Primer		Hybrid	*Pulsatilla patens*	*Pulsatilla pratensis*	*Pulsatilla vernalis*
ISJ4	Number of bands	11	8	11	14
	Number of specific bands	1	2	9	6
ISJ5	Number of bands	8	12	15	13
	Number of specific bands	2	8	7	8
ISJ11	Number of bands	7	9	12	11
	Number of specific bands	0	1	7	3
Total	Number of bands	25	29	38	38
	Number of specific bands	3	11	23	17

results of molecular analyses presented in this study clearly point to the hybrid origin of the evaluated species. The presence of two NOR genotypes characteristic of *P. patens* and *P. pratensis* in the hybrid genome suggests that those species are the parents of the analyzed specimen, which was identified as *Pulsatilla* × *hackelii*. The analyzed hybrid was often identified in Europe based on the distribution of *P. patens* and *P. pratensis* and similar habitat preferences. To date, the hybrid origin of *Pulsatilla* × *hackelii* was usually confirmed based on its morphology, which revealed

Table 3. Number of bands shared by the hybrid and the analyzed *Pulsatilla* species.

Primers	*P. patens*	*P. pratensis*	*P. vernalis*	Total
ISJ4	1	7	2	10
ISJ5	2	2	4	8
ISJ11	4	6	3	13
Total	7	15	9	

that hybrids had characteristics intermediate between *P. pratensis* and *P. patens*. Detailed morphological evaluations (Hegi and Weber, 1975; Holub, 1978) demonstrated that *Pulsatilla* × *hackelii* cannot be differentiated based on the color of its flowers, the number of leaf blade segments, or the length of the leaf base.

The hybrid genome did not harbor two different copies of the plastid genome, which points to the maternal inheritance of cpDNA, the most common variant in angiosperms (Sears, 1980). The sequenced plastid genome of the hybrid was most similar to *Pulsatilla pratensis* (Figure 2), which suggests that the *P. pratensis* specimen was pollinated by *P. patens*. The hybrid's chloroplast genome differed from the genomes previously identified in *P. pratensis* (Szczecińska and Sawicki, 2015) by more than 150 SNPs, all of which were observed in plastome intergenic regions. Deep sequencing of the genome did not reveal any SNPs in the analyzed cpDNA regions with frequency higher than 5%, which points to the presence of copies of the chloroplast genome from one parent. Lee et al. (2010) analyzed three chloroplast loci and concluded that *P. tongkangensis* has a hybrid origin, which was not confirmed by analyses of the nuclear ITS region (Sun et al., 2014). The presence of SNPs in plastid sequences of *P. tongkangensis* was examined to verify the hybridization hypothesis, but maternal inheritance of cpDNA was not taken into account. Duplication of those regions in the chloroplast genome and heteroplasmy cannot be ruled out. These processes have never been identified in the genus *Pulsatilla*, but they are increasingly often reported in other plant genera (Sabir et al., 2014; Szczecińska et al., 2014).

The pollinated plant of the parental generation is relatively easy to identify, but the identification of the pollinating plant is far more complicated. *P. patens* and *P. vernalis*, the species noted in the vicinity of the hybrid's locality, are very closely related. They share many morphological characteristics during inflorescence and are bound by a close phylogenetic relationship (Ronikier et al., 2008; Szczecińska and Sawicki, 2015).

Close relationships between *P. patens* and *P. vernalis* were also noted at the level of the chloroplast genome, where the *trn*L-F and *trn*H-*psb*A regions, which are usually polymorphic and are used as markers for barcoding (Kress et al., 2005), differed only in the length of the homopolymer regions (Ronikier et al., 2008). A comparative analysis of complete plastid genomes revealed only 49 SNPs characteristic of *P. patens*, 41 SNPs characteristic of *P. vernalis*, and 294 SNPs characteristic of *P. pratensis* (Szczecińska and Sawicki, 2015). Reliable identification of hybrids requires an analysis of the nuclear genome, which should contain copies of both parent genomes. Previously identified nuclear material of the *Pulsatilla* species was limited to SSR markers (Szczecińska et al. 2013, 2016; Bilska and Szczecińska, 2016), ISJ markers (Bilska and Szczecińska, 2016), and rRNA clusters (Szczecińska and Sawicki, 2015).

An analysis of the complete 18s-ITS1-5, 8S-its2-26S region with the size of 5762 bp revealed only one SNP in the ITS2 region, and this information was used to discriminate between *P. patens* and *P. vernalis*, but the analysis did not account for intergenic spacer (IGS) regions between 26S rRNA and 18S rRNA (Szczecińska and Sawicki, 2015). The IGS spacer is by far the most variable part of rRNA, and it is highly useful in microevolutionary phylogenetic studies (Hamby and Zimmer, 1992). Despite the absence of universal amplification primers, these regions often supported the resolution of species boundaries between closely related taxa (Tucci et al., 1994). The expansion of the NOR region by 1752 bp in the 5' direction and by 1899 bp in the 3' direction supported the identification of 26 SNPs differentiating *P. patens* and *P. vernalis* (Figure 3) and enabled the identification of *P. patens* as the pollinating species.

SSR markers are often used in hybridization research (Snow et al., 2010), but they failed to produce the anticipated results in this study. The origin of the analyzed hybrid was not clarified by SSR markers. The evaluated loci were not heterozygous, and they harbored alleles characteristic of *P. pratensis* or *P .patens*/*P. vernalis*. The examined specimens of *P. patens* and *P. vernalis* harbored SSR alleles of the same size, which prevented the identification of species-specific alleles. The absence of heterozygosity in the evaluated hybrid could be attributed to hybrid incompatibility in one of the chromosomes (Huang et al., 2015) or the presence of null alleles in parents.

SSR markers provided additional evidence that the evaluated species were closely related, because they are generally effective in molecular delimitation of species, even at early stages of divergence (Karlin et al., 2008). The presence of hybridization was also validated by the ISJ markers that revealed amplicons originating from both parents. The potential of dominant ISJ markers in molecular differentiation of closely related species was reported in previous research (Szczecińska et al., 2006; Sawicki et al., 2012) and in studies of allopolyploid species (Sawicki and Szczecińska, 2011).

The negative influence of hybridization on the survival of rare and endangered plant species has been reported by many authors (Levin et al., 1996; Antilla et al., 1998). Hybridization can lead to genetic introgression from common species to rare species with a narrow geographic range (Petit et al., 1997), and it can contribute to the loss of features characteristic of rare species (Levin et al., 1996; Rhymer and Simberloff, 1996). However, the effects of hybridization are determined by many factors, including the initial hybridization rate and the relative fitness of the resulting hybrid individuals.

Hybridization has negative connotations in plant protection biology, but this common and natural phenomenon is not always provoked by human-caused habitat disturbance. Hybridization can increase genetic diversity and set the template for adaptive evolution and hybrid speciation, a major evolutionary force in the diversification of plants (Stebbins, 1950; Reiseberg et al., 2003). There is no clear evidence to indicate that hybridization can lead to the extinction of a population at a faster rate than self-extinction (Wolf et al., 2001). Hybridization is very important for the development of new evolutionary pathways, and instead of eliminating hybrids whenever they come into contact with rare species, conservation biologists should develop new methods for predicting the outcomes of interactions between species (Wolf et al., 2001).

The presence of natural hybrids in the genus *Pulsatilla* does not yet pose a threat for any of the species evaluated in this study. Over the centuries, many species have developed natural hybridization zones in peripheral areas of their geographic range that do not affect the genetic structure of populations remaining within the range of the parental species. *P. patens* and *P. pratensis* populations generally colonize different habitats, and most localities where both species were found also differed in the phenology of flowering. Further research into the taxonomic status of *P. patens* and *P. vernalis* is required, with the involvement of specimens from the entire geographic range.

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