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GENETIC STRUCTURE OF THE RARE MOSS SPECIES *RHODOBRYUM ONTARIENSE* IN VOJVODINA (SERBIA) AS INFERRED BY ISOZYMES

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Abstract - Rhodobryum ontariense (Kindb.) Kindb. (Bryaceae, Bryophyta) is a rare moss, only recently discovered in Serbia (at Deliblatska Sands). After a revision of the genus *Rhodobryum* in Serbia, it was concluded that all high-mountain records belong to *R. roseum*, while *R. ontariense* is confined to the one known locality at Deliblatska Sands. It is listed in the bryophyte red-list of Serbia and Montenegro. Within the single known locality we have counted 15 small sub-populations over a total surface area of 6 hectares. The species is always in sterile condition and has been recorded only on dunes exposed to the north, at the edge of shrub-grassland transition interspersed with fragments of steppe vegetation. No propagules are known. This raised the question of whether the population was once continuous, or whether vectors exist that spread detached plants or fragments to establish new subpopulations. To answer this question an isozyme analysis was performed to estimate the genetic structure of this isolated population. Based on the isozyme forms of superoxide dismutase and peroxidase at least six haplotypes were determined within the population. It can be concluded that the present patches of the moss do not derive from one subpopulation. Some kind of short-distance dispersal exists, but it remains unclear what structures act as propagules and what is the vector for them.

Key words: Rhodobryum ontariense, moss, bryophytes, isozymes

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INTRODUCTION

Rhodobryum ontariense (Kindb.) Kindb. (Bryaceae, Bryophyta) is a rare moss in Serbia. It was recently identified from a single population at Deliblatska Sands in Vojvodina (Sabovljević and Cvetić, 2001; Sabovljević, 2003), consisting of 15 patches dispersed along two dune slopes exposed to the north in bushy vegetation over a surface area of cca. 6 hectares (UTM 34TEQ60). It is included in the red list of bryophytes of Serbia but its threat status requires revision (Sabovljević et al., 2004).

R. ontariense is a moss with a wide but very fragmented distribution (Dierssen, 2001). It usually grows on dry, warm, calcareous soils and can be classified as subneutrophytic, meso- to moderately thermophytic, considerably adapted to shade (and therefore present in open, woodland formations), anitrophytic and ahemerobous. Within SE Europe it is recorded in Bulgaria, Croatia, Hungary, Romania and Slovenia (Erzberger and Papp, 2004; Sabovljević, 2006; Sabovljević et al., 2008a).

The plant is dioecious and it has never been seen in Serbia with sporophytes. Male plants have rarely been observed.

The principal goal in the study of bryophyte population biology can be said to be the determi-

nation of the relationships between different reproduction patterns and genetic composition, as well as an understanding of the degree of variability at the population level. Persistent vegetative reproduction in bryophytes influences spatial structure, defined by allele and genotype frequency within the area occupied by individual colonies that compose a given population.

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; Jevremović et al., 2010). However, isozymes are capable of solving questions of population biology, conservation biology and ecology equally well (Zeidler, 2000).

Electrophoretic analyses of isozyme markers have been applied quite often to solve taxonomic relationships, especially where morphological characteristics overlap or where there are variables within the genus or species (Micales et al., 1998), as well as to assess intra- and inter-specific genetic variability.

There is rather poor evidence of the genetic structure of bryophytes. Stenoien and Sastrad (1999) suggested that one reason is the rather traditional view of bryophyte population biology, where 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 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, the early isozyme studies performed on bryophytes revealed unexpectedly high levels of genetic variation (Cummins and 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 and 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.

To solve the question of whether colonies represent clones that have arisen by the vegetative reproduction of individual genotypes, and whether other factors shape spatial distribution of genotypes in a population, two isozyme markers were chosen.

The present paper describes the genetic structure of the *R. ontariense* population in Deliblatska Sands based on the isoenzymatic electrophoretic patterns of the enzymes peroxidase (POD) and superoxide dismutase (SOD).

MATERIAL AND METHODS

The plant material consisted of 35 shoots (young gametophyte tips) collected on 20 November 2009 from each patch considered to be a clone/subpopulation, and placed in plastic bags prior to laboratory examination. Voucher specimens are deposited in the bryophyte collection at BEOU (nos. 4708, 4912). After mechanical cleaning, the material was kept at -20°C until protein extraction and electrophoretic analyses were performed. The subpopulations/clones were sampled with a minimum distance of 10 m between each subpopulation, and numbered to indicate the distance from the first sampling point.

Protein extraction

Up to 20-30 gametophyte tips (cca. 250 mg) per extraction were used. The plants were carefully cleaned under a dissecting microscope and washed three times in de-ionized water. The moss material was then paper-dried and transferred to ice. The material

was treated with liquid nitrogen and then homogenized, after which 750 µl of ice-cold extraction buffer Tris-HCl (50 mM, pH 7.5), containing 1mM DTT, 2 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) with the addition of 1% (w/v) polyvinylpyrrolidone (PVP), was added to each sample. After extraction, all samples were transferred to a centrifuge previously cooled to +4°C and further centrifuged at +4°C for 15 min 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 electrophoresis was run at a voltage of 200 V for 3 h at 4°C.

Peroxidases

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. After color-staining, the gel was analyzed with the Quantum-ST4 system (Vilber Lourmat, France).

Superoxide Dismutase

Superoxide dismutase isoforms were detected by the riboflavin-NBT method (Beauchamp and 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 staining, the gel was washed with distilled water under light.

The 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 two selected isozyme patterns show that there is genetic variability within the population of *R. ontariense* at Deliblato Sands in Serbia. All 15 subpopulations/clones express the same isozymatic pattern for SOD (Fig. 1) and so provided no informative characters. However, the pattern for POD (Fig. 2) gave enough information to construct a relationship tree among the subpopulations.

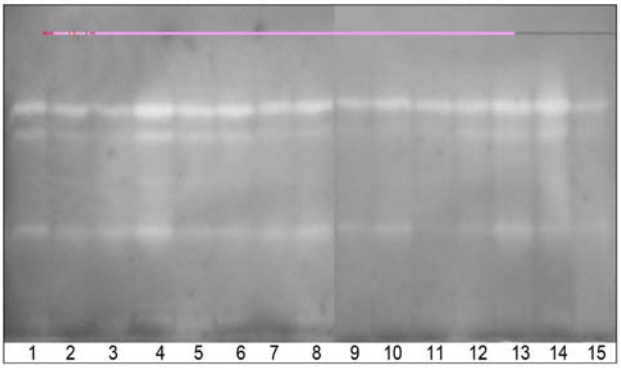

Fig. 1. Native PAGE, stained for SOD isoenzymes within the Deliblato Sands subpopulations of *R. ontariense*

Six isotypes are present in the Deliblatska Sands population of *R. ontariense*:

- isotype 1: subpopulations 1, 3, 4, 5
- isotype 2: subpopulation 2
- isotype 3: subpopulations 6, 7, 8
- isotype 4: subpopulations 9, 11, 12, 15
- isotype 5: subpopulation 10
- isotype 6: subpopulations 13, 14.

The phylogram (Fig. 3) shows the relationships between the subpopulations studied.

Sporophytes have never been seen at the site of *R. ontariense* at Deliblato Sands during a 15-year period of study. It was previously identified as *R. roseum* at this locality and there are no herbarium specimens or written notes from the 1950s onwards to provide data on the presence of sporophytes and sex organs.

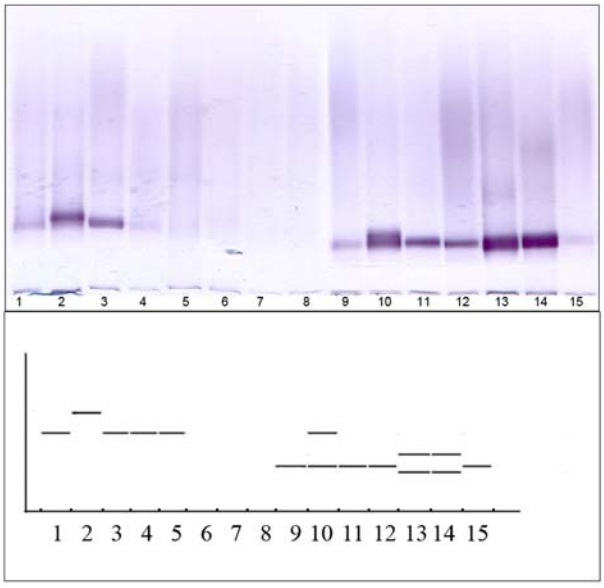

Fig. 2. Twin-Gel electrophoresis and zymogram of peroxidase isoforms within the Deliblato Sands subpopulations of *R. ontariense*

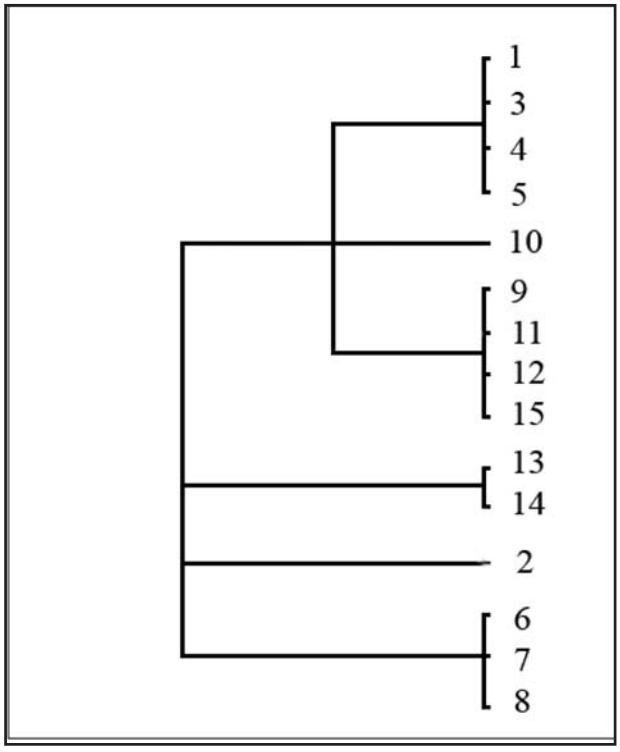

Fig. 3. Phylogram of Serbian *R. ontariense* subpopulations inferred from peroxidase isoforms.

R. ontariense spreads by means of subterranean stolons, but only in close proximity to the mother plant. It is not clear what structures are appropriate in this species for asexual dissemination. No obvious propagules are known.

In the studied population, the subpopulations occur exclusively on the north-exposed slopes of sand dunes in the ecotones of wood-steppe fragments. The distances between the subpopulations are such that the possibility of spreading by stolons can be excluded. In any case, there is an ecological barrier between the two adjacent north-facing dune slopes.

However, the genetic diversity inferred by POD is present within the subpopulations at Deliblato Sands, indicating a polyphyletic origin and/or sexual reproduction in the ancestor population.

Since spreading by stolons can be excluded and the zymogram offers clear evidence of genetic diversity, it can be assumed that events involving sexual reproduction and distance dispersal have occurred in the near past. It is possible that environmental factors induced sexual reproduction only during exceptional seasons and that such events are rare but not impossible.

The genetic diversity of the studied population raises questions about the nature of the asexual propagules for meso- and macro-distance dispersal, and their vectors. A similar pattern is shown in other bryophyte species such as *Hilpertia velenovskyi* (Schiffn.) Zander, *Dichelyma capillaceum* (With.) Myr., *Campylopus oerstedianus* Mitt. and *Rhytidium rugosum* (Hedw.) Kindb. (Sabovljević et al., 2006, 2008b; Sabovljević and Frahm, 2008, 2009, 2011).

Considering the rarity and high conservation value of this species, the first evidence of genetic structure provided by this study provides insight into the importance of active protection of the vulnerable population at Deliblato Sands.

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