Phylogenetic and expression analysis of CENH3 and APOLLO genes in sexual and apomictic Boechera species

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Abstract: Apomictic plants (reproducing via asexual seeds), unlike sexual individuals, avoid meiosis and egg cell fertilization. Consequently, apomixis is very important for fixing maternal genotypes in the next plant generations. Despite the progress in the study of apomixis, molecular and genetic regulation of the latter remains poorly understood. So far APOLLO (Aspartate Glutamate Aspartate Aspartate histidine exonuclease) is one of the very few described genes associated with apomixis in Boechera species. The centromere-specific histone H3 variant encoded by CENH3 gene is essential for cell division. Mutations in CENH3 disrupt chromosome segregation during mitosis and meiosis since the attachment of spindle microtubules to a mutated form of the CENH3 histone fails. This paper presents in silico characteristics of APOLLO and CENH3 genes, which may affect apomixis. Also, we characterize the structure of CENH3, study expression levels of APOLLO and CENH3 in gynoecium/silique of the natural diploid apomictic and sexual Boechera species at the stages of before and after fertilization. While CENH3 was a single copy gene in all Boechera species, the APOLLO gene have several polymorphic alleles associated with sexual and apomictic reproduction in the Boechera genera. Expression of the APOLLO apo-allele during meiosis was upregulated in gynoecium of apomict B. divaricarpa downregulating after meiosis until 4th day after pollination (DAP). On the 5th DAP, expression in apomictic siliques increased again. In sexual B. stricta gynoecium and siliques APOLLO apo-allele did not express. Expression of the APOLLO sex-allele during and after meiosis in gynoecium of sexual plants was several times higher than that in apomictic gynoecium. However, after pollination the sex-allele was downregulated in sexual siliques to the level of apomicts and increased sharply on the 5th DAP, while in apomictic siliques it almost did not express. At the meiotic stage, the expression level of CENH3 in the gynoecium of apomicts was two times lower than that of the sexual Boechera, decreasing in both species after meiosis and keep remaining very low in siliques of both species for several days after artificial pollination until the 4th DAP, when the expression level raised in sexual B. stricta siliques exceeding 5 times the level in apomictic
B. divaricarpa siliques. We also discuss polymorphism and phylogeny of the APOLLO and CENH3 genes.

Keywords: Boechera; Brassicaceae; CENH3; APOLLO; apomixis associated genes; cell division; apomeiosis

1. Introduction

Sexual reproduction is the main mode of reproduction of flowering plants. The major features of sexuality are meiosis and fertilization, the latter occurs through the fusion of haploid female and male gametes, giving rise to the formation of the genetically variable progeny. Genetic mutations and meiotic recombination provide permanent genetic changes, which are the source for evolution and adaptation of the population, as well as the basis for selection in agriculture. Despite the fact that sexual reproduction is energetically very expensive, the advantage of sex is that it forms a combination of useful mutations that are absent in asexual organisms [1,2]. Thus, the benefit of sexuality is that it helps to get rid of harmful mutations (Hill-Robertson effect) [3,4] and produces useful traits and variability. However, apart from the high energy costs, the disadvantage of sexual reproduction is the segregation of beneficial traits in subsequent generations, so that the offspring can lose useful combinations of their parental genes [5,6].

One of the mechanisms that can produce “clones” of mother plants is apomixis, which is a mode of asexual reproduction through seeds that has been identified in over four hundred of plant species [7,8]. In apomicts, meiosis and fertilization are modified or completely absent. Consequently, the embryo is formed without prior meiosis (by apomeiosis) and fertilization (i.e. by parthenogenesis), while endosperm development occurs either autonomously, i.e. without fertilization, or pseudogamously (by fertilization of the central cell) [6,8–13]. During double fertilization, intrinsic to all Angiosperms, pollen tube transports two sperm cells (male gametes) to the embryo sac, one of which fertilizes the egg cell (female gamete) and the second one fertilizes the central cell, giving rise to a 2n embryo and a 3n endosperm, respectively. In sexuals departure from the 2 maternal:1 paternal (2m:1p) genome ratio in endosperm nuclei results in seed abortion. While in pseudogamous apomicts, endosperm ploidy varies according to the ploidy of the sperm and central cell. Deviations from 2m:1p genome ratio sometimes occur, demonstrating that the apomictic system is more resilient compare to sexuals [14].

Apomorphic plants, forming genetically identical offspring, dramatically influence the structure of the population, thereby being excellent models for studying the mechanisms of the onset of meiosis and its replacement by apomeiosis, the formation of a seed in the absence of fertilization or under pseudogamy; as well as the important ecological role of apomixis in the origin of polyploids and speciation [15,16].

However, so far, little is known about the molecular background of apomixis and the genes associated with its triggering [17].

