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GENETIC VARIABILITY WITHIN SERBIAN POPULATIONS OF THE RARE AND ENDANGERED POTTIOID MOSS *HILPERTIA VELENOVSKYI* (SCHIFFN.) ZANDER INFERRED BY ISOZYME ANALYSES

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Abstract — Genetic variability within Serbian populations of the pottioid moss *Hilpertia velenovskyi* (Schiffn.) Zander was studied. Eight populations of this rare and endangered moss species were chosen for peroxidase isozyme analyses. From the data obtained, it can be inferred that four haplotypes are present among *H. velenovskyi* populations in Serbia, the Banat population being distinctive from all others.

Key words: Moss, *Hilpertia velenovskyi*, genetic variability, isozyme analysis, Serbia

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INTRODUCTION

Erected for a moss measuring only a few milimeters, the pottioid genus *Hilpertia* is monospecific. It is based on material collected in the vicinity of Prague in the Czech Republic and described by Schiffner (1893) as *Tortula velenovskyi* Schiffner. Later, Zander (1989) erected the genus *Hilpertia* for it, based on the strongly recurved laminal margins forming a hollow tube of thin-walled and papillose cells. The given species is characteristic of cold and dry loess habitats. The type locality remained for a long time the only known locality for this species. Some fifty years later, Boros (1944) found *H. velenovskyi* in northeastern Hungary. Subsequently, Podpera (1954) recorded it from Serbia, Pilous (1958) from the Czech Republic, Peciar (1960) from Slovakia, and Waclawska (1958) and Kuc (1960) from Poland. Later, Pócs (1999) added many new localities in Hungary and neighbouring regions. Müller (2000) reported this species as new for Germany based on a collection from the vicinity of Dresden (East Germany), and Frahm (2000) confirmed its presence in Germany based on a collection from another loess cliff in Rheinhessen (West Germany). Finally, it was newly found in Romania and Bulgaria (Pócs *et al*., 2002; Natcheva and Ganeva, 2005, 2006; Sabovljević et al., in press.). The record in Rheinhessen is the westernmost locality in Europe and quite distant from any other continental population. The present European range of *Hilpertia velenovskyi* is very scattered, but it is mainly known from Central and Eastern Europe (Czech Republic, Poland, Slovakia, Hungary, Eastern Germany, Romania, Ukraine, and Serbia). Some additional localities are in West Germany and Bulgaria, and detailed investigation can be expected to reveal its presence in Austria and Croatia on loess cliffs along the Danube. In Serbia, *Hilpertia velenovskyi* grows on the southern boundary of its range, and many small populations are known from cliffs of the Titel loess plateau in Vojvodina (Bačka) (Sabovljević and Stevanović, 2006), one is known from the Alibunar loess cliffs in Banat (Sabovljević, 2003a), and three are known from loess cliffs in Srem (Surduk, Slankamen, Zemun; Sabovljević, 2003b).

It was originally thought that *Hilpertia (Tortula) velenovskyi* is a European endemic species. However, another *Hilpertia* species [*Hilpertia scotteri* (R. H. Zander & Steere) R. H. Zander] was described from

the Northwest Territories of Canada as *Tortula scotteri* R. H. Zander & Steere (Zander and Steere, 1978). Tan and Zhao (1997) treat *H. scotteri* as a synonym of *H. velenovskyi,* which extends its range in the Northern Hemisphere, but makes it even more scattered. During the last few decades, many new sites in the Northern Hemisphere were recorded: in China (Bai, 1987; Tung, 1963; Tan and Zhao, 1997); in Sibiria (Ignatov and Afonina, 1992); in Northern Canada (Zander, 1989); in dry areas of British Columbia (McIntosh, 1989); and on Ellesmere Island (Mogensen and Zander, 1999).

Hilpertia velenovskyi is a continental-subarctic moss species, as suggested by Müller (2000), and an element of cold, insolated, and dry loess cliffs.

The habitats of *Hilpertia* and accompanying bryophytes (*Aloina, Crossidium, Didymodon cordatus, Pterygoneuron,* etc.) in Europe suggest a xerothermic origin (Sabovljević, 2004; Pócs *et al*., 2004). For a map of the complete Holarctic distribution of *Hilpertia*, see Müller (2000).

Hilpertia velenovskyi is a threatened species, listed in the European Bryophyte Red Data Book (ECCB, 1995). An understanding of genetic diversity within the species is essential in order to develop strategies of collection, conservation, and germ plasm formation. Genetic variation within a taxon is thought to be crucial for the long-term survival and continued evolution of populations or species (Franklin, 1980; Beardmore, 1983; Frankel, 1983; Huenneke, 1991). Thus, an accurate estimate of the level and distribution of genetic diversity of threatened and endangered species is an important element in proper conservation (Hamrick et al., 1991; Shaal et al., 1991; Chalmers et al., 1992; Cardoso et al., 1998; Kim *et al*., 2005).

In this study, the genetic variability of the *Hilpertia velenovskyi* in Serbia is considered as part of a national action plan for conservation of this globally rare and endangered moss.

Hilpertia velenovskyi is nationally protected by law in many countries (including Serbia) because of its rarity and vulnerability (Pospisil, 1977; Rajczy, 1990; Ochyra, 1992; Kučera and Váňa, 2003; Sabovljević et al., 2004).

MATERIALS AND METHODS

Moss samples

Eight samples (representing entities, i.e., subpopulations) from the Serbian range of *Hilpertia velenovskyi* were chosen for our study:

Bačka:

1. Mošorin - UTM: 34TDR41
2. Lok - UTM: 34TDR40
3. Vilovo - UTM: 34TDR31
4. Titel - UTM: 34TDR40

Srem:

5. Surduk - UTM: 34TDQ84
6. Stari Slankamen - UTM: 34TDQ84
7. Zemun - UTM: 34TDQ56

Banat:

8. Alibunar - UTM: 34TDQ99

Since the moss is very small, only plants appearing in small patches, i.e., densely attached to each other, were used for isozyme analyses. The plants were collected by the senior author in the spring of 2003 and kept dry at room temperature until the beginning of the analysis. Six weeks before protein extraction, the plants were rewatered and restored to full physiological activity by regular spraying with water every three days under constant light of 1500 Lux in a 16 L/8 D regime slightly modified from Cronberg (1995, 1997), Quandt et al. (2000), and Ahmed and Frahm (2003).

Protein extraction

Up to 15 plants per extraction were used. The plants were carefully cleaned under a dissecting microscope and washed three times in deionizied

water. The moss material was then paper-dried and transfered to ice. The material was treated with liquid nitrogen and then homogenized, after which 100 µl of ice-cold extraction buffer was added to each sample. We used a slightly modified version of the buffer used for extraction of enzymes from phenolic-rich plants by Cronberg (1995) and Wendeel and Weeden (1989). Cold extraction buffer [0.1 M Tris-HCl (pH=7.0) 33 mM citrate, 2% (w/v) Triton X-100, 5% (w/v) polyvinylpyrrolidone (PVP-40), 46 mM sucrose, and 43 mM 2-mercapto-ethanol] was added shortly before extraction. After extraction all samples were transfered to a centrifuge previously cooled to +4°C and further centrifuged at +4°C for 30 minutes at 15000 rpm. The upper phase containing proteins was transfered to new test tubes and kept on ice until electrophoresis.

To judge from the zymogram with four peroxidases (Fig. 1), Serbian metapopulations of *Hilperia velenovskyi* contain four different enzymatic genotypes. The relationship among the eight examined populations was obtained using PAUP (Swofford, 2001) with a matrix constructed from the zymogram for neighbor-joining (Saitou and Nei, 1987) (Fig. 2).

Electrophoresis

Native polyacrylamide gel electrophoresis (Native-PAGE). A Tris-glycine system with pH 8.8 (25 mM Tris / 192 mM glycine) was used as the running buffer. The starting voltage was 40 V. After the probes entered the gel, the voltage was raised to 100 V for the next 4 hours at an ambient temperature of 10°C.

Peroxidases

The gel was flushed with 50 ml of color buffer [50 mM Na-acetate buffer (pH=5.0) with 50 mg $CaCl_2$] and transfered to 3-amino-9-ethylcarbazole (25 mg dissolved in 2 ml of N,N-dimethylformamide) for 20 minutes at room temperature. After incubation, 0.25 ml of 3% H_2O_2 was added. Coloration with benzidine as substrate was used as a control. The first colored bands (red or blue) appear within 5 min. After full coloration, the gel was placed between two pieces of cellophane and dried at 40°C for easier analyses.

RESULTS AND DISCUSSION

The populations examined do not exhibit a well defined geographic pattern. The populations from Lok and Titel in Bačka and Surduk and Stari Slankamen in Srem represent one enzymatic genotype, while the Mošorin and Vilovo populations in Bačka represent a second one well separated from the previous one. The population from Zemun has a different genotype in relation to the Mošorin and Vilovo populations. The population from Alibunar (Banat) is a completely different genotype. In general, genetic structure among bryophytes is rather varied (Bischler and Boisselier-Dubayle,

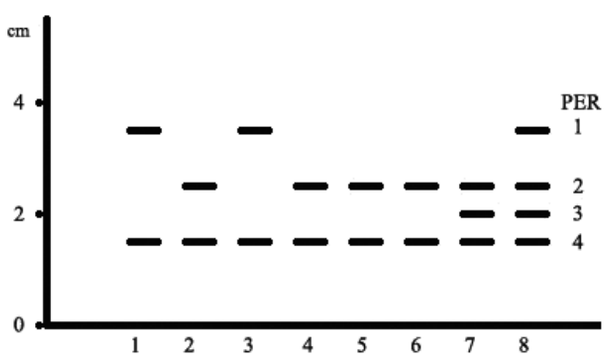

Fig. 1. Zymogram of the relative positions of four peroxidases (1-4) from Serbian Hilpertia velenovskyi populations in 7.5% polyacrilamide gels (Native PAGE, 100V, 4 h, 10°C). 1-8) Populations from Material and Methods.

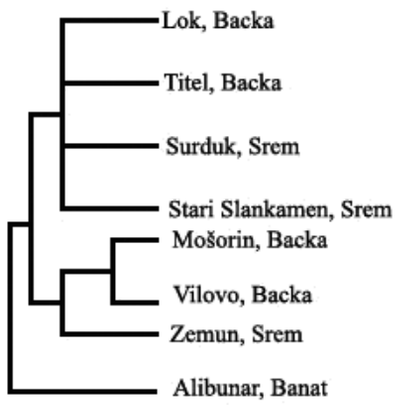

Fig. 2. Dendrogram representing relationships among Serbian populations of Hilpertia velenovskyi.

1997). Distinctiveness of the Banat population is probably a result of long-distance dispersal and recent establishment rather than relictivity, although the latter cannot be excluded.

Hilpertia velenovskyi in Serbia has recently been rarely observed with sporophytes. However, the establishment of new populations is possible by means of spore dispersal (Van Zanten, 1978; Frahm, 2007) from neighboring or even distant far areas or from long-lasting dispore banks (Miles and Longton, 1992; During, 1997). Sabovljević et al. (2006) showed that *Hilpertia velenovskyi* can be dispersed over long distances.

The Serbian population from Mošorin is related to South Hungarian populations, to judge from the ITS region of the nrDNA (Sabovljević et al., 2006). It would be interesting to examine the relation of Serbian populations with other populations of *Hilpertias* worldwide.

Further investigation of the establishment and survival of this species on the southern boundary of its range is needed for its proper conservation and protection of its genetic pool. Boundary metapopulations in a metropolitan system are very important for the survival of a species (Husband and Barrett, 1996). A knowledge of sexual versus asexual behavior of *H. velenovskyi* is needed for its preservation, as in the case of other bryophytes (Kimmerer, 1994).

Inasmuch as long-distance dispersal is present in bryophytes (McDaniel and Miller, 2000; Nathan, 2001) including *H. velenovskyi* (Sabovljević et al., 2006), the probability is low that cryptic speciation takes place among Serbian populations as has been shown for some other mosses (Shaw, 2001).

Genetic diversity with four haplotypes in Serbia and knowledge of its ecology (Kürschner and Wagner, 2005) provide a good basis for active protection of *H. velenovskyi.* However, further investigation is needed to establish characteristics of sexual and asexual reproduction, population genetics, and diaspore survival.

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ГЕНЕТИЧКА ВАРИЈАБИЛНОСТ РЕТКЕ И УГРОЖЕНЕ ПОЦИОИДНЕ МАХОВИНЕ *HILPERTIA VELENOVSKYI* (SCHIFFN.) ZANDER У СРБИЈИ НА ОСНОВУ АНАЛИЗЕ ИЗОЗИМА

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У овом раду је изучавана генетичка структура српских популација поцоидне маховине *Hilpertia velenovskyi* (Schiffn.) Zander. Обзиром на угроженост и малу величину ове маховине, у раду је коришћено осам узорака популација за пероксидазну изозимску анализу. Добијени подаци указују на постојање четири различита хаплотипа у Србији, као и на то да је банатска популација јасно дистинктивна од свих других у Србији.