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Morphometric variation of two endangered Bulgarian medicinal plant species Alchemilla mollis (Buser) Rothm. and Alchemilla achtarowii Pawł. (Rosaceae)

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Abstract. Morphometric variation of the protected in Bulgaria species *Alchemilla mollis* (Buser) Rothm. and *A. achtarowii* Pawł. (Rosaceae) was investigated based on ten morphometric characters under in-situ and ex-situ environmental conditions. Plant materials originating from Bulgarian localities of *A. mollis* and *A. achtarowii* in Central Balkan Mountains (1100-2000 m a.s.l.) were compared to others, harvested from experimental live collection, grown in Sofia (570 m a.s.l.). Abiotic factors like water regime, and temperature had strong influence on the performance of both species in-situ and ex-situ. A distinctive difference in some morphometric characters of *A. mollis* in nature over the past 40 years was found. When exposed to prolonged temperature and drought stress *A. achtarowii* showed considerable variability regarding flower diameter and epicalyx indentation. The presented study should be used as a practical example of how some high mountain apomictic species react to changes in the environment and what are the resulting implications related to their traditional taxonomic treatment and conservation.

Key words: *Alchemilla,* apomicts, plant stress, climate change.

Introduction

Genus *Alchemilla* includes more than 300 species distributed in Europe, Asia, Africa and North America (Tihomirov et al., 1995) and it is famous for the limited number of morphological characters for species determination, interspecific hybridization, polyploidy and apomictic reproduction manner, all resulting in a complicated systematic structure (Fröhner, 1995). There are 35 *Alchemilla* species in Bulgaria (Assenov, 1973). The species *A. mollis* (Buser) Rothm. and *A. achtarowii* Pawł. are strictly protected according to Bulgarian Biological Diversity Act (2002). They are larger in size compared to common species like *A. crinita* Buser, *A. monticola* Opiz and *A. serbica* (Paulin) Pawł. (Gavrilova & Vitkova 2010), and possess medicinal properties linked to their phenolic content (Vitkova, 1996; Trendafilova et al., 2011; Trendafilova et al., 2012). That gives them perspective for cultivation to be used sustainably while preserving the natural populations.

A. mollis is native for Romania, Bulgaria, Montenegro, Turkiye, Armenia and Georgia. The species is widely introduced in many European countries mainly as ornamental plant, but in some northern parts of the continent it has become naturalized (Buia, 1956; Walters & Pawlowski, 1968; Davis, 1972; Strid, 1986; Kurtto et al., 2007; 2009). In Bulgaria the species is critically endangered according to the Bulgarian Red Data Book (Peev, 2015) and is known to have a single small locality in the vicinity of Rai chalet (Kamenlivitsa area, 1158 m a.s.l.), Central Balkan Mountains (Assenov, 1973; Vitkova, 1997; Peev, 2015).

The Bulgarian local endemic species *A. achtarowii* is spread only on the southern slopes of Central Balkan Mountains (Pawlowski, 1953; Vitkova et al., 2008). *A. achtarowii* is an endangered species according to the IUCN criteria (Petrova & Vladimirov, 2009).

It is widely presumed that the low rate of variation of the morphological characters of *Alchemilla* species is due to obligate apomixis and extremely rare hybridization (Pihu et al., 2009). According to Fröhner (Fröhner, 1995, 1999) the interspecific differences are small, but they are stable and allow clear discrimination between the species. Other studies based on random amplified polymorphic DNA (RAPD) method show high intraspecific variability of morphological characters and lack of clear discrimination between some microspecies (Sepp & Paal, 1998, 2001). Traditionally, it is assumed that the morphological variations of genus *Alchemilla* can be both genetically determined and environmentally induced (Bradshaw, 1963, Bradshaw et al., 1964). Modern concepts upgrade this notion and perceive the change of morphological characteristics as environmentally related response to epigenetic variation (Hojsgaard & Hörandl, 2015; Hodač et al., 2023). However, only a few studies test the variation of the characters of some *Alchemilla* species under different abiotic factors (Turesson, 1943, 1956, 1957, 1958; Bradshaw et al., 1964; Pihu et al., 2009) but they all serve as evidence of the uneven nature of this variation between species.

The aim of this study is to describe the morphometric variation of the protected in Bulgaria species *A. mollis* and *A. achtarowii*, developing under in-situ and ex-situ conditions, and based on the results to outline the most critical abiotic factors that could influence their development.