In this paper, we characterized the protein structure, evolution, and expression patterns of the APOLLO (for APomixis-Linked LOcus; encodes Aspartate Glutamate Aspartate Aspartate
histidine exonuclease) gene, one of the important genes associated with apomixis in *Boechera* genus. It was shown that *APOLLO* have several polymorphic sex- and apo-alleles [18,19]. Apo-alleles of this gene are missing in sexual ovules and are up-regulated in apomeiotic ovules in *Boechera* plants. It was reported that genomes of apomictic plants are always heterozygous carrying at least one of the apo-alleles, while sexual genotypes were always homozygous for sex-alleles [18,19]. We showed that while sex-alleles were upregulated during meiosis in sexual plants and downregulated at the same stage in apomictic *Boechera* plants. Evolutionary *APOLLO* gene analysis presented in this study shows that sexual and apomictic species of *Boechera* are clustered in different clades in the phylogenetic tree based on the multiple protein sequence alignment. This could be partly because the *APOLLO* apo-alleles present in the genomes of apomictic species could acquire a new function [19]. Expression analysis of the *APOLLO* presented here showed that the expression levels of the gene dramatically decreased during meiosis in gynoecia of both apomictic and sexual species, however after meiosis and fertilization, the expression of the *APOLLO* was upregulated in apomictic siliques compared to sexual ones.

We also characterize *CENH3* gene, which is essential for cell division [20,21] and might affect the apomictic events. *CENH3* encodes the centromere-specific histone H3 variant. The accumulation of *CENH3* provides an assembly site for a protein complex called kinetochore. The main function of the kinetochore is to bind chromosomes to spindle fibers during chromosome segregation in meiosis and mitosis [22]. *CENH3* harbor two domains, a DNA-binding histone fold domain (HFD) and N-terminal tail domain. HFD is structurally similar to the same domain in H3 and is highly conserved across higher eukaryotes. However, the N-terminal tail of the *CENH3* protein is highly variable even among closely related species and has meiosis-specific function [23]. Mutations at the N-terminal tail of *CENH3* disturb chromosome segregation in meiosis and often lead to sterility [20,24]. Null mutations in *CENH3* also cause chromosome elimination and were proposed as a tool to produce haploid plants [25–28].

In *Arabidopsis thaliana*, the *CENH3* mutants act like a haploid inducer parent when cross with a wild type plant [25,27] and the chromosomes in these mutants were lost in the developing embryo. The resulting plant contains a haploid set of the wild-type parent chromosomes, which can be induced to double its number. We investigated the structure and the expression profiles of *CENH3* in apomictic and sexual *Boechera* species (Brassicaceae) to further understand its role in meiotic chromosome segregation.

Plants from the *Boechera* genus are attractive models for research because both sexual and apomictic accessions are present within this genus. Moreover, plants from the *Boechera* genus are close relatives of *Arabidopsis thaliana*, which is very well studied in terms of molecular genetics and functional gene annotation [29–31]. In apomictic *Boechera* species, the *Taraxacum* type diplospory with pseudogamous endosperm development that requires fertilization of the central cell is reported [31–34]. Recently, the Hieracium type apospory and antennaria-type diplospory have also been reported in various *Boecehra* taxa [35]. A special attention deserves apomictic accessions of *Boechera divaricarpa*, which is known as an interspecific hybrid between sexual species *B. stricta* and *B. retrofracta* or a closely related species [36]. Although diploid apomixis is an extremely rare condition in plants [37], both diploid and triploid apomictic *B. divaricarpa* lineages have been reported [31,38].
Our study revealed CENH3 expression levels in the gynoecia/siliques during sexual and apomictic development. During meiosis and pollination CENH3 expression demonstrated decreasing levels in both sexual and apomictic gynoecia and in the first day after pollination showed an increase in expression but in the gynoecia of sexual plants expression was almost two-fold higher than that in the gynoecia of apomicts. These results may indicate to a possible role that CENH3 might play in apomictic development in *Boechera* species.

2. Materials and Methods

2.1 Plant material

The diploid sexual *B. stricta* (ES6, ID 500206; DG; DQ013050) and apomictic *B. divaricarpa* (ES9, 500209; BS) seeds were kindly obtained from Dr. Eric Schranz (Wageningen University & Research, Netherlands). *Boechera* plants were grown as described in [38, 39]. Seeds of ES6 and ES9 accessions were germinated on moist filter paper after vernalization at 4°C for 3 weeks in the dark to break seed dormancy. Then petri dishes containing the seeds were transferred into a growth chamber under the condition of 16 h light: 8 h dark (21°C). Germinated seeds were transferred to a peat: perlite mix (1:4) for 4 weeks. Then the plants were grown at 10° C for 6 weeks. Flowering of the plants started in 6 weeks after the vernalization.

Emasculation was performed on unopened flower buds by removing anthers with fine forceps. Hand pollination of stigmas was carried out with pollen from the same plant. Gynoecia/ siliques were collected one day before pollination (during meiosis according to gynoecium size, see [40]), right after pollination (after meiosis), on the 1st, 3rd, 4th and 5th days after pollination. Anthers were collected during and after meiosis (see Figure 1 [40]). The collected samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.
2.2 APOLLO and CENH3 genes retrieval and pre-processing

Sequences of the APOLLO (Aspartate Glutamate Aspartate Aspartate histidine exonuclease) and CENH3 (histone H3-like centromeric protein) genes in various Boechera species were obtained from the sources listed in table 1 (also see in [31]). For APOLLO phylogenetic analysis, additionally 10 accessions from [18] listed in table 2 (5 apo-alleles, 5 sex-alleles) were used.

Table 1. Genome sequencing data for Boechera genus

| Species      | Reproduction mode | Raw data NCBI accession | Genome assembly | Genome annotation | Reference |
|--------------|-------------------|-------------------------|-----------------|-------------------|-----------|
| B. stricta   | Sexual            | SRR396760, SRR396762, SRR396756 | Yes            | Yes               | [41]      |
| B. retrofracta | Sexual          | SRR3929707              | Yes            | Yes               | [19]      |
| B. puberula  | Sexual            | ERX2578777, ERX2578776  | Yes            | No                | [42]      |
| B. spatifolia | Sexual           | SRR5116719              | No             | No                | [43]      |
Boechera accessions used for investigation of APOLLO gene (from [18])

| GenBank accession | Sample ID | Allele type |
|-------------------|----------|-------------|
| KF705583.1        | 369S2_S3 | Sex-allele  |
| KF705582.1        | 376S2_S5 | Sex-allele  |
| KF705581.1        | 355S2_S3 | Sex-allele  |
| KF705569.1        | 329S2_S1 | Sex-allele  |
| KF705572.1        | 385S2_S11| Sex-allele  |
| KF705596.1        | 43A3_A3  | Apo-allele  |
| KF705598.1        | 1A2_A6   | Apo-allele  |
| KF705600.1        | 11A2_A1  | Apo-allele  |
| KF705599.1        | 11A2_A3  | Apo-allele  |
| KF705588.1        | 33A2_A5  | Apo-allele  |

For the species with available assembled and annotated genome a BLAST search over extracted proteins datasets was performed using *A. thaliana* peptide sequences as a query. For the other species (all *B. spatifolia* accessions from research [43] as well as *B. arcuata* and *B. divaricarpa* accessions) a local assembly using SRAssembler v1.0 was performed [44]. Consistency of the loci assembly results were checked with another approach, in which a whole-genome draft assembly was performed using Platanus software v1.2.1 [45] with a consequent genes search via Exonerate v2.2 run in protein2genome mode [46]. All the mentioned tools were run with default parameters.

The resulting parameters of the obtained assemblies are listed in Table 1S (Supplement). As one can see these assemblies have rather modest characteristics (N50 of about a few kilobases) as predicted earlier in [31]. Insufficient genome coverage in the datasets (30x in average), short reads length and high complexity of the genomes resulted in the fact, that most of the *B. spatifolia* accessions reported in [43] were excluded from the further analysis. In the excluded datasets, a significant divergence of the obtained sequences between two assembly
methods and abnormally low their similarities with homologs in the other Brassicaceae species were detected. Thus, only Rosita3 accession was accepted for CENH3 analysis. For APOLLO analysis Rosita3, Tiesiding2, Chicago2 and Royal2 accessions were accepted. In B. puberula and B. perrenas assemblies APOLLO gene was incomplete and thus these accessions were also excluded from the further gene analysis. It is worth noting that a short read length didn’t allow us to perform unambiguous diploid assemblies and distinguish different copies and alleles of the considered genes, potentially presenting in the genomes. Thus, all the downstream analysis was performed with consensus variants of the assembled genes. Sequences for non-Boechera species were retrieved directly from Phytozome v. 12.1 [47] and Uniprot [48] databases.

2.3 General characteristics of CENH3 gene

CENH3 gene length and exon structure were directly extracted from both SRAssembler and Exonerate output. The molecular weight and isoelectric point of the proteins were calculated with Expasy’s ProtParam server [49]. Subcellular localizations of the Boechera proteins were predicted via CELLO v.2.5: subcellular localization predictor [50]. Conserved regions were investigated with CDD (Conserved Domain Database) [52]. The promoter regions were analyzed for regulatory motifs using PLANTCARE database [52].

2.4 Evolutionary analysis of the CENH3 and APOLLO genes

Multiple alignment of the obtained amino acid sequences was performed with MUSCLE algorithm [53] and then visualized with Jalview v2 [54]. The evolutionary history was inferred by the Maximum Likelihood method and w/freq. model [55]. As a result, the tree with the highest log likelihood was reconstructed. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1000 iterations were performed). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X [56]. Heatmaps visualization was performed by R package Pheatmap [57].

2.5 CENH3 and APOLLO genes expression studies

Total RNAs were isolated from gynoecium and anthers during meiosis and siliques 1-5 days after pollination (DAP) according [40] with Purelink RNA mini kit (Invitrogen – 12183018A) and TRIzol reagent (Invitrogen, 15596018). RNA quality was checked on 1% agarose gel electrophoresis, the concentration of RNA was measured with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Before the cDNA synthesis, 1 µg of total RNA was treated with Rnase-free Dnase I (Thermo Scientific, EN0521) for 30 minutes at 37 °C. Reverse transcription reactions were performed using 1 µg of total RNA and High-Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Expression of the CENH3 and APOLLO genes were analyzed with the 7500 real-time PCR system (Applied Biosystems) according to the manufacturer’s instructions. The efficiency of the primers used in the study was evaluated with the standard curve experiments, only the primers with 90%-100% efficiency were used. UBQ (Polyubiquitin) gene was used as the endogenous control. No template (water instead of
the template) and – RT (water instead of reverse transcriptase enzyme in cDNA synthesis) controls showed negative amplification. Primers sequences were BOECHUBQ_F: 5’-GGCTAAGATCCAGGACAAGGAAGGTAT-3’, BOECHUBQ_R: 5’-CTGGATGTTTATAGTCAGCCAAAGTGCG-3’ for UBQ amplification; APOLLO_F: 5’-CGGAGTTCTCTCTGCACCTAC 3’ and APOLLO_R: 5’-TTCGTCCGTGGAGAATGTCG-3’ for APOLLO analysis; Cenh3_F 5’-CAACTCCTACAACCTCACCAGCTACTG-3’ and Cenh3_R 5’-TTGTGAACCTTGTGGCCTAGCATATC-3’ for CENH3 analysis. All reactions were performed in 3 biological and 3 technical replicates. Reactions included 10 µl SYBR Green PCR Master Mix (Applied Biosystems – 4367659), 0,6 µl primers (10 µM stock), 1 µl cDNA template (50 ng/µl), 7,8 nuclease-free water. The PCR cycling conditions were as follows: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s for 40 cycles. The data were analyzed with expression suite software (Applied Biosystems) with the ∆∆Ct method.

3. Results

3.1. Characteristics of CENH3 gene and protein isoforms

General characteristics of the CENH3 gene and CENH3 protein in seven studied Boechera species is given in table 3. In all these species CENH3 protein isoforms showed high similarity to each other with similarity index not less than 97% (at both the nucleotide and protein levels). All Boechera CENH3 protein sequences contained a Histone H3/CENP-A (IPR000164) conserved domain, were 177 a.a. long and predicted to be localized in the nucleus. CENH3 protein alignment results are represented in Fig. 2.

Table 3. General characteristics of CENH3 gene and CENH3 protein in studied seven Boechera species.

| Parameter                  | Value                           |
|----------------------------|---------------------------------|
| Gene length                | 2231 - 2298 b.p.                |
| Number of exons            | 10                              |
| Protein length             | 177 a.a.                        |
| Molecular weight           | 19616.82 ± 60.4                 |
| Theoretical pl             | 11.25 ± 0.10                    |
| Subcellular Localization   | Nuclear                         |
| Conserved Domains          | Histone H3/CENP-A (from 57 a.a. to 172 a.a.) |
Among the seven investigated Boechera species 10 polymorphic amino acid positions in CENH3 protein were detected (listed in Table 4). B. stricta and B. divaricarpa, which were further subjected to the comparative expression analysis of CENH3 gene, had only two polymorphic amino acids in N-tail positions 7 and 16, which are located out of the Histone H3 conserved domain. Variability within the N-terminal tail might affect the chromosome segregation in meiosis [20,24] and lead to apomeiosis.

Table 4. Polymorphic positions in CENH3 protein
CENH3 similarity heatmap matrix and reconstructed phylogenetic tree are shown in Fig. 3, A and B respectively. The heatmap demonstrates that CENH3s in all Boechera species are almost identical, which is consistent with the protein alignment analysis (Fig. 2) and are very similar (≥80%) to Arabidopsis and most of the other representatives of Brassicaceae studied. CENH3 gene is represented by only one copy per genome of each investigated species. The evolutionary history was estimated with the help of the highest log likelihood. The reconstructed phylogenetic tree (Fig. 3B) was rooted with Capsella spp. as an out-group. All Boechera species were grouped in one clade, which was separated from Arabidopsis spp., Eutrema spp., Cardamine sp., suggesting that unlike APOLLO (see paragraph 3.5) CENH3 protein sequences do not reflect the mode of propagation within the Boechera spp.
Figure 3. (A) Similarity matrix and (B) Phylogenetic tree of CENH3 (histone H3-like centromeric protein) in seven species of interest. Sequences of *Capsella* species were used as an outgroup. All *Boechera* species are grouped in one clade. Numbers near nodes represent corresponding bootstrap support. Phylogenetic tree was reconstructed using Maximum Likelihood method.
3.2 CENH3 promoter characteristic

Since it was found that genomes of all studied species contain only a single copy of the CENH3 gene, we decided to investigate and compare the structure of the CENH3 gene promoters in two species, sexual B. stricta and apomictic B. divaricarpa. 1000 bp upstream regions from the first codon (ATG) of BsCENH3 and BdCENH3 genes were analyzed. Several regulatory elements and core promoter regions like TATA and CAAT-box within both CENH3 promoters were found. Among the regulatory motifs, MYB transcription factors recognition and binding sites were common for both CENH3 promoters. MYB transcription factors are encoded by several genes that control plant development, differentiation, stress resistance, and defense [58]. The presence of several MYB binding motifs might indicate to a role of MYB transcription factors in CENH3 regulation. E2F transcription factor binding sites at -163 positions of BsCENH3 and BdCENH3 were found as well. At -115 site BdCENH3 and BsCENH3 had a single polymorphic site (GCGGGAAA/ GCGGGAAG). Promoter regions also included several light response motifs like Box 4, GT1-motif and TCT-motif. Hormone response elements like gibberellin responsive element (GARE-motif) and auxin-responsive elements (TGA-element) were detected in the CENH3 promoters of both species. A cis-acting regulatory element involved in the MeJA-responsiveness (CGTCA-motif), cis-acting element involved in low-temperature responsiveness (LTR), cis-acting regulatory element involved in circadian control, cis-acting regulatory element related to meristem expression (CAT-box) and cis-regulatory element involved in endosperm expression (GCN4_motif) were also found in the CENH3 promoter (Fig. 4). Our research of promoter regions of BsCENH3 and BdCENH3 genes did not reveal significant differences (Fig. 4). Therefore, we decided to check the expression levels of BsCENH3 and BdCENH3 genes in the meiotic gynoecia and young siliques just after fertilization.

![Figure 4. Motifs found in 1000 bp upstream of the transcription start site of BsCENH3 and BdCENH3](image)

3.3 CENH3 expression during and after meiosis in gynoecia/siliques and anthers

Comparative expression analysis of CENH3 gene was performed in gynoecia and anthers from unopened flower buds (before pollination) and in siliques 1-5 DAP (Fig. 5A; Table 2S in Supplement) to cover developmental stages around meiosis, gametophyte development, and seed set in diploid apomict B. divaricarpa and diploid sexual B. stricta. For investigation after the pollination, only the extending siliques were used to make sure that the pollination has
occurred, and seed development has started. It was found that, during meiosis/ apomeiosis, the CENH3 gene is intensely expressed in gynoecium of both sexual B. stricta and apomictic B. divaricarpa, over two folds higher expression level was observed in B. stricta. CENH3 was significantly downregulated after meiosis, yet the expression level in B. stricta was higher than that in B. divaricarpa (Fig. 5A). During three days after pollination, the level of CENH3 expression in siliques remained low in both studied species. On the 4th DAP, the expression of CENH3 significantly increased in siliques of B. stricta and was more than 6 times higher than that in B. divaricarpa, while in the latter it remained practically unchanged. The same trend persisted in siliques of both species on the 5th DAP, however, the difference between sexual and apomictic species was less and differed by 3.7 times (Fig. 5A).

We also investigated the expression levels of the CENH3 gene in B. stricta and B. divaricarpa anthers around meiosis. During meiosis in anther tissues the level of the CENH3 expression in B. stricta was 1.5 times lower than in B. divaricarpa. By the end of meiosis, expression of CENH3 was down regulated almost completely in anthers of both species (Fig. 5A).

The observed difference in the level of CENH3 gene expression between sexual and apomictic accessions could be either due to differences in regulation of expression by the transcription factors and genes responsible for their expression or due to epigenetic factors that might regulate the expression at a post-translational level. A decreased level of expression of CENH3 during and after meiosis in the generative organs of apomictic B. divaricarpa compared with sexual B. stricta may be one of the reasons of the meiosis disturbance in apomicts.
Figure 5. Expression levels of CENH3 and APOLO genes in diploid apomict and sexual Boechera plants: B. divaricarpa and B. stricta in (A) Expression patterns of CENH3; (B) Expression patterns of APOLO: 1) Expression of APOLO using universal primers for both sex- and apo-alleles. 2) Expression of APOLO apo-alleles in anthers, gynoecium, and siliques of apomictic B. divaricarpa and sexual B. stricta plants. 3) Expression of APOLO sex-alleles in anthers, gynoecium, and siliques of apomictic B. divaricarpa and sexual B. stricta plants. Bars indicate ± standard errors of fold change levels. Days After Pollination (DAP).

3.4 APOLO expression during and after meiosis in gynoecia/siliques and anthers
Along with the analysis of the CENH3 gene dynamics, the expression of the APOLLO gene apo- and sex- alleles were studied during and after meiosis in gynoecium and anthers and after pollinations in siliques of sexual diploid *B. stricta* and apomictic diploid *B. divaricarpa* 1-5 DAP (Fig. 5B; Table 2S in Supplement). Since *B. stricta* has no presence of apo-allele in the genome we used it as a negative control.

In apomeiotic gynoecium of *B. divaricarpa* the level of APOLLO apo-allele expression was 4 times higher compared to its expression after meiosis. During four DAP, apo-allele expression was downregulated in siliques of *B. divaricarpa* almost to zero, however, on the 5th DAP in *B. divaricarpa* siliques, the expression of the APOLLO apo-allele increased sharply, while in *B. stricta*, as expected, the expression has not been observed. (Fig. 5B). Regarding the expression of sex-allele, the opposite picture was found. During meiosis gynoecium of apomictic *B. divaricarpa* demonstrated 6 times lower expression of the APOLLO sex-allele compared to the sexual *B. stricta*. Whereas after meiosis, the expression of the APOLLO sex-allele was strongly downregulated in gynoecium of both species, nevertheless, the expression level in *B. stricta* remained 6 times higher than that in *B. divaricarpa*. After pollination, the expression of the sex-allele almost completely declined in siliques of both species, and then from the 3 DAP start gradually increasing to a sharp rise in the expression on the 5th DAP, while in siliques of sexual *B. stricta*, the expression was higher than that in apomicts of *B. divaricarpa* in 12 times.

The expression levels of the APOLLO apo-allele in meiotic anthers of *B. divaricarpa* was slightly upregulated after meiosis, whereas in the anthers of sexual *B. stricta* there was no expression as it should be (Fig. 5B). As for the APOLLO sex allele expression in the anthers, its level remained rather high during and after meiosis in the sexual *B. stricta* and was very low during and after meiosis in anthers of apomictic *B. divaricarpa* (Fig. 5B).

Using universal primers to detect the expression of both sex- and apo-alleles, we found that in anthers during meiosis, the total expression of APOLLO alleles was high in sexual and apomictic species. It was strongly downregulated in the anthers of both species soon after the end of meiosis (Fig. 5B). As for the expression of the combined APOLLO alleles in gynoecium and siliques of both species, noticeable differences between sexual *B. stricta* and apomictic *B. divaricarpa* began to be observed only by the 5th day after pollination. Namely, during meiosis in gynoecium of both species, the expression of APOLLO was approximately the same, which was strongly downregulated after meiosis. After pollination the expression levels remained low up to the 4th DAP, then the level of gene expression in siliques of both species began to increase on the 5th DAP. In this stage, the expression of APOLLO remained still low for the apomicts, while for the sexual plant it jumped almost 5 times up (Fig. 5B).

Thus, for the first time, we distinguished between the expression levels of apo- and sex-alleles of APOLLO in the generative organs of two *Boechera* species, differing in the mode of reproduction. Our studies have shown that the APOLLO apo-allele is upregulated in gynoecium and anthers during meiosis and in siliques on the fifth DAP in apomicts, while this allele is not expressed in sexual plants. The sex-allele showed the opposite
picture: in sexual *B. stricta*, it was upregulated in the gynoecium and anthers during meiosis and on the fifth DAP, while the expression of this allele in *B. divaricarpa* remained at a very low-level during/after meiosis in anthers and the first days after pollination. Revealed interesting patterns of *APOLLO* expression were somewhat different from those previously published [18], the difference observed in *APOLLO* expression in our study, may also be tissue and species specific.

### 3.5 Phylogenetic analysis of *APOLLO* gene

A preliminary analysis of *APOLLO* gene and protein orthologs we reported previously [19], therefore, the objective of this research was to study the phylogeny of *APOLLO* gene using additional available in open access sequenced genomes of *Boechera* species with sexual and apomictic mode of reproduction by translating *APOLLO* nucleotide sequences into proteins and applying Multiple alignment method for obtaining amino acid sequences using MUSCLE software and Maximum Likelihood method to construct a phylogenetic tree. The heatmap shown in Fig. 6A demonstrates that all compared *APOLLO* proteins of *Boechera* are rather similar (≥92.5%) despite the presence of 5 apo-alleles and 5 sex-alleles of that gene (see table 2 in Methods). Phylogenetic tree of *APOLLO* (Fig. 6B) shows that the *Boechera* species are clustered in two separate subclades according to the mode of reproduction, i.e., branches in the tree were grouped by genes rather than by species: the first subclade contains the homozygous for sex-alleles accessions reproducing sexually and the second contains accessions with apo-alleles that reproduce via apomixis. At the same time, all *Boechera* species represented in the phylogenetic tree are clustered in a common clade, separating them from the other genera of the Brassicaceae family (similarity ≥82.5%).
**Figure 6.** Similarity matrix (A) and Phylogenetic tree (B) of APOLLO in species of interest. Numbers near nodes represent corresponding bootstrap support. Phylogenetic tree was reconstructed using Maximum Likelihood method. The *Boechera* species are clustered in two separate subclades according to the mode of reproduction: the first subclade contains the homozygous accessions for sex-alleles and the second contains accessions with apo-alleles.
4. Discussion

Apomixis via clonal seeds produces the offspring, that are genetically identical to the maternal plant. Understanding of the genetic components that regulate apomixis is very important for studying plant development and evolution, in addition, the introduction of apomixis in agricultural plants would allow a long-term fixation of complex genotypes, including F1 hybrids, often used in agriculture. Yet, the molecular mechanisms underlying apomixis are poorly understood. Namely, the factors inducing avoidance or modification of meiosis (apomeiosis) and parthenogenesis. To study the genes regulating meiosis and embryogenesis in comparison of sexual vs. apomictic plants we use a convenient model plants from the *Boechera* genus, that comprise species naturally reproducing both by sexual and apomictic ways and show features of hybrid origin [31].

In this paper we studied similarity of homologs and phylogeny of *CENH3* and *APOLLO* genes that might affect some components of apomixis and also compared the expression patterns of these genes in apomictic and sexual plants of *Boechera*.

The *CENH3* gene plays an important role in cell divisions and genome elimination when mutated. Mutations in *CENH3* of *Arabidopsis thaliana* cause disturbed meiotic chromosome segregation [20,21] that was also used to induce genome elimination in *A. thaliana* and rice. In hybrids of *cenh3* mutant lines with diploid wild type plants the *cenh3* line genome was eliminated [59]. We showed that *CENH3* is a single-copy gene and that its structure is almost identical among the seven studied *Boechera* species irrespective of the reproduction (sexual or apomictic) mode. Polymorphic sites were mostly found at the N-tail protein regions, although *B. retrofracta* and *B. arcuata* had one site at 91 a.a. and two polymorphic sites at 67 a.a. and at 96 a.a. respectively in conservative Histone H3/CENP-A domain. Variability within the N-terminal tail might lead to apomeiosis, since it influences the chromosome segregation in meiosis [20,24], although this assumption still needs to be tested on mutant lines with the replacement of the corresponding polymorphic sites. Still the similarity index between all studied *Boechera CENH3* was ≥ 97% (both at nucleotide and protein levels). Also, in the *CENH3* phylogenetic tree, performed by multiple alignment, all studied *Boechera* species are clustered into the same clade, although being very close to other Brassicaceae species from *Arabidopsis*, *Eutrema*, *Cardamine* genera. *CENH3* expression profile analysis showed that during meiosis expression levels of *BsCENH3* in gynoecium of sexual *B. stricta* was more than twice as high compared with *BdCENH3* in gynoecium of apomictic *B. divaricarpa*. By the meiosis time expression levels of both these genes dramatically dropped in gynoecia. After pollination, the expression *CENH3* significantly increased in *B. stricta* siliques by 4th DAP still remaining low in siliques of *B. divaricarpa*. The lower expression levels before and after pollination in *B. divaricarpa* (ES9) could indicate to a feasible *CENH3* role in apomeiosis and initiation of parthenogenesis. In the meiotic anthers of *B. divaricarpa*, the expression of *CENH3* was 1.5 times higher than that in *B. stricta*, which may be associated with apomeiosis during pollen maturation. After meiosis, the level of gene expression dropped to zero in the anthers of both species.

