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Biodiv. Res. Conserv. 1-2: 50-53, 2006

Genetic variation within and between an isolated population of *Phyteuma spicatum* L. ssp. *coeruleum* R. Schulz near Mrągowo (northeastern Poland) and populations of *P. spicatum* L. ssp. *spicatum*

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Abstract: The aim of this study was to determine genetic variation within populations of two *Phyteuma spicatum* subspecies, and to estimate genetic differences between them. An analysis of 5 enzyme systems, i.e. AAT, EST, GDH, MDH and LAP, revealed 11 loci for ssp. *coeruleum* and 10 for ssp. *spicatum*. Four RAPD markers revealed 25 loci. The population of *P. spicatum* ssp. *coeruleum* examined in the study was found to be genetically variable, and the level of its variation did not differ from that estimated for the typical subspecies. Genetic similarity was very high between the two populations of the typical subspecies but low between any of them and the population of *P. spicatum* ssp. *coeruleum*. Some of the isoenzymatic and DNA markers enable identification of both taxa.

Key words: *Phyteuma spicatum*, Campanulaceae, RAPD, genetic diversity, isoenzymatic electrophoresis, genetic similarity

1. Introduction

Spiked rampion *Phyteuma spicatum* L., which belongs to the family Campanulaceae, is represented in Poland by two subspecies: ssp. *spicatum* and ssp. *coeruleum* R. Schulz. The main feature that enables to distinguish between them is the color of flowers. *P. spicatum* ssp. *spicatum* has a cream-colored or whitish-green corolla, whereas the flowers of ssp. *coeruleum* are blue or violet. The former taxon is widespread in Central Europe, mainly in hardwood and mixed forests, as well as in meadows and woodland glades. It is also fairly common in the mountains, but scattered in the lowlands. In contrast, the distribution range of the latter taxon is limited mostly to southern Europe and the northern part of the Balkan Peninsula.

The taxonomic status of *P. spicatum* ssp. *coeruleum* remains unclear. According to Kovanda (1981), the populations with blue and violet flowers found in the Czech Republic and Slovakia are hybrids of the typical subspecies and *P. nigrum* F.W. Schmidt. Huber (1988) suggests that ssp. *coeruleum* is a result of hybridization between *P. spicatum* and *P. ovatum* Honck. The lack of molecular data makes it impossible to explicitly determine the taxonomic position of *P. spicatum* ssp. *coeruleum*.

Only the typical subspecies (ssp. *spicatum*) has been recorded from Poland until quite recently, but in 1994 a locality of *P. spicatum* ssp. *coeruleum* was found near Mrągowo. This is the only locality of this subspecies in Poland, and the northernmost in Europe (Korniak & Hołdyński 1998). The origin of this only Polish population of this species is unknown. According to historical data, *P. nigrum* occurs in the region of Mrągowo (Abromeit *et al.* 1903), but leaf morphology excludes the possibility that the currently known population belongs to this taxon.

The aim of the present study was to determine the genetic variation of populations of two *P. spicatum* subspecies, and to estimate their genetic divergence.

2. Material and methods

Sampling. The experimental materials originated from two *P. spicatum* ssp. *spicatum* populations and the only *P. spicatum* ssp. *coeruleum* population recorded from Poland. The location and characteristics of these

populations are presented in Table 1. All of them are located in northeastern Poland, within a distance of up to 65 km from Olsztyn city. Leaves of 10 individuals of *P. spicatum* ssp. *spicatum* and 20 individuals of *P. spicatum* ssp. *coeruleum* were collected in each locality, depending on population size. The leaves were taken from plants spaced at least 1 m apart. The collected materials were stored at -20°C.

Table 1. List of compared *Phyteuma* populations from Poland

Taxon	Abbreviation	Locality	Habitat	Population size
P. spicatum ssp. *coeruleum*	Ph-s-c	Zalec near Mrągowo	*Molinio-Arrhenatheretea*	186
P. spicatum ssp. *spicatum*	Ph-s-s1	Welski Landscape Park	*Tilio-Carpinetum*	10
P. spicatum ssp. *spicatum*	Ph-s-s2	Kortowo Forest	*Tilio-Carpinetum*	16

Isoenzymatic electrophoresis. Allozyme analysis was carried out for the 40 individuals by using horizontal starch electrophoresis. The following 5 enzyme systems were assayed: aspartate aminotransferase (AAT), esterases (EST), glutamic acid dehydrogenase (GDH), leucyloaminopeptidase (LAP), and malate dehydrogenase (MDH). Staining was performed as described by Soltis & Soltis (1989).

DNA analysis. A sample (1 g) of leaves from each individual was taken for the analysis. DNA was isolated by the modified CTAB procedure (Doyle & Doyle 1990). The purity of DNA samples was assessed spectrophotometrically and reached 90-92%. The sequences of RAPD (Random Amplified Polymorphic DNA) primers used for DNA amplification in this study are given in Table 2. The PCR reaction was conducted

Table 2. Primers used in the RAPD analysis of *Phyteuma spicatum*

Primer	Primer sequence
OPB-08	5' AGGGAACGAG 3'
OPB-19	5' ACCCCCGAAG 3'
OPD-06	5' ACCTGAACGG 3'
OPD-17	5' TTTCCCACGG 3'

in a volume of 20 ml, containing 1 µl PCR buffer [400 mM $(NH_4)_2SO_4$ and 1 M Tris-HCl, pH 9 at 25°C], 2 mM $MgCl_2$, 200 µM each dATP, dGTP, dCTP, dTTP, 0.3 µM primer, 1 unit of Taq polymerase (Eppendorf) and 60 ng of template DNA. The reaction was processed at 94°C for 3 min., followed by 45 cycles at 94°C for 1 min., 36°C for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min. PCR samples were loaded on a 1.2% agarose gel containing 0.5 µg/ml ethidium bromide and separated in 1 × TBE (TRIS – borate – EDTA) buffer at 100 V constant power. Gels were observed in UV light of 312 nm and photographed.

Data analysis. The percentage of polymorphic loci (*P*) and Nei's (1973) gene diversity (*H*) were calculated by using POPGENE-1.32 (Yeh & Boule 1999). A locus was considered polymorphic if the frequency of its most common allele did not exceed 0.95. The degree of genetic similarity (*I*) was determined with Nei & Li's (1979) formula. Principal component analysis and UPGMA grouping were performed in the STATISTICA 7.0 software (Statsoft 2004).

3. Results

Electrophoretic patterns. The analysis of 5 enzyme systems, i.e. AAT, EST, GDH, MDH and LAP, revealed 11 loci for *P. spicatum* ssp. *coeruleum* and 10 for the typical subspecies. The highest number of loci (6) was shown by EST, 2 loci by GDH and LAP, and 1 locus by AAT and MDH. The analysis of 3 populations of *P. spicatum* by using 4 RAPD primers enabled to distinguish 28 loci: 7 loci were revealed by OPB-08 and OPD-06 primers, 8 by OPD-17, and 6 by OPB-19.

Genetic variation and diversity. All of the examined populations were genetically differentiated and 33 of the identified loci were polymorphic. The mean percentage of polymorphic loci in *P. spicatum* was *P*=67.4. Among the 40 analyzed specimens, 39 had different genotypes. Only in the *P. spicatum* ssp. *coeruleum* population 2 plants were found to be identical. Nei's (1973) coefficient of gene diversity (*H*) for both subspecies of *P. spicatum* was 0.270. All values of the genetic variation coefficients are given in Table 3. The mean gene diversity in the *P. spicatum* ssp. *coeruleum* population was 0.142. A similar mean value (0.137) was observed in the *P. spicatum* ssp. *spicatum* population from the Welski Landscape Park, whereas the population from the Kortowo Forest was less diverse (0.126).

Table 3. Sample size (N), number of different genotypes (G), percentage of polymorphic loci (P), gene diversity (H, Nei 1973)

Population	*N*	*G*	*P*	*H*
Ph-s-s1	10	10	35	0.137
Ph-s-s2	10	10	35	0.127
Ph-s-c	20	19	41	0.142
Total	40	39		
Mean			36.7	0.135

Genetic divergence between *P. spicatum* ssp. *coeruleum* and ssp. *spicatum*. The coefficient of Nei & Li's (1979) genetic similarity (*I*), calculated on the basis of enzymatic and RAPD data, was 0.94 between populations of the typical subspecies, whereas between them and ssp. *coeruleum*, it reached only 0.65 in the case of the population from the Welski Landscape Park and 0.64 in that of the population from the Kortowo Forest. Population grouping by UPGMA (Fig. 1) and principal component analysis (Fig. 2) confirmed the taxonomic distinctness of *P. spicatum* ssp. *spicatum* and *P. spicatum* ssp. *coeruleum*. Isoenzymatic electrophoresis enabled molecular identification of the taxa. Marker alleles were found in the following loci: EST-1, EST-2, EST-5 and GDH-1. RAPD markers also enabled molecular identification of the taxa analyzed in the study. Among 25 identified RAPD loci, 8 made it possible to distinguish between the 2 subspecies. Four specific loci were revealed by OPD-06 and OPD-17 primers. OPB-08 and OPB-19 primers revealed monomorphic bands only. None of the DNA markers revealed polymorphism within *P. spicatum* ssp. *spicatum* populations.

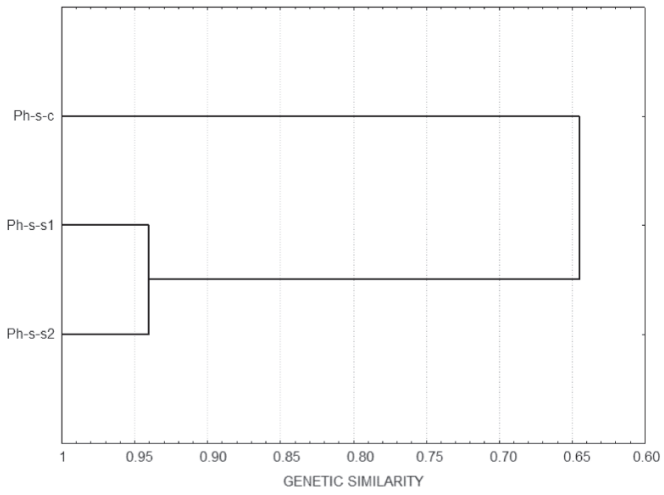

Fig. 1. UPGMA dendrogram of 3 populations of *Phyteuma spicatum*, based on Nei & Li's (1979) genetic similarity

Hamrick & Godt (1990) for vascular plants (*P*=34.2, *H*=0.113). Such a high genetic variation of *P. spicatum* may result from open pollination. Similar parameters of genetic variation were also recorded in the open-pollinated *Lolium perenne* and *L. multiflorum* (Charmet & Balfourier 1994).

The values of genetic variation coefficients obtained for the *P. spicatum* ssp. *coeruleum* population did not differ from those determined for the typical subspecies. The high level of intrapopulation variation could be affected by the size of this population, which was relatively large. However, it should be noted that this is the only population of this subspecies recorded from Poland, and that it is located beyond the continuous distribution range. The origin and age of this population are unknown, so its anthropogenic genesis cannot be excluded.

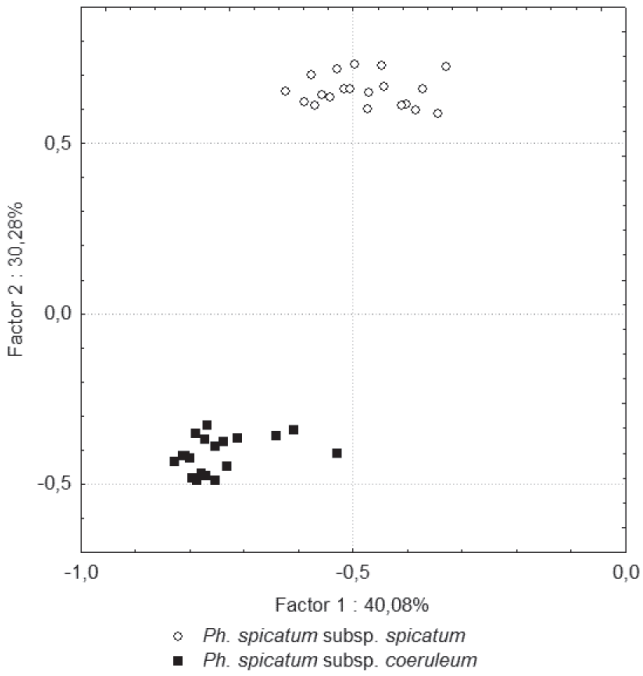

Fig. 2. Principal component analysis of 3 populations of *Phyteuma spicatum*, based on Nei & Li's (1979) genetic similarity

4. Discussion

Genetic diversity in populations located beyond the continuous range of distribution is generally lower than in populations under optimum habitat conditions. This is caused by a strong selection pressure of the factors limiting the natural range of the taxon. In the case of the *P. spicatum* ssp. *coeruleum* population, another factor reducing its genetic diversity could be geographic isolation. In this study, *P. spicatum* shows high genetic variation. Both its mean percentage of polymorphic loci (*P*=67.4) and Nei's (1973) gene diversity (*H*=0.270) were significantly higher than the mean values obtained by

While analyzing the parameters of genetic variation it should be kept in mind that they concern populations belonging to 2 subspecies whose taxonomic status has not been the object of genetic studies so far. The genetic similarity, calculated on the basis of isoenzymatic and RAPD data (*I*=0.65), suggests that the subspecies have separate gene pools. Similar values of this coefficient were obtained for *Polygonatum odoratum* and *P. multiflorum* (*I*=0.57), which are good biological species (Polok *et al.* 2005). Taxonomic distinctness was also proved by the presence of isoenzymatic and DNA markers, enabling molecular identification of *P. spicatum* subspecies. Molecular data seem to confirm the hypothesis

proposed by Kovanda (1981) and Hubner (1988), concerning the hybrid origin of *P. spicatum* ssp. *coeruleum*. The color of flowers, different than in the typical subspecies, may be a consequence of hybridization with the species with blue flowers, and not a result of mutation of the gene or genes responsible for color. According to Kovanda (1981), hybridization took place between *P. spicatum* and *P. nigrum,* whereas Huber (1988) believes ssp. *coeruleum* is the *P. spicatum* × *P. ovatum* hybrid.

A reliable determination of the taxonomic status of *P. spicatum* ssp. *coeruleum* requires further investigations, both molecular and ecological, performed on a greater number of populations and taxa of this genus.

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