Materials and methods

The plant material for the presented morphometric study was collected in 2010 from the natural clone populations of *A. mollis* and *A. achtarowii* in Central Balkan Mountains and from cultivated plants in the experimental field of IBER-BAS in Sofia (570 m a.s.l.). The Central Balkan Mountains are part of the physical geographical region of Stara planina, where the climate is represented by the mountain version of the temperate-continental climate. At the foothills of the region the average annual temperature is 8°C, while at Botev Peak (2376 m) it is -0.7°C. In the same sense the average monthly temperature in January is -1°C and -9°C respectively, while in June is 21°C and 7.5°C respectively. The annual precipitation varies between 700-1000 mm with maximum in May-June, and minimum in February. The snow cover given in percent from the total annual precipitation varies between 12% at the foothills and 30% at the ridges. The river runoff maximum is in April-May, and its minimum – in September (Yordanova et al., 2002). The city of Sofia falls within the region of the Sub-Balkan basins, where the climate is temperate-continental. The average annual, January and June temperatures are 10-11°C, 0-(-1)°C and 20-21°C respectively. The annual precipitation is 550-600 mm with May-June maximum and January-February minimum. The share of snow cover of the total annual precipitation is 15%. The river runoff is one of the lowest in the country (Yordanova et al., 2002).

Regarding the high conservation status of both species, which are included in Appendix 3 of the Bulgarian Biodiversity Act (2002), all plant parts gathered from the natural clone populations were acquired in compliance with written permission letters from the Minister of Environment and Water, after fulfilling the legal procedure for requesting the use of genetic material from protected plants for scientific studies.

The plant material of *A. mollis* originated from the single known Bulgarian locality which occupies a small open beech forest glade of 200 m^2, on a micaceous shale substrate with an almost absent soil horizon. The size of all samples used for characterization of wild *A. mollis* plants are relatively small due to objective constraints arising from the high conservation status of the species, such as limited in number ramets, occurring on very small area in the single Bulgarian locality.

For *A. achtarowii* the plant material was gathered from several sites near Rai chalet, Vasil Levski chalet and Echo chalet between 1604-1806 m a.s.l. The natural habitats of *A. achtarowii* occupy relatively steep stony slopes with shallow soils (rankers). The plants grow along high-mountain water streams with snow feed.

The cultivation experiment was carried out in the period 2007-2010 at the experimental field of the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Science (IBER-BAS), in Sofia. The climate falls under the temperate-continental climatic region. The transplant material consisted of 2-3 cm long rhizome segments, originating from different ramets. The transplants were cultivated for 3 years for *A. achtarowii* and 4 years for *A. mollis* respectively. The field plots were regularly watered, weeded out and hoed when necessary. The cultivation experiment included 32 plants of *A. mollis* and 16 plants of *A. achtarowii*.

All plant materials (basal leaves and flowering stems) for the morphometric study were gathered in 2010 in one and the same phenological stage of mass bloom. All measurements were taken with accuracy to 0.1 cm, except for the flowers where it was 0.01 mm. Ten characters with taxonomic significance were studied. Three of them are related to the flowering stem – length, total number of stem leaves, and number of stem leaves with axillary inflorescences. Five basal leaf characters were considered – length and width of basal leaf lamina, length of basal leaf petiole, number of basal leaf lobes, and number of teeth in a leaf half-lobe. Regarding the flowers their diameter and the presence of typical epicalyx indentation were determined. As main reference source for the morphometric characteristics of *A. mollis* and *A. achtarowii* is used the monograph of genus *Alchemilla* in Flora of PR Bulgaria (Assenov, 1973). Two characters, total number of leaves on a stem and number of stem leaves with axillary inflorescences were described with specific values for the first time.

For statistical data analysis, Excel 2000 and SPSS Statistics 21 were used. After normality data testing it was determined that almost all data samples deviate from the standard normal distribution pattern. Therefore, the nonparametric Mann–Whitney U test was applied. The comparisons of the data samples are visualized through box and whisker plots.

Results and Discussion

Our results show that the size of the wild *A. mollis* plants established in the current study significantly differs from the reference source, which presents a characteristic of the same species from 40 years ago (Assenov, 1973). Table 1 uses seven morphometric characters to compare value range of the reference data with data established in the current study. Although the confidence intervals of the mean for the morphometric characters of stem and leaf in-situ cannot be correctly calculated due to small sample size, the maximum values of the observed wild *A. mollis* plants are around the minimum reference values. On the contrary, regarding the *A. mollis* plants under ex-situ conditions all value ranges of the studied characters are like those indicated in the reference source. The only character that shows steady value range across both species is the number of leaf lobes.

Table 1. Comparison of the value ranges of seven morphometric characters of *A. mollis* according to Flora of PR Bulgaria (Assenov, 1973) and the current study.

Character	Value range according to Flora of PR Bulgaria	Ex-situ results			In-situ results		
		Min	95%- CI	Max	Min	95%- CI	Max
Stem length (cm)	(25)50÷70(80)	40.00	48.66÷52.39	**63.20**	**24.00**	**23.31÷30.25**	**31.50**
Length of basal leaf petiole (cm)	(12.5) 20÷30 (40)	**10.10**	20.76÷23.37	**32.80**	**11.40**	*	**14.40**
Basal leaf lamina length (cm)	8÷13	**6.00**	9.82÷10.59	13.00	**6.20**	*	**7.60**
Basal leaf lamina width (cm)	10÷15	**7.50**	11.10÷11.87	**14.00**	**7.20**	*	**9.10**
Number of basal leaf lobes	9÷11	9	11	11	11	*	11
Number of teeth in a leaf half-lobe	7÷9	**6**	7÷9	**12**	7	*	**8**
Flower diameter (mm)	3.5÷5	**3.01**	3.88-4.13	4.67	3.50	*	4.50

Legend: CI – confidence interval; min – minimum value; max- maximum value.
* For conservation reasons the sample size was very small and therefore these values have not been calculated.

Identical morphometric measures were taken for *A. achtarowii* (Table 2). It is noticeable that the values of the cultivated *A. achtarowii* plants generally are around or below the lower part of the reference range, while those regarding the wild *A. achtarowii* plants fit in its upper part. The most stable characteristic similarly to the results for *A. mollis* is the number of basal leaf lobes.

Table 2. Comparison of the value ranges of seven morphometric characters of *A. achtarowii* according to Flora of PR Bulgaria (Assenov, 1973) and the current study.

Character	Value range according to Flora of PR Bulgaria	Ex-situ results			In-situ results		
		Min	95%- CI	Max	Min	95%- CI	Max
Stem length (cm)	35÷45(50)	**17.00**	**31.82÷35.49**	**44.30**	**20.50**	42.11÷46.99	**66.80**
Length of basal leaf petiole (cm)	(10)15÷30	**7.40**	**12.79÷14.69**	**21.30**	**8.90**	17.54÷22.47	**34.10**
Basal leaf lamina length (cm)	5÷10	**4.50**	5.97÷6.55	**8.60**	**5.80**	7.86÷8.83	**10.50**
Basal leaf lamina width (cm)	6÷12	**5.50**	7.23÷7.91	**10.30**	**7.20**	9.70÷10.65	**12.10**
Number of basal leaf lobes	9÷11	9	11	11	9	11	11
Number of teeth in a leaf half-lobe	(7)8÷10(12)	**4**	8	**14**	7	10	**13**
Flower diameter (mm)	(4)4.5÷6.5(7.2)	**2.77**	**3.24÷3.46**	**4.11**	**2.81**	**3.73÷4.16**	**5.12**

Legend: CI – confidence interval; min – minimum value; max- maximum value.

The following results include various comparisons of the studied plant samples of *A. mollis* and *A. achtarowii* by origin and age. Table 3 shows the statistical significance of the tested differences. Some comparisons do not prove statistically significant difference between the studied samples because of the non-random sampling of the initial plant material, non-normal data distribution, and heterogeneity of variance. In the case of *Alchemilla* species due to their apomictic manner of reproduction in combination with genetic polymorphism of the infraspecific clones the random data sampling in most cases is unfeasible. The spatial structure of these clonal species is highly complicated and the distinguishable in the field units are ramets from several genetic cytotypes (progenitors) (Cain, 1990; Lájer, 2007; Hojsgaard & Hörandl, 2015).

Table 3. Statistical significance of the differences in some morphometric characters by plant species and age.

Morphometric character	Statistical significance of the differences		
	A. mollis, in-situ vs. ex-situ	3- vs. 4-year *A. mollis* plants, ex-situ	*A. achtarowii*, in-situ vs. ex-situ
Length of flowering stem	sig.	sig.	sig.
Total number of stem leaves	not sig.	not sig.	sig.
Number of leaves with axillary inflorescences	not sig.	sig.	sig.
Length of basal leaf petiole	not sig.	sig.	sig.
Length of basal leaf lamina	not sig.	sig.	sig.
Width of basal leaf lamina	not sig.	sig.	sig.
Number of teeth in leaf half-lobe	not sig.	not sig.	sig.
Number of basal leaf lobes	not sig.	not sig.	sig.

The comparisons of stem length, basal leaf lamina length and width, basal leaf petiole length, and flower diameter of *A. achtarowii* and *A. mollis*, observed in-situ and ex-situ, are visualized in Fig. 1. The results generally confirm the summary in Tables 1 and 2 that the plants of *A. achtarowii* are in better condition in their natural habitats, while those of *A. mollis* are under cultivation conditions.

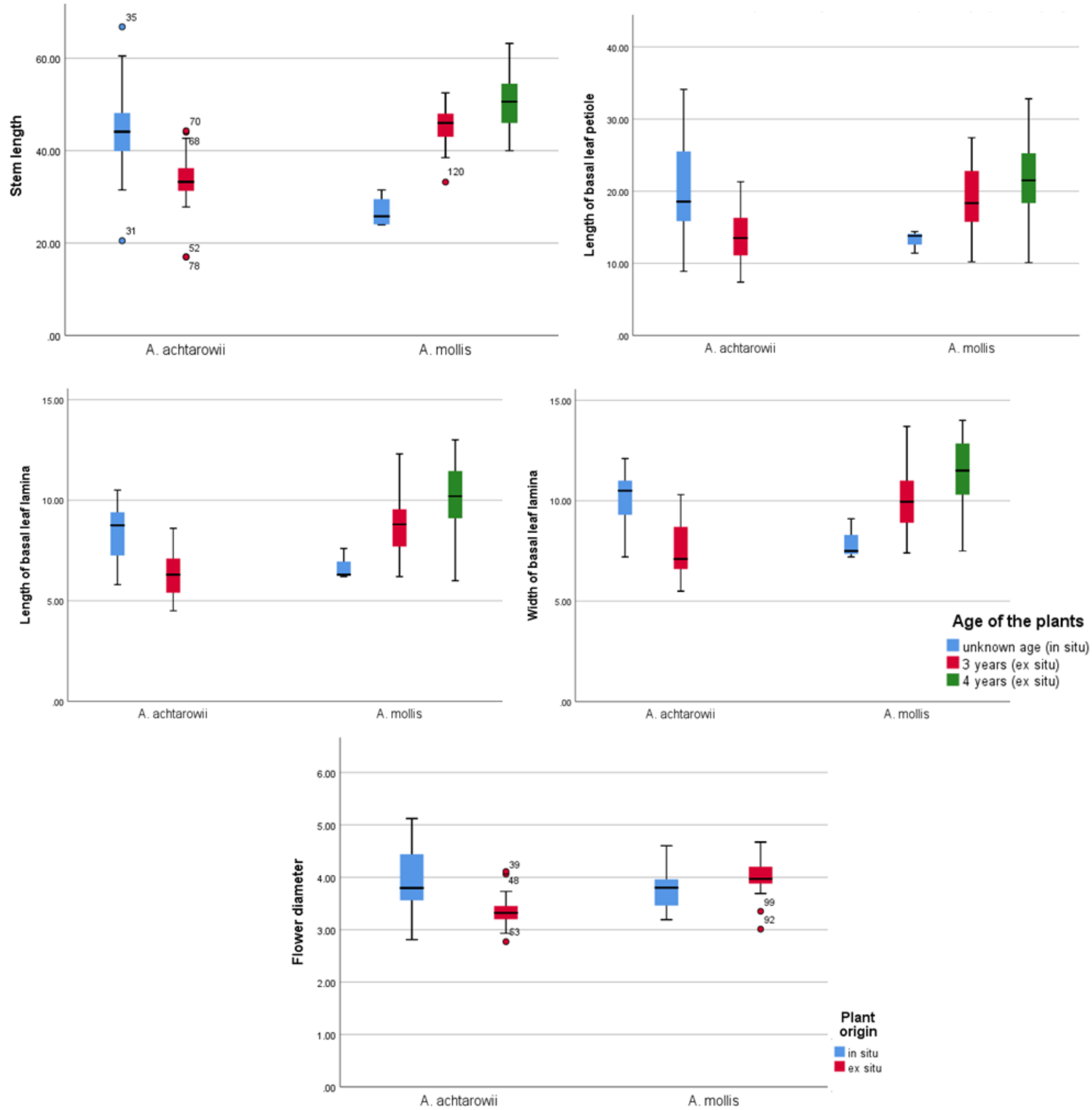

Fig. 1. Comparison of the stem length (cm), basal leaf lamina length (cm) and width (cm), basal leaf petiole length (cm) and flower diameter (mm) of *A. achtarowii* and *A. mollis*, observed in-situ and ex-situ.

Some classical studies also confirm that the variation in size and habit of many *Alchemilla* plants is due not only to genetic factors, but also to environmental factors, correlated to climate, temperature, exposure and their effects on the soil and habitat type (Bradshaw, 1963). The increase in size of the cultivated plants due to soil enrichment stands out as common opinion across authors (Turesson, 1943, 1956; Bradshaw, 1963; Pihu et al., 2009). Most likely the significant boost in plants' size of *A. mollis* under the ex-situ experimental conditions can be attributed to irrigation and richer soil substrate in contrast to the conditions of its natural locality. The smaller size of the plants of the natural clone population of *A. mollis* can be assumed as a sign of drought and temperature stress, which is indirectly supported by the results of systematic hydrological investigations for the territory of Bulgaria which claim that the periods 1982–1983, 1993–1994, 1997, and 2001–2011 are characteristic with significant drought events that last in most cases from June to November (Chilikova-Lubomirova, 2020).