In sexually reproduced plants, two sperms enter the embryo sac, while one of them fuses with the central cell nucleus that further forms endosperm, the second fertilizes the egg, in contrast, during pseudogamous apomixis, the embryo develops without fertilization, which is
the cause of the "spare sperm problem" [60,61]. In apomicts fertilization of the central cell with haploid sperm generally leads to a 4m:1p genomes ratio in endosperm cells, which causes seed abortion [62]. Apomictic species can tolerate such deviations in endosperm via changing their imprinting systems, however, preventing the ‘spare sperm’ from fusing with the central cell nucleus, might also be important [63]. The fusion of reduced or unreduced ‘spare sperm’ to the central cell can potentially affect the parental genomic ratio. Therefore, it is not known how the central cell in apomicts can avoid fertilization by enhancing a polyspermy barrier for that ‘spare sperm problem’ [61]. Thus, different expression behavior of CENH3 before and after pollination in B. divaricarpa vs B. stricta gynoecium/silique might suggest its involvement in apomictic development such as elimination of the chromosomes from male gamete during endosperm development. However, this assumption requires the further proof.

Promoter analysis revealed the presence of several MYB transcription factor binding and recognition motifs within the promoter regions of BsCENH3 and BdCENH3. These motifs in Boechera CENH3 promoters might suggest a regulation of these genes by a MYB family proteins. A study on Arabidopsis CENH3 promoter and its regulation revealed two E2F binding region at -163 and -115 sites [62]. Our analysis of these regions in Boechera species detected the motif at -163 site in BsCENH3 and BdCENH3. At -115 site BdCENH3 and BsCENH3 had a single polymorphic site (GCGGGAAA/ GCGGGAAG). Further functional studies on the Boechera CENH3 gene and its epigenetic and transcription regulation could elucidate a functional difference of CENH3 between apomictic and sexual Boechera.

Concerning APOLLO, it is the only so far found gene comprising the alleles with apomixis-associated polymorphism in Boechera species [18]. Thus, identification of APOLLO apo-alleles might be used as molecular markers to spot apomictic individuals among the Boechera species. Earlier it was shown that APOLLO encodes the exonuclease NEN3 and suggested an evolutionary scenario where after series of duplications one of the NEN3 protein copies of the Boechera ancestors acquired an alter function leading to apomictic development from the sexual state [19]. Moreover, it was demonstrated that apo-alleles are under a positive selection [19]. In the current study we retrieved 5 apo- and 5 sex-alleles from [18] to screen the genomes of ten Boechera species with different reproduction modes as well as other Brassicaceae species from the Arabidopsis, Eutrema, Cardamine, and Capsella genera and to perform APOLLO gene phylogenetic analysis. The results showed that sexual and apomictic Boechera species are clustered into the separate sub-clades, while being very similar to each other and to other Brassicaceae species. As well as for the CENH3 gene, functional studies of the APOLLO alleles and their putative epigenetic and transcriptional regulation in Boechera species is required in order to find out if there are functional differences between sexual and apomictic accessions. However, the latter will only be possible when good quality diploid genome assemblies of the studied species have been implemented. Investigation of APOLLO expression in pre- and after meiotic gynoecium and anthers and also after meiosis in siliques of sexual diploid B. stricta and apomictic diploid B. divaricarpa 1-5 DAP have been performed. The upregulation of the APOLLO apo-allele in B. divaricarpa apomicts after meiosis in the anthers and gynoecium and the decrease in the level of its expression with the onset of embryo sac formation may indicate to a certain relationship between the expression of the APOLLO apo-allele in apomicts and apomeiosis. This is consistent with the previously published data that the APOLLO apo-allele is exclusively expressed in ovules of apomictic Boechera species.
around stage of meiosis [18]. The upregulation of the APOLLO sex-allele in anthers and gynoecium of sexual B. stricta during and after meiosis and very low levels of its expression in apomicts is possibly associated with a connection of the sexual allele of the Aspartate Glutamate Aspartate Aspartate histidine exonuclease gene (APOLLO) with meiosis and further postmeiotic processes. However, the increase in the expression of apo- and sex-allele in siliques on the 5th DAP in apomicts andsexuals, respectively, requires further explanation. Upregulation of APOLLO apo- and sex-alleles in meiotic anthers in apomictic B. divaricarpa two folds higher than in sexual B. stricta may indicate to a role of APOLLO in apomeiosis or meiosis during pollen formation, moreover the male gametophyte development in apomictic B. divaricarpa were reported to produce both reduced and nonreduced gametes [38,39]. Therefore, we infer that APOLLO may have species specific function for the regulation of meiosis/apomeiosis in Boechera and have diverse functions in pseudogamous apomicts compared to sexual relatives.

In conclusion, a detailed knowledge of the structure, phylogeny of genes related to apomixis and the dynamics of their expression can presumably help to better understand the nature and regulation of apomixis vs sexual reproduction and facilitate further study of the evolutionary, ecological, and population role of apomixis. However, for more accurate studies on the phylogeny and evolution of the Boechera species, it is necessary to have a good quality diploid level whole genome assembly for these species, which we are now actively working on.

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