



Morphological and anatomical studies on hill clover (*Trifolium alpestre*) (Fabaceae) from Sharri Mountain, Kosovo

¹Kimete Lluga-Rizani, ²Dubravka Šoljan, ³Smajl Rizani, ¹Kemajl Kurteshi

¹ Department of Biology, Faculty of Mathematical Natural Sciences, University of Prishtina „Hasan Prishtina“, Republic of Kosovo; ² Department of Biology, Faculty of Mathematical Natural Sciences, University of Sarajevo, Bosnia and Hercegovina; ³ Faculty of Food Science and Biotechnology, University of Business and Technology, Prishtina, Kosovo. Corresponding Author: K. Lluga-Rizani, kimete.lluga@uni-pr.edu

Abstract. *Trifolium alpestre* L. species was investigated with the aim to evaluate the level of morphological and anatomical variability between populations from different habitats. The research covered a considerable number of morphological and anatomical traits. The investigation results indicate that *Trifolium alpestre* L. was not frequent in Brezovica (Sharri Mountain-Kosovo) and has very limited vertical distribution (1047-1256 m). However, there is significant variability for most of the investigated parameters, but there are some parameters that are more consistent to the change of environmental conditions of habitats such as the length of calyx tube, maximum fruit width, fruit length.

Key Words: morphological variability, narrow vertical distribution, *Trifolium alpestre*.

Introduction. Genus *Trifolium* L. is one of the largest genera in the Fabaceae family, with 237 recorded species, including several cultivated species of high agronomic importance (Coombe 1972; Zohary & Heller 1984). The species studied in this research was *Trifolium alpestre* L. The aim was to ascertain the scale of individual, population and inter-population morphological variability of some parameters from individuals from 9 different locations, in order to determine the most consistent and variable morphological traits which can contribute to the taxonomic analysis of this genus, either by completion and addition of new scientific facts, or revision of this taxon. In comparison with often broad and poorly defined latitudinal distribution in the lowlands, the distribution of this species in mountain regions is typically restricted to relatively narrow altitudinal bands (Jump et al 2009). The distribution of *T. alpestre* in our study site has a very narrow altitudinal band. But the results of this investigation have shown that some morphological features correlate positively or negatively to the increase of altitude, even though the difference in altitude between populations was not very high (1047-1256 m). In the case of altitudinal gradients, by increasing altitude, plants must cope with fluctuating environmental conditions (Korner 2007). The distribution of plant species along elevation gradients is governed by a series of interacting biological, environmental and historical factors (Colwell & Lees 2000). Elevation represents a complex gradient where many environmental variables change simultaneously (Campbell & Xia 2002).

Material and Method. In order to have a more comprehensive and complete research on morphological and anatomical differentiation of some parameters of *T. alpestre*, a considerable number of individuals (30) from 9 locations was researched. Samples or plant material were collected in different topo-geographical-ecological localities on the vertical profile of Brezovica that reaches an altitude up to 2500 m. The study period extended from 2011 to 2012. The study area belongs to the highland regions, with average temperatures of 10⁰C, down to 4⁰C above 2000 m on the West part, whereas on the East part from 9.3⁰ C down to 2.5⁰C above 2000 m (Pllana 2015). Locations were nominated by numbers (1 to 9), where 1 represents the lowest altitude (1047 m),

whereas 9 represents the highest altitude (1256 m). One part of the plant material was dried and placed in a herbarium, making it ready for further research, whereas the other part of plant material was preserved in alcohol 70%, destined for preparation of microscope slides and investigation of anatomical features.

In order to come up with more reliable results regarding the morphological variability of this species, the research covered a considerable number of parameters: total plant height, total leaf length, length and width of the first and middle leaflet, length of the leaf petiole, corolla length, calyx length, length of the calyx tube, number of flowers in inflorescence, length of inflorescence peduncle, length of the short and long calyx teeth, length, width and weight of the fruit, number of stomata per mm² at the upper and lower leaf epidermis, length and width of stomata at the upper and lower leaf epidermis, length of trichomes at the upper leaf epidermis, the diameter of the midrib vascular bundle, the length of palisade and spongy tissue cells. The values obtained during measurements were analyzed using statistical methodologies. The evaluation and interpretation of the level of variability of the above-mentioned morphological parameters, the degree of correlation, respectively the connection between certain features and ecological parameters, as well as the analysis of the result significance was carried out using Sigma software.

Results and Discussion. The investigation results indicate that *T. alpestre* was not frequent in Brezovica (Sharri Mountain-Kosovo) and has very limited vertical distribution (1047-1256 m). Usually, this species inhabits dry open woods, pastures, dry grassy places, with very high inclination. The maximum average value of the total plant height was found in Location 2 (37.9 cm), whereas the minimum average value was found in Location 9 (20.3 cm) (Table 1). Since the sampling populations were from various altitudes, there is a negative correlation between the plant height and increase of altitude.

Table 1

Trifolium alpestre height (cm)

	Locations								
	1	2	3	4	5	6	7	8	9
Number of samples	10	10	10	10	10	10	10	10	10
AV	30.1	37.9	24.2	28.7	30.6	27.9	36.9	26.0	20.3
SD	6.09	4.24	3.05	5.21	4.11	2.06	5.45	5.97	3.78
Max	38.8	45.9	29.3	39.6	36.8	32.3	48.8	35.3	28.2
Min	19.5	32.3	18.8	22.1	23.5	25.3	26.4	18.8	15.5

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

Our results are in concordance with results of Yaqoob et al (2015) and Mekonnon et al (2014). Also Roblek et al (2008) studied *Hypericum perforatum* L. morphological characteristics in different altitudes, and they found that plant height decreased at high altitudes due to the reduction in number of stem internodes, and the decrease of leaf chlorophyll content. The maximum value encountered was 45.9 cm, whereas the minimum value was 15.5 cm. The significant variability in plant height between populations in some plant species was also found by many other scientists (Tabarki et al 2017; Yemataw Z et al 2017).

The leaf morphology and anatomy. The leaf of *T. alpestre* is composed of three leaflets. The results of measurements of the total leaf length show that the highest average value was found to be in Location 7 (3.7 cm), whereas the lowest in Location 1 (2.5 cm). The maximum value of the total leaf length is 4.8 cm, whereas the minimum value is 1.9 cm (Table 2). Another element of the compound leaf is the petiole. Investigation results show that the highest average value of the petiole length is in Location 7 (3.82 cm), whereas the lowest is in Location 3 (1.87 cm). The maximum

length and width of the first and middle leaflets were investigated. The results have shown that the highest average value of the first leaflet length is 3.5 cm (Location 7), whereas the lowest is 2.4 cm (Location 1). The highest average value of the first leaflet width is 1.02 (Location 8), whereas the lowest average value is 0.69 cm (Location 9).

The width of the middle leaflet ranges from 0.8 to 1.14 cm, while the length ranges from 2.61 to 3.93 cm. The epidermal cells of upper and lower epidermis are arranged in a single layer. Our results are in accordance with Zoric et al (2009). The upper leaf epidermis of *T. alpestre* is made of polygonal cells, mostly hexagonal (Figure 1A). Lower leaf epidermis of *T. alpestre* has hexagonal cells with undulate cell walls (Figure 1B). Both upper and lower leaf epidermis have stomata (Figure 1A and 1B), but regularly the number of stomata on the upper leaf epidermis is lower compared with the one in the lower leaf epidermis. On the upper leaf epidermis, stomata are mostly situated at the top of the leaf and around the leaf midrib. Sometimes, the upper leaf epidermis stomata are so rare that no stomata can be observed on the light field of the microscope.