Regardless of the increased soil richness under cultivation, *A. achtarowii* appeared to be in a suppressed state. We suggest that it was mainly due to the unfavorable amount of air humidity and moisture, as well as higher temperature levels in culture in comparison to the condition of the same factors in the high-mountain natural habitats of the species. Throughout the growing season, the *A. achtarowii* plants in Sofia were regularly and abundantly watered by sprinkler irrigation to meet their water needs. However, as can be seen in Fig. 1, their basic morphometric parameters decreased compared to the wild plants. The significant reduction in size of the cultivated plants was accompanied by the observation of additional morphological changes in their overall coloring and texture of the aerial parts. The leaf color specifically turned from fresh green to a paler shade with a yellowish to grayish tint. The texture of the leaves has changed and became coarser. These results are consistent with already known from the reference plant morphological adaptations to high temperature and water stress, such as decrease of leaf thickness and surface area, cuticle and wax layer thickening, shoot growth reduction, an increase in the density of stomata. All these adaptations improve the evaporative cooling capacity of the plant (Schulze et al., 2005; Lambers et al., 2008; Fritz et al., 2018). The change in the leaf color lightness is linked to its reflectance and thus reduces leaf temperature (Monteiro et al., 2016).

An interesting observation about the most significant taxonomic character of the flowers of *A. achtarowii* was made during the presented study. A distinctive species characteristic of the flowers is the epicalyx indentation, which includes all 4 sepals of the epicalyx (rarely 3 or very rarely only 2) having (1) 2-4 (5) teeth (Pawlowski, 1953; Assenov, 1973). We found that for 100 random flowers per stem the number of flowers with toothed epicalyx was 9±0.79 for the wild plants in comparison to 3±0.46 for the ex-situ grown plants. In summary, regardless of their origin all *A. achtarowii* plants studied in 2010 showed very small number of flowers with typical for the species epicalyx indentation and in ex-situ this number was even smaller than under natural conditions. We assume that the decrease in size of the flower diameter (Table 2, Fig. 1) together with the reduction in the number of flowers with epicalyx indentation is a significant finding since the general notion is that the flower features across genus *Alchemilla* are more stable than the vegetative ones (Glazunova & Myatlev, 1990; Pihu et al., 2009). More research is needed to determine the role of abiotic plant stress in this variability of flower morphology.

Some authors hold the opinion that apomictic plants like *Alchemilla* possess general-purpose genotype which makes them less sensitive to variation in the environment (Bierzychudek, 1989). On the contrary, others support the hypothesis that apomicts follow the frozen niche variation model as they inhabit the total suitable niche space through broad arrays of highly specialized individual clones, derived from few genotypes (Kenny, 1996; Hojsgaard & Hörandl, 2015). In this sense, although *A. mollis* shows better adaptability under ex-situ conditions compared to *A. achtarowii*, both species can be assumed to be representatives of the frozen niche model. It is currently known that morphological variation in apomictic plants is related to epigenetic rather than to genetic variation, and epigenetic markers are at least partially heritable and directly influenced by abiotic environments (Hojsgaard & Hörandl, 2015). This notion may bear a possible explanation for the differences in the ecological plasticity of the two species.

Many authors advocate that one of the main plant adaptations to climate change in the future will be towards polyploidization and apomixis (Soltis et al., 2015; Hörandl, 2011; Levin, 2019; Heslop-Harrison et al., 2023). However, this view serves best to the coarse large-scale predictions of plant evolution in general. At smaller scale in terms of current biodiversity loss mitigation, it does not necessarily mean that the group of alpine and sub-alpine highly specialized apomictic polyploid species will survive the presently unfolding climate changes. This is especially true for apomictic species like *Alchemilla*, which are believed to have lost their diploid sexual ancestors long ago (Asker & Jerling, 1992; Briggs & Walters, 2016). Some of them, such as *A. achtarowii*, are assumed to be "young" species that are related to currently active speciation loci (Kozhuharov, 1977). This group of apomictic species is bound to the upper mountain parts and many of them are closely dependent on the water resources with snowpack feed. According to Spinoni et al. (2017) after the 1950s a general drying tendencies over the Southern European regions and the Carpathians is represented. The same authors claim that the turning point of these climate change effects can be attributed somewhere between the late 1970s and the early 1980s. Some systematic studies on climate variability and its impact on water resources in Bulgaria point out the periods 1982–1983, 1993–1994, 1997, and 2001–2011 are characteristic with significant drought events and "heat waves" that last in most cases from June to November (Alexandrov & Genev, 2003; Chilikova-Lubomirova, 2020; Nikolova et al., 2024). A relevant study analysed temporal variability of the extreme monthly temperature in Rilla Mountain during the period 1960-2012 and found that the number of extremely warm months have increased significantly during the period 1987-2012 in comparison to 1961-1986 (Nikolova et al., 2018). Prognostic models have already pointed out some mountainous European regions that would face disproportional species loss due to climate change such as mid-altitude Alps and Pyrenees, central Spain, French Cavennes, Balkans and Carpathians (Bakkenes et al., 2002, 2006; Thuiller et al., 2005). In terms of temperature and water regime, climate change has affected precipitation patterns and caused greater frequency and intensity of climatic extremes (Lee & Romero, 2023). With reduction of winter snow precipitation and duration of snowpack retention, many high mountain streams and peat lands are in danger of drying out. These relatively small snow fed water bodies sustain many habitats that harbor a significant amount of biodiversity, including species of conservational importance like *A. achtarowii*. We think that *A. achtarowii* and other highly specialized high-mountain apomictic species that fit their behavior towards moisture and humidity can be good indicator species for climate change dynamics in prognostic modeling.

Conclusions

The main conclusion of our study is that abiotic factors related to water regime and temperature influenced significantly the studied morphometric parameters of *A. mollis* and *A. achtarowii.* It was found that there is a distinctive difference in some morphometric characters of the rare and endangered for Bulgaria species *A. mollis* in the nature over the past 40 years. That can be attributed to the unfavorable climate change events, especially the variations of temperature and water regime. The species *A. achtarowii* is also vulnerable in terms of these factors in its natural habitats. When exposed to prolonged drought stress under ex-situ conditions *A. achtarowii* showed considerable variability in key structures, important for its traditional taxonomic determination such as flower diameter and epicalyx indentation.

Cultivation of *A. mollis* and *A. achtarowii* is recommended to preserve their genetic resources. The results of our research may find application in planning suitable cultivation conditions in this sense. The study adds another perspective to the discussion whether asexual plant species adopt general purpose genotype or follow the model of the frozen niche. Apomictic species may have different survival strategies in this sense and there is a need of more research in the field. More biochemical and physiological studies to describe the specific response of apomicts to abiotic stress are needed. Species like *A. achtarowii* can be good indicators for climate change dynamics in prognostic modeling.

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