AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not. Bot. Hort. Agrobot. Cluj 38 (1) 2010, 37-40

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

Isoenzyme Pattern in Selected Taxa of the *Primulaceae*

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Abstract

Primula leucophylla Pax is endemic to the Romanian Carpathians and has a very controversial taxonomical status, with no molecular research on its populations genetic structure and taxon identification. Based on morphological traits, many authors considered this entity as a new taxon as well as a subspecies of *Primula elatior* (L.) Hill. In the present study the isoenzyme pattern of four enzymes: esterase Est, 6-phosphogluconate dehydrogenase 6-PGDH, shikimate dehydrogenase SKDH and superoxide dismutase SOD was investigated to show whether these isozymes can be useful as discriminatory taxonomic markers between *P. leucophylla* and the closely related species *P. elatior*. No genetic intra- and inter-specific variability was detected, in spite of all the morphological variations distinguished between these two taxa. The failure in the present study to detect polymorphic loci which could be applied in a possible identification and delimitation of *P. leucophylla* from *P. elatior*, does not rule out the possibility of an existing polymorphism in other isoenzymes.

Keywords*:* isoenzyme, genetic variability, taxonomic status

Introduction

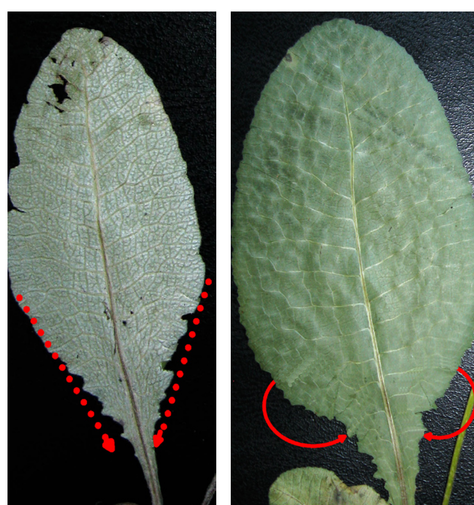
P. leucophylla Pax is endemic to the Romanian Carpathians, being first described by Pax in 1897. He described this entity as a new taxon belonging to the eastern Carpathians, being very similar to the species *P. elatior* (L.) Hill but presenting some morphological differences such as: a more dense indumentum and a specific shape of leaf lamina (Fig. 1). Pax denominated this entity as a new taxon *P. leucophylla* (gr. leukos = white; gr. phyllon = leaf), with *locus classicus* in Giurgeu-Hasmas Mountains.

In 1946, Nyárády performed a thorough and unbiased study of this variable group and identified several varieties. Moreover he distinguished a continuous morphological intergradation between the two taxa and pointed numerous intermediates based on the thickness of the indumentum (Fig. 2).

In Fl. Europaea (vol. III, 1972) this taxon is considered as a subspecies of *P. elatior*. Ciocarlan (2000) considered the entity described by Pax as a subspecies of *P. elatior*, as well.

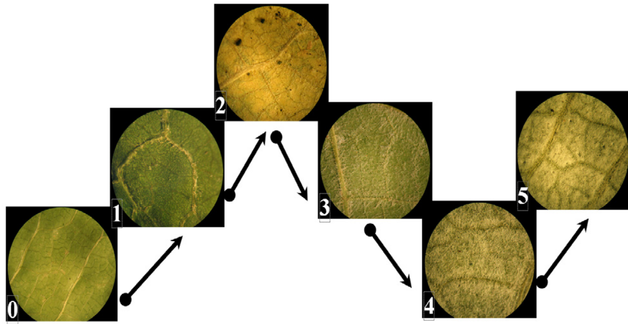
The main morphological traits that separates *P. leucophylla* from *P. elatior* are represented by the more dense indumentum of the inner face of the leaf, the shape of the leaf and the length of the calyx. Based on these morphological traits, authors considered the taxonomic status of *P. leucophylla* differently.

Clearing the taxonomical status of an entity, based only on subtle morphological traits is unsatisfactory, since it can lead to contradictory opinions. Isoenzyme markers analyzed by means of electrophoresis have been applied quite often to solve taxonomic problems, 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 (Borza *et al*., 1996; Butiu-Keul *et al*., 2007). The effectiveness of using isoenzymes as molecular markers for resolving taxonomic disputes among different closely related taxonomic units, have been already reported in plants (Angelov, 2006), parasites (Snabel *et al*., 2004), insects (Scarpassa and Hamada, 2003), crustaceans (Gusmaõ *et al*., 2006), mollusks (Gallardo *et al*., 2003) and mammals (Smit and Van der Bank, 2001), among others.



P. leucophylla Pax *P. elatior* (L.) Hill

Fig. 1. The differences in leaf morphology among the two taxa



glabra-communis-villosiuscula-cinerea-subviridis-villosula-subleucophylla-euleucophylla

Fig. 2. The variability of the indumentum and intermediate varieties identified between the two taxa (*P. elatior* (L) Hill, *P. leucophylla* Pax)

The present paper describes monomorphisms in the isoenzymatic electrophoretic patterns of the enzymes: esterase Est, 6-phosphogluconate dehydrogenase 6-PGDH, shikimate dehydrogenase SKDