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Genetic diversity within selected European populations of the moss species *Atrichum undulatum* as inferred from isozymes

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Summary: The moss species *Atrichum undulatum* (Hedw.) P. Beauv. (Polytrichaceae, Bryophyta) is widespread throughout the northern hemisphere. Selected accessions from Europe (23) have been studied based on 5 enzymes and their isoforms with aim to reveal genetic diversity. The results obtained clearly show that there is a high genetic diversity in *Atrichum undulatum* within the samples tested. However, the results do not document any distributional pattern or any special gradient based on genetical analyses.

Keywords: *Atrichum undulatum*, moss, bryophytes, isozymes

Genetic variability within the same species is a one of the biodiversity components which enables survival of living entities. Although, bryophytes are often regarded as slow evolving taxa, they accumulate genetic diversity within populations and thus increase the ability to cope with changing environment (e.g. Rosengren et al. 2015).

Atrichum undulatum (Hedw.) P. Beauv. (Polytrichaceae, Bryophyta) is a moss species widely distributed throughout the northern hemisphere, inhabiting shaded soil in temperate and boreal forests. It is among the biggest terrestrial mosses in Europe. It produces sporophytes relatively often and the spores are 13–18 µm large. The spore size of *A. undulatum* is slightly bigger than that of the bryophyte average, and therefore a long distance dispersal happens probably more rarely.

Atrichum undulatum is common in most European countries, also in SE Europe (Sabovljević et al. 2008a). It was recorded only recently in Albania (Marka & Sabovljević 2011), though it prefers temperate climate.

This species is also used for developmental studies due to its size and easier manipulation in lab conditions compared to other bryophytes (Bijelović et al. 2004; Sabovljević et al. 2005, 2006, 2018).

Isozymes (or isoenzymes) are a powerful tool for the investigation of gene variability within and between populations of plants and animals, but new molecular techniques based on DNA are now commonly used (Sabovljević et al. 2008b, 2011). However, isozymes are capable of solving questions of population biology, conservation biology and ecology rather well (Zeidler 2000).

Electrophoretic analyses of isozyme markers have been applied quite often to solve taxonomic relationships, especially if morphological characteristics overlap, or if there are variables within a genus or species (Micales et al. 1998), as well as to assess intra- and interspecific genetic variability. However, genetic diversity within bryophyte species is rarely documented (e.g.

Cronberg 2000; Sabovljević et al. 2008b, 2011; Sabovljević & Frahm 2008, 2009, 2011; Mikulaškova et al. 2015).

There is rather poor evidence of the genetic structure of bryophytes. Stenoien & Sastrad (1999) suggested that one reason is the rather traditional view of bryophyte population biology, in which genetic variability is severely restricted in bryophytes by the dominant haploid phase of their life cycle, their widespread asexuality and the assumed predominant inbreeding in bisexual taxa. Crum (1972) states that bryophytes are a genetically depleted group with a limited evolutionary potential. This is supported by the view that bryophytes have evolved early and have remained morphologically unchanged through geological time, and / or by the existence of highly disjunct conspecific populations with little or no morphological divergence.

However, early isozyme studies performed on bryophytes revealed unexpectedly high levels of genetic variation (Cummins & Wyatt 1981; Yamazaki 1981). It is uncertain, whether the high genetic variability found in bryophytes affects their evolutionary rate.

The majority of isozyme loci in bryophytes behave in a selectively neutral manner (Stenoien & Sastrad 1999). Neutrality may thus explain the high isozyme variability of bryophytes in general. For neutral loci, the balance between mutation and random genetic drift is critical in determining the patterns of genetic variation at each locus and, together with the amount of gene flow, the multilocular genetic structure within the species.

Five isozyme markers were chosen to solve the question of genetic diversity among different populations of *A. undulatum* from Europe.

The present paper describes the genetic diversity within various randomly chosen accessions of *A. undulatum* populations from across Europe based on the isoenzymatic electrophoretic patterns of the enzymes: guaiacol peroxidase (GPX), phenol peroxidase (PPX), ascorbat peroxidase (APX), catalases (CAT) and superoxide dismutase (SOD).

Materials and methods

A total of 22 populations from across Europe was studied (Table 1). The unrelated bryophyte *Marchantia polymorpha* was used as control sample. Voucher specimens are kept in BEOU.

Prior to the experiment, plant material was cleaned, rinsed and revived. Each patch was considered as a clone/population. The revived material was then kept under constant conditions of light (16 h) and temperature (18±2°C) during one week before protein extraction.

Abbreviations: BSA – bovine serum albumin; TEMED – *N,N,N',N'*-tetramethylenediamine; EDTA – ethylenedinitrilo-tetraacetic acid; NBT – nitroblue tetrazolium; DTT – Dithiothreitol.

Protein extraction

The plants were washed three times in deionizied water. The moss material was then paper-dried and transferred to ice. Crude extracts of soluble proteins for all enzyme assays were prepared by grinding 1 g of fresh gametophyte tips in liquid nitrogen and homogenization in 1 ml of extraction buffer containing 1000 µl of ice-cold extraction buffer Tris-HCl (50 mM, pH 7.5), containing 1 mM DTT, 2 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) with addition of 1% (w/v) polyvinylpyrrolidone (PVP). After extraction, all samples were transferred to a centrifuge previously cooled to +4°C and further centrifuged at +4°C for

Table 1. Selected accessions of *Atrichum undulatum* (1–22) and *Marchantia polymorpha* (23) from Europe.

No.	Locality (origin) of tested specimens	Date and collectors
1.	Divcibare, W Serbia	20.10.2011 (Sabovljević M. & Vujičić M.)
2.	Vlasina, E Serbia	19.10.2011 (Sabovljević M. & Vujičić M.)
3.	Kosmaj, C. Serbia	18.04.2009 (Sabovljević M. & Sabovljević A.)
4.	Szent György, W Hungary	02.04.2010 (Sabovljević M.)
5.	Shishtavec, N Albania	25.05.2009 (Marka J.)
7.	Weibersbrunn, C. Germany	23.09.2011 (Sabovljević M. & Sabovljević A.)
8.	N Köln, W Germany	21.09.2011 (Sabovljević M. & Sabovljević A.)
9.	Herzogenhorn, S Germany	13.09.2011 (Sabovljević M. & Sabovljević A.)
10.	Frankfurt, C. Germany	19.09.2011 (Sabovljević M. & Sabovljević A.)
11.	Sremcica, C. Serbia	20.10.2011 (Sabovljević M. & Vujičić M.)
12.	S Köln, W Germany	08.12.2007 (Sabovljević M. & Sabovljević A.)
14.	Kopaonik, C. Serbia	12.07.2002 (Sabovljević M.)
15.	Djerdap, E Serbia	12.07.2001 (Sabovljević M.)
16.	Put Beograd-Zrenjanin, N Serbia	15.11.2000 (Sabovljević M.)
17.	Derventa, Bosnia-Herzegovina	05.10.2003 (Šarčević Lj.)
18.	Djerdap, E Serbia	28.09.2011 (Sabovljević M. & Sabovljević A.)
19.	Avala, C. Serbia	From in vitro stable culture
20.	Köln, W Germany	From in vitro stable culture
21.	Ljubljana, C. Slovenia	05.12.2011 (Sabovljević M., Sabovljević A. & Vujičić M.)
22.	Brnik, W Slovenia	05.12.2011 (Sabovljević M., Sabovljević A. & Vujičić M.)
23.	Belgrade, Serbia	From in vitro stable culture

15 minutes at 10 000 rpm (Heraeus Biofuge Stratos Centrifuge, Thermo electron corporation, Kendro, Germany). The upper phase containing proteins was transferred to new test tubes and kept on ice until electrophoresis.

Electrophoresis

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed. A Tris-glycine system with pH 8.3 (25 mM Tris / 192 mM glycine) was used as the running buffer. Protein concentration was determined by Bradford assay (Bradford 1976), with BSA solutions as standards. For the gel assays, proteins (50 mg/lane) were separated on 7% discontinuous non-denaturing polyacrylamide slab 16×16 cm gels, using Hoefer SE600 unit at 200V and 4°C. For SOD, GPX, PPX and APX, the electrophoresis was run until the dye front reached the bottom of the gel (5–6 h), while the CAT isoforms were separated for 18 h.

Ascorbate peroxidase (APX)

For ascorbate peroxidase (APX) detection, a sensitive and specific method based on the ability of APX to prevent the ascorbate dependent reduction of NBT in the presence of H_2O_2 was used (for details please see Mittler & Zilinskas 1993), resulting in white activity bands on a purple background of the formed formazan.

Guaiacol peroxidase (GPX)

Guaiacol peroxidase (GPX) isoforms, separated by native PAGE, were detected using universal peroxidase substrate guaiacol. The staining solution contained 6 mM guaiacol and 12 mM H_2O_2 in 100 mM citrate buffer (pH 4) (for details please see Rothe 2002), so the visualized isoforms are mostly acidic. The gels were stained for ~ 20 min, until bright red bands of tetrahydroguaiacol appeared.

Phenol Peroxidases (PPX)

The gel was flushed with 50 ml of color buffer [50 mM Tris-HCl buffer (pH 7.2) with 10 mg 4-chloro-α-naphthol and 30% H_2O_2] for 15 min at room temperature (Chance & Maehly 1955). After full coloration, the gel was analyzed at the Quantum-ST4 system (Vilber Lourmat, France).

Catalases (CAT)

Catalase (CAT) gel assay involved a preincubation of the gels in 0.01% H_2O_2 for 25 min at room temperature, followed by a brief wash and incubation in a mixture of 2% $K_3Fe(CN)_6$ and 2% $FeCl_3$ for 100 s until the white bands on a dark green background appeared, as described by Woodbury et al. (1981).

Superoxide Dismutase (SOD)

Superoxide dismutase isoforms were detected by riboflavin-NBT method (Beauchamp & Fridovich 1971). The gel was flushed with 50 ml of color buffer (NBT buffer (pH = 7.8) with 1.8 mg riboflavin, 0.012 ml TEMED, 0.12 ml 0.5M EDTA and K-P buffer) for 30 minutes at room temperature under dark conditions. After full coloration, the gel was washed with distilled water under light.

Data analyses

The gel analyses allowed to construct the matrix, which was used afterwards for statistical analyses. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm for pairwise distance matrix was used to construct a relationship tree, assuming a constant rate of evolution.

Results and discussion

The results obtained clearly show the high genetic diversity within the studied accessions of *A. undulatum*. Different isozymes point to genetic diversity to different extent within tested representatives. Undoubtedly, there is a genetic diversity among selected populations of this species in Europe. Figures 1–5 show that 3–14 isotypes can be detected within the selected populations. This is the consequence of existing isoforms within each of isozymes applied. Once, the matrix was made out of these 5 isozymes tested (with all detected isoforms), we were able to construct dendrogram implicating the relatedness among each of the accessions from Europe (Fig. 6).

The accession from Serbia (loc. Kosmaj), sample no. 3, showed maximal detected isoforms among all tested samples (in total 15).

Based on the gel analyses (zymograms), we can confirm the existence of 3 isoforms of guaiacol peroxidase and additional 3 of catalase, 2 isoforms of ascorbate peroxidase, 6 isoforms of phenol peroxidases, 9 isoforms of superoxide dismutase in tested accessions of *Atrichum undulatum*.

Table 2. Similarity index after Sørensen, based on the matrix made by isoforms detected.

	1	2	3	4	5	7	8	9	10	11	12	14	15	16	17	18	19	20	21	22	23
1	1.000																				
2	0.842	1.000																			
3	0.480	0.500	1.000																		
4	0.737	0.889	0.583	1.000																	
5	0.526	0.444	0.750	0.444	1.000																
7	0.632	0.667	0.500	0.667	0.444	1.000															
8	0.778	0.941	0.522	0.941	0.471	0.706	1.000														
9	0.667	0.800	0.692	0.900	0.600	0.600	0.842	1.000													
10	0.720	0.750	0.533	0.667	0.333	0.667	0.696	0.615	1.000												
11	0.632	0.778	0.667	0.778	0.444	0.556	0.824	0.700	0.667	1.000											
12	0.727	0.762	0.741	0.857	0.667	0.571	0.800	0.957	0.593	0.667	1.000										
14	0.154	0.333	0.333	0.500	0.167	0.000	0.364	0.429	0.222	0.333	0.400	1.000									
15	0.526	0.444	0.750	0.556	0.778	0.444	0.471	0.700	0.333	0.444	0.762	0.333	1.000								
16	0.526	0.556	0.500	0.667	0.333	0.444	0.588	0.600	0.583	0.556	0.571	0.333	0.556	1.000							
17	0.571	0.700	0.692	0.800	0.500	0.700	0.737	0.727	0.692	0.700	0.696	0.429	0.600	0.700	1.000						
18	0.636	0.667	0.593	0.762	0.476	0.667	0.700	0.696	0.593	0.571	0.750	0.267	0.571	0.667	0.696	1.000					
19	0.421	0.556	0.500	0.667	0.222	0.667	0.588	0.600	0.583	0.444	0.571	0.500	0.444	0.444	0.700	0.571	1.000				
20	0.778	0.824	0.435	0.824	0.471	0.588	0.875	0.737	0.696	0.706	0.700	0.364	0.353	0.471	0.632	0.600	0.471	1.000			
21	0.720	0.667	0.800	0.750	0.667	0.583	0.696	0.846	0.600	0.667	0.889	0.333	0.750	0.500	0.692	0.741	0.583	0.696	1.000		
22	0.696	0.818	0.500	0.727	0.455	0.727	0.762	0.667	0.714	0.636	0.640	0.250	0.455	0.545	0.750	0.720	0.636	0.667	0.643	1.000	
23	0.316	0.444	0.250	0.444	0.222	0.222	0.471	0.400	0.333	0.333	0.381	0.333	0.111	0.222	0.300	0.286	0.444	0.471	0.333	0.364	1.000

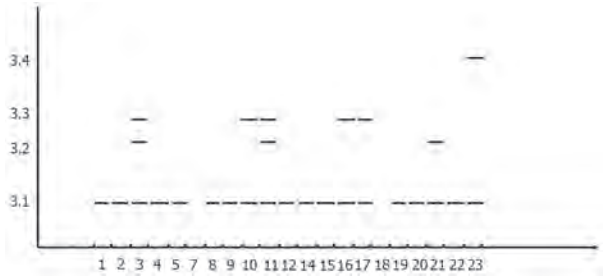

Figure 1. The zymogram of GPX in European accessions of *A. undulatum* with 3 isoforms and the 4 isotypes. Sample 23 represents GPX of *M. polymorpha* (control sample).

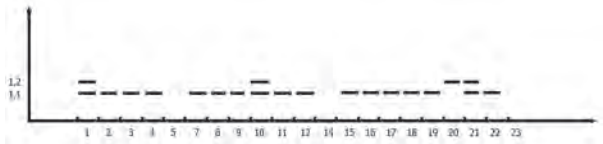

Figure 2. The zymogram of APX in European accessions of *A. undulatum* with 2 isoforms and the 3 isotypes. Sample 23 represents APX of *M. polymorpha* (control sample).

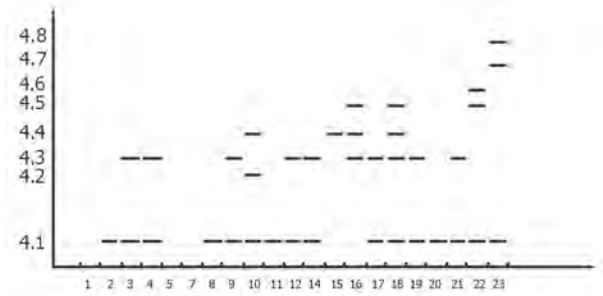

Figure 3. The zymogram of PPX in European accessions of *A. undulatum* with 6 isoforms and the 6 isotypes. Sample 23 represents PPX of *M. polymorpha* (control sample).

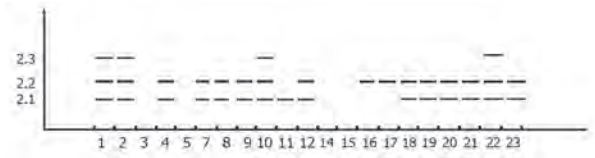

Figure 4. The zymogram of CAT in European accessions of *A. undulatum* with 3 isoforms and the 4 isotypes. Sample 23 represents CAT of *M. polymorpha* (control sample).

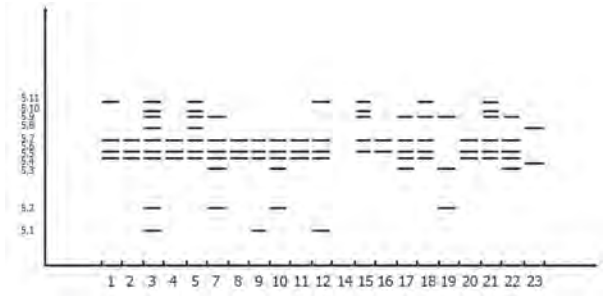

Figure 5. The zymogram of SOD in European accessions of *A. undulatum* with 11 isoforms and the 14 isotypes. Sample 23 represents SOD of *M. polymorpha* (control sample).

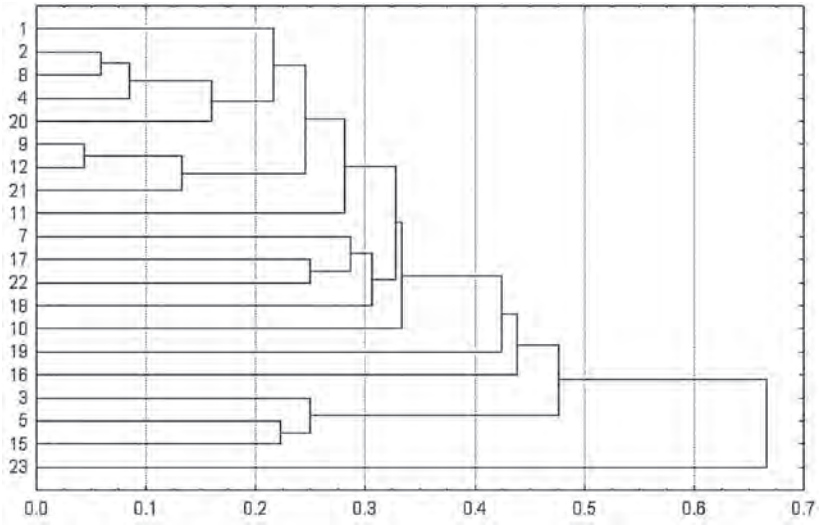

Figure 6. Similarity UPGMA dendrogram of selected European accession of *Atrichum undulatum*.

Table 2 allows us to judge on the relatedness among samples tested according to Sørensen index. High similarity is detected among pair samples 2-8, 4-8, 4-9 and 9-12, but also between 4-2, 4-12 and 12-21. Sørensen index also showed sample 14 to be weakly genetically similar to sample 1 and 5, due to a very low isozyme diversity. The performed isozyme analyses document a rather huge genetical diversity within European *Atrichum undulatum*, but no distributional pattern could be inferred.

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