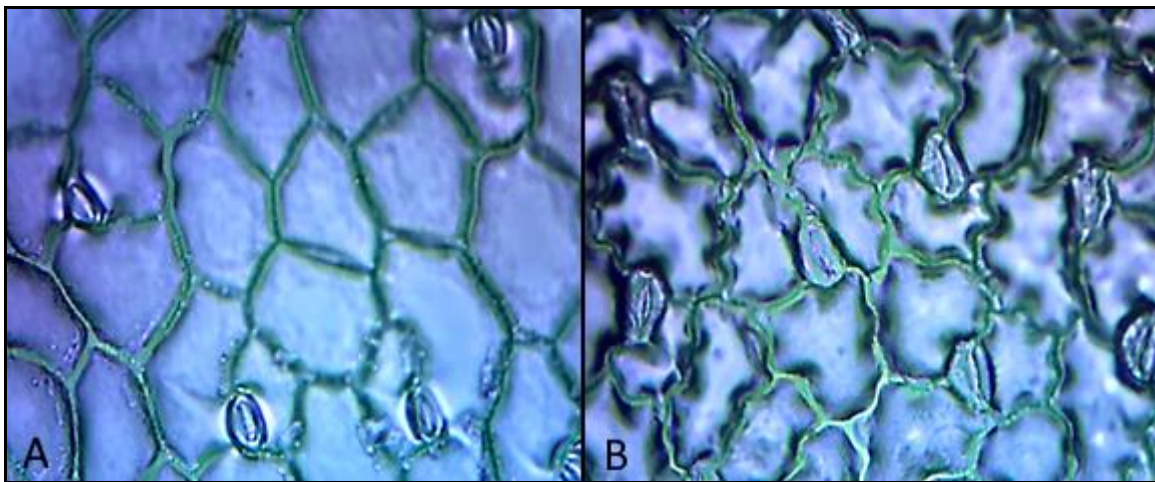


Figure 1. A - the upper leaf epidermis; B - the lower leaf epidermis and stomata.

The number of stomata per mm^2 on the upper leaf epidermis varies from 9 to 35 (Table 3). Population 9, which inhabits a dry habitat has the highest number of stomata. Higher stomata density and smaller stomata size is an adaptation to dry, more insolated habitats, because it enables plants to regulate water transport and transpiration more effectively (Dickison 2000; Fahn & Cutler 1992; Selvi & Bigazzi 2001). The number of stomata on the lower leaf epidermis varies from 62 to 104. Also from the measurements of the length and width of stomata on both abaxial and adaxial leaf epidermis it was found the following: the length of stomata on the upper leaf epidermis varies from 17 to 23 μm , whereas the width ranges between 12.3 and 15 μm .

The average values of length of stomata on the lower epidermis vary from 15 to 24 μm , whereas the average values of stomata width vary from 10.6 to 16.3 μm . The lowest values for these two parameters were found in Location 9 at the highest altitude. The lower leaf epidermis is covered by trichomes (Figure 2). Trichomes are single-celled, thin and long.

Table 2

Trifolium alpestre leaf morphological traits

<i>Morphological trait</i>	<i>Location</i>									
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	
Number of samples	10	10	10	10	10	10	10	10	10	
The total leaf length (cm)	AV	2.5	3.2	2.6	3.2	3	2.9	3.7	3.2	3
	SD	0.29	0.48	0.3	0.5	0.51	0.44	0.75	0.83	0.39
	Max	2.9	4.1	3.1	4	3.9	3.6	4.8	4.4	3.7
	Min	2.1	2.7	2.1	2.4	2.1	2.2	2.8	1.9	2.4
The length of leaf petiole (cm)	AV	1.9	1.9	1.8	2.1	2.6	2.8	3.8	2.2	2
	SD	0.21	0.45	0.37	0.75	0.68	0.74	0.77	0.84	0.96
	Max	2.1	2.4	2.6	3.7	3.5	4.0	5.0	3.3	3.4
	Min	1.5	1.2	1.5	1.1	1.7	2.0	2.5	1.0	0.8
The length of middle leaflet (cm)	AV	2.6	3.3	2.9	3.3	3.2	3.1	3.9	3.3	3.2
	SD	0.26	0.47	0.47	0.51	0.59	0.42	0.77	0.88	0.4
	Max	3	4.2	3.5	4.2	4.3	3.7	5.0	4.7	3.9
	Min	2.3	2.9	2.2	2.6	2.3	2.4	3.0	2	2.6
Maximum width of middle leaflet (cm)	AV	0.99	1.08	1.07	1.14	1.04	1.12	1.04	1.14	0.8
	SD	0.12	0.11	0.12	0.14	0.13	0.12	0.17	0.25	0.08
	Max	1.2	1.2	1.3	1.4	1.3	1.3	1.3	1.6	1
	Min	0.8	0.9	0.9	0.9	0.8	1	0.8	0.9	0.7
The length of the first leaflet (cm)	AV	2.4	3.1	2.4	3	2.9	2.8	3.5	3.1	2.9
	SD	0.32	0.5	0.24	0.49	0.45	0.46	0.74	0.78	0.39
	Max	2.9	4	2.8	3.8	3.6	3.5	4.7	4.3	3.6
	Min	2	2.6	2.1	2.3	2	2.1	2.7	1.9	2.3
Maximum width of the first leaflet (cm)	AV	0.89	0.97	0.88	0.95	0.95	0.93	0.9	1.02	0.69
	SD	0.12	0.09	0.1	0.09	0.15	0.11	0.12	0.16	0.05
	Max	1	1	1.1	1.1	1.2	1.1	1.1	1.3	0.8
	Min	0.7	0.8	0.7	0.8	0.7	0.8	0.7	0.7	0.6
The length of trichoma on lower epidermis (μm)	AV	909	1395	1003	879	989	908	960	783	1086
	SD	292	1395	242	202	180	185	262	219	448
	Max	1290	2029	1532	1392	1277	1307	1343	1336	1859
	Min	476	852	689	586	818	729	556	572	638

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

Table 3

Trifolium alpestre - stomata

Parameters	Location									
	1	2	3	4	5	6	7	8	9	
Number of samples	10	10	10	10	10	10	10	10	10	
Stomata length on the upper epidermis (μm)	AV	22	23	22	23	21	21	20	21	17
	SD	1.96	1.62	1.36	1.43	1.43	1.33	1.11	2	1.65
	Max	24	25	24	25	24	23	22	22	21
	Min	18	20	20	21	19	19	18	15	16
Stomata length on the lower epidermis (μm)	AV	20	24	20	23	21	22	21	21	15
	SD	2.05	1.38	1.14	1.27	1.09	2.5	1.54	1.71	1.65
	Max	24	26	22	24	23	25	25	23	18
	Min	17	22	19	20	20	18	20	18	13
Stomata width on the upper epidermis (μm)	AV	14.1	14.8	14.2	14.4	14.6	14.3	13.8	15	12.3
	SD	0.9	0.47	0.66	0.68	1.05	0.96	0.5	4.02	1.23
	Max	16	15.5	15.2	15.2	16	16.3	14.5	26.3	14.4
	Min	13	14	13.1	13	12.7	12.6	12.8	12.7	11.1
Stomata width on the lower epidermis (μm)	AV	14.6	16.3	15.2	15.1	15.4	15.4	15.8	14.8	10.6
	SD	0.84	0.98	0.85	0.71	0.72	0.91	0.99	1.02	0.99
	Max	15.8	18.4	16.2	16.2	16.6	17.3	17.1	15.8	13.2
	Min	13.4	15.3	13.7	13.9	14.3	14.2	14.1	12	9.5
The number of stomata per mm^2 on upper epidermis	AV	13	11	20	17	9	10	15	24	35
	SD	5.98	8.29	6.96	12.3	6.27	5.14	9.17	7.74	15.8
	Max	28	25	28	40	18	17	29	36	59
	Min	5	1	9	2	3	0.88	2	14	11
The number of stomata per mm^2 on lower epidermis	AV	104	79	84	84	73	62	73	98	101
	SD	10.7	9.05	21.2	5.73	12	6.8	11.3	9.63	20.5
	Max	120	93	121	93	94	71	97	113	123
	Min	84	66	62	74	57	52	58	87	66

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

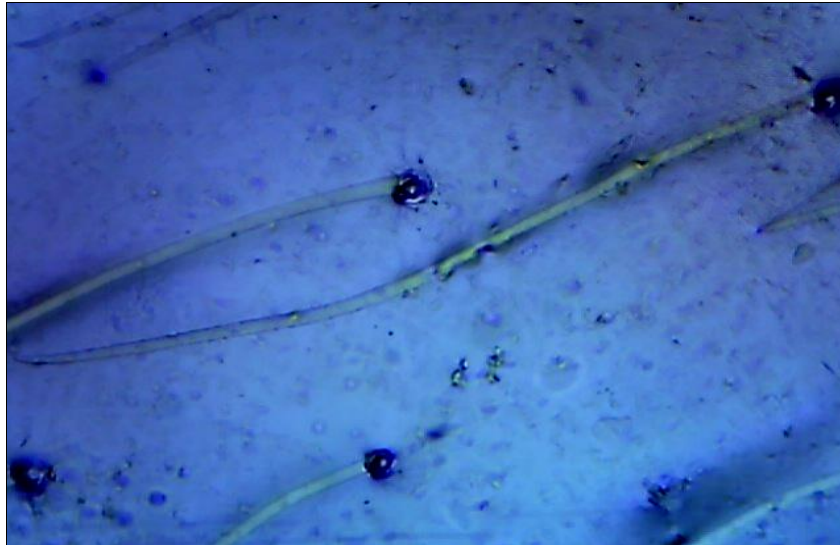


Figure 2. *Trifolium alpestre* trichomes.

The average value of trichomes length varies between 783 and 1395 μm . The leaf mesophyll is differentiated in palisade and spongy tissue (Figure 3). Usually, there is one layer of palisade cells, but sometimes two layers can be seen. Palisade cells are long and cylindrical, while the spongy cells are also cylindrical, but placed horizontally. The vascular bundles of *T. alpestre* leaves are collateral (Figure 4). Mechanical tissue is well developed and covers, as a cape, the two poles of the collateral vascular bundle (Figure 4B). Cells are polygonal and show very thick walls. In some cases, the merging tendency of the midrib vascular bundle with lateral bundles was observed.

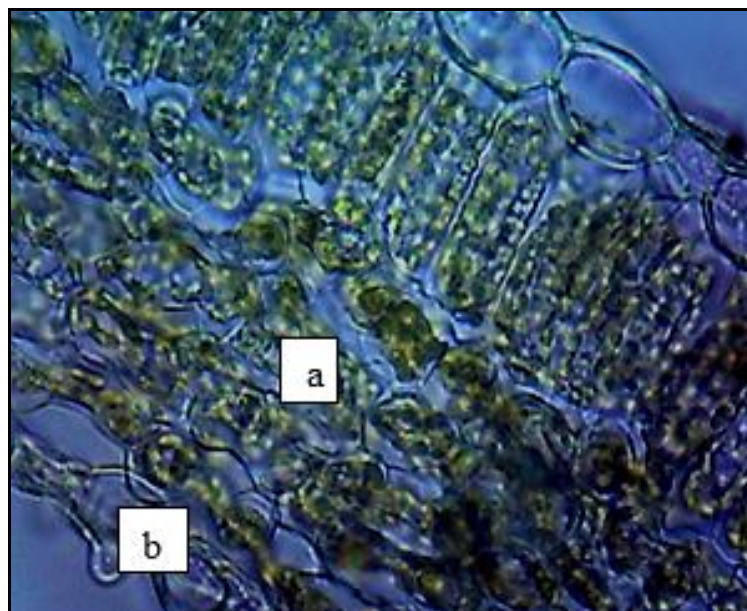


Figure 3. *Trifolium alpestre* - the mesophyll. a - palisade tissue; b - spongy tissue.

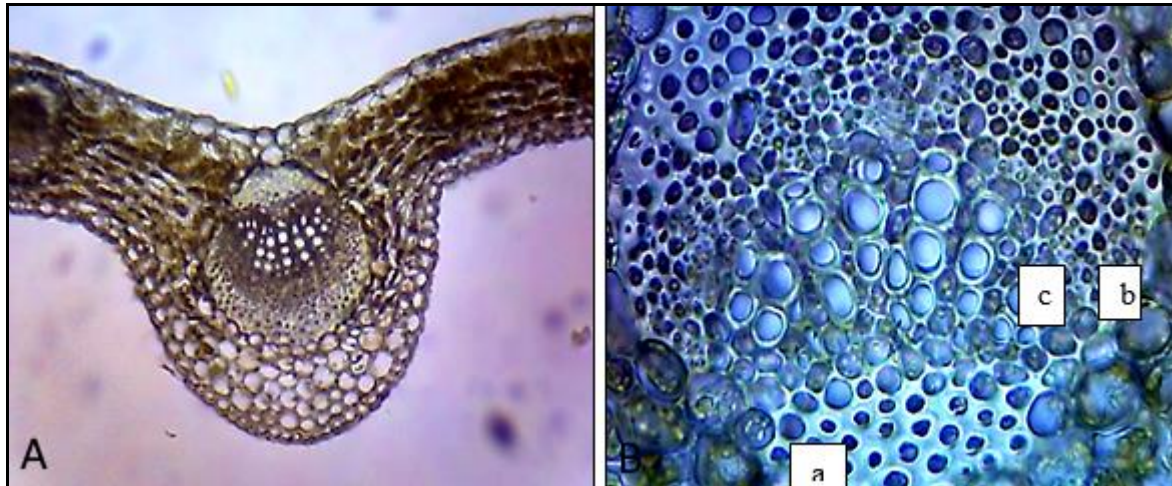


Figure 4. *Trifolium alpestre*. A - the leaf midrib collateral vascular bundle; B - vascular bundle; a - mechanical tissue; b - phloem; c - xylem.

Table 4

Trifolium alpestre - leaf mesophyll

Parameters	Location									
	1	2	3	4	5	6	7	8	9	
Number of samples	10	10	10	10	10	10	10	10	10	
The length of palisade tissue cells (μm)	AV	95	79	98	77	85	93	80	91	84
	SD	5.1	9.3	9.6	16.4	8.45	6.3	16.1	10.8	9.9
	Max	104	94	116	99	97	103	100	110	106
The length of spongy tissue cells (μm)	AV	65	65	74	59	69	78	60	63	48
	SD	6.8	4.8	10.3	9.2	8.1	4.6	7.1	9.7	5.7
	Max	72	74	86	74	85	85	85	78	57
The diameter of collateral vascular bundle (μm)	AV	223	252	264	238	175	206	241	263	278
	SD	39.8	20.1	55.9	49.7	37.3	42.4	55.2	40.6	12.8
	Max	285	282	335	323	216	268	312	318	320
	Min	167	218	168	194	101	114	149	187	217

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

The average values of the length of palisade cells, obtained from investigating the individuals from various locations range from 77 to 98 μm . The average values of spongy cell length vary from 48 to 78 μm . The lowest average value was found to be in Location 9 at the highest altitudes. The midrib vascular bundle was investigated by analyzing its diameter. The results show that the average values of this parameter vary from 175 to 278 μm . The highest average value was noted in Location 9 at the highest altitude.

The flower morphology. Flowers are purple. The calyx is composed of calyx tube and calyx teeth. There are five calyx teeth (Figure 5) covered with dense trichomes, with different length, two of them being longer than the remaining three. The detailed examination included the corolla length, calyx tube, calyx teeth and the total calyx length, and the number of flowers in inflorescence (Table 5).



Figure 5. *Trifolium alpestre* calyx; a - long calyx teeth; b - short calyx teeth.

Table 5

Trifolium alpestre - flower morphological traits

Morphological trait		Locations								
		1	2	3	4	5	6	7	8	9
Number of samples		10	10	10	10	10	10	10	10	10
The total calyx length (mm)	AV	8.1	7.3	7.4	8.7	8.8	9.2	8.7	8.7	8.4
	SD	1.04	0.58	1.02	1.31	1.02	0.7	0.92	1.29	0.76
	Max	9.5	8	9.5	10.5	10.5	10	10	10	9.5
	Min	6	6.5	6	6.5	7	7.5	7.5	6	7
The length of calyx tube (mm)	AV	3.7	3.8	3.8	3.9	3.9	3.9	3.9	4	3.8
	SD	0.35	0.35	0.24	0.21	0.39	0	0.15	0	0.24
	Max	4	4	4	4	4.5	4	4	4	4
	Min	3	3	3.5	3.5	3	3	3.5	4	3.5
The length of long calyx teeth (mm)	AV	4.3	3.5	3.4	4.8	4.8	5.2	4.8	4.7	4.4
	SD	0.78	0.57	1.01	1.25	0.94	0.58	1.1	1.29	0.68
	Max	5.5	4	5.5	6.5	6	6	7	6	5
	Min	3	2.5	2	3	3	4	3.5	2	3
The length of short calyx teeth (mm)	AV	2.6	1.9	2	3.2	3.3	3.4	3.4	3	1.3
	SD	0.76	0.93	0.72	0.71	0.62	0.56	0.43	0.72	0.42
	Max	4	3.5	3.5	4	4.5	4.5	4	4	2
	Min	1.5	1	1.5	2	2.5	2.5	3	1.5	1
The total corolla length (mm)	AV	9.3	9.5	8.7	8.5	10.9	11.1	9.6	4.7	7.7
	SD	0.81	0.68	0.85	1.2	0.59	0.58	0.58	1.29	0.42
	Max	11	10.5	10	10	12	12	10.5	6	8.5
	Min	8	8	7.5	6.5	10	10.5	8.5	2	7
Number of flowers in inflorescence	AV	54	37	69	38	52	49	51	66	65
	SD	20.1	12.8	13.5	14	15	10.2	10.9	22	14
	Max	88	65	93	65	70	63	68	109	97
	Min	31	22	52	25	25	36	35	32	47

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

The average values of the total calyx length vary from 7.3 to 9.2 mm. The investigation of the length of the calyx tube showed that the average values of this parameter vary from 3.7 to 4 mm. The average values of the corolla length vary from 4.7 to 11.1 mm. Since the calyx teeth are unequal, the investigations of the short and long calyx teeth were made separately. The average values of the long calyx teeth vary from 3.4 to 5.2 mm. The average values of short calyx teeth vary from 1.3 to 3.4 mm. The inflorescence of *T. alpestre* has a considerable number of flowers. The number of flowers was investigated and the results show that the average values vary from 37 to 69.

The fruit morphology. The fruit of *T. alpestre* is a legume (Figure 6) with ovoid shape. Fruit has one triangle shaped light brown seed. The fruit length, width and weight were investigated (Table 6).



Figure 6. *Trifolium alpestre*; a - legume; b - seed.

Table 6

Trifolium alpestre - The fruit morphological traits

Morphological trait	Locations									
	1	2	3	4	5	6	7	8	9	
Number of samples	10	10	10	10	10	10	10	10	10	10
The fruit width (mm)	AV	1.9	1.5	1.8	1.5	1.8	1.6	1.4	1.8	1.6
	SD	0.21	0.49	0.42	0.52	0.42	0.51	0.51	0.42	0.45
	Max	2	2	2	2	2	2	2	2	2
	Min	1.5	1	1	1	1	1	1	1	1
The maximum fruit length (mm)	AV	2.6	2.4	2.1	2.2	2.6	2.6	2.3	2.5	2.6
	SD	0.51	0.51	0.56	0.42	0.51	0.51	0.48	0.7	0.51
	Max	3	3	3	3	3	3	3	3	3
	Min	2	2	1	2	2	2	2	1	2
The fruit weight/g	AV	0.0175	0.0199	0.0197	0.0166	0.0174	0.0196	0.0117	0.0216	0.0135

Note: AV - mean; SD - standard deviation; Max - maximum value; Min - minimum value.

The average values of fruit width vary from 1.4 to 1.9 mm, while the averages of fruit length ranged between 2.1 and 2.6 mm. Results of fruit weight investigations show that average values vary from 0.0135 to 0.0216 g. The correlation between morphological traits of *T. alpestre* was analyzed using the Spearman methodology.

Positive correlation was established between these morphological parameters:

- total plant height and stomata length on lower epidermis (0.683) and stomata width on the lower epidermis (0.700);
- total leaf length and middle leaflet length (0.996) and length of the first leaflet (0.967);
- leaf petiole length and total calyx length (0.913), calyx tube length (0.802), long calyx teeth (0.954), short calyx teeth (0.867);
- length of the first and middle leaflet (0.186);
- width of middle and first leaflet (0.734) and stomata width on the upper leaf epidermis (0.74);
- width of the first leaflet and stomata length on the lower leaf epidermis (0.72) and stomata width on the upper leaf epidermis (0.929);
- calyx length and calyx tube length (0.767), long calyx teeth (0.780), short calyx teeth (0.828);
- length of calyx tube and long calyx teeth (0.78) and short calyx teeth (0.709);
- long calyx teeth and short calyx teeth (0.862);
- corolla length and stomata width on the lower leaf epidermis (0.433);
- fruit width and length of the palisade tissue cells (0.809);
- number of flowers in inflorescence and length of palisade tissue cells (0.7);

- length of palisade tissue cells and length of spongy tissue cells (0.7);
- diameter of vascular bundle and number of stomata on the upper leaf epidermis (0.867);
- number of stomata on the upper epidermis and number of stomata on the lower epidermis (0.7).

Negative correlation was established between the following investigated morphological parameters:

- stomata length on the upper leaf epidermis and number of stomata on the lower leaf epidermis (-0.667); this result is in concordance with the results of Miller-Rushing et al (2009);
- total plant height and number of flowers in inflorescence (-0.7) and number of stomata on the upper leaf epidermis (-0.7);
- total leaf length and fruit width (-0.749) and length of palisade tissue cells (-0.783);
- length of the middle leaflet and fruit width (-0.795) and length of palisade tissue cells (-0.619);
- length of the first leaflet and length of the palisade tissue cells (-0.7);
- length of short calyx teeth and diameter of collateral vascular bundle (-0.683);
- corolla length and diameter of midrib vascular bundle (-0.733), and number of stomata on the upper epidermis (-0.9) and number of stomata on the lower leaf epidermis (-0.8);
- stomata width on the lower leaf epidermis and number of stomata on the lower leaf epidermis (-0.817).

Conclusions. Based on the conducted research on *T. alpestre* on the vertical profile of Brezovica (Sharri Mountain), it has been established that this species has a very narrow vertical dispersion. This species inhabits open dry areas, areas close to the foot of *Quercus* sp. trees, or pastures with higher humidity, at altitudes from 1047 m to 1256 m. During the process of adaptation to different habitats with different environmental conditions and different altitudes, most of the morphological and anatomical parameters have shown a significant difference, although the difference in altitude was not very high. It can be concluded, based on the results obtained when investigating different parameters of plant individuals from 9 locations, that the variation found is related more to the microclimate and less to the influence of the altitude gradient. The following morphological parameters are found to be more consistent: the length of calyx tube, maximum fruit width, fruit length. Plant height varied from 15.5 to 45.9 cm. The fruit of *T. alpestre* is a globose legume at the base (1-3 mm long), becoming ovate at the top. Leaves are amphistomatous with very few stoma (1.76–59.84/mm²) on the upper epidermis. Flower's corolla is red, calyx is hairy; number of flowers in inflorescence is 22-109. Leaves have a total leaf length between 1.95 and 4.85 cm; upper leaf epidermis is hairless; trichomes of the lower leaf epidermis are long from 476.8 to 2029.650 µm; diameter of midrib vascular bundle is 101.4 – 335.3 µm. A visible positive correlation with increase of altitude was observed between the stomata number and the diameter of collateral bundle.

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Authors:

Kimete Lluga-Rizani, Department of Biology, Faculty of Mathematical Natural Sciences, University of Prishtina, Mother Theresa Str., 10000 Prishtina, Kosovo; e-mail: kimete.lluga@uni-pr.edu

Dubravka Šoljan, Department of Biology, Faculty of Mathematical Natural Sciences, University of Sarajevo, Zmaja od Bosne 33-35, 71000 Sarajevo, Bosnia and Herzegovina, e-mail: dr.dsoljan@gmail.com

Smajl Rizani, Faculty of Food Science and Biotechnology, University of Business and Technology, Lagja Kalabria Str., 10000 Prishtina, Kosovo; e-mail: smajl.rizani@ubt-uni.net

Kemajl Kurteshi, Department of Biology, Faculty of Mathematical Natural Sciences, University of Prishtina, Mother Theresa Str., 10000 Prishtina, Kosovo, e-mail: kemajl.kurteshi@uni-pr.edu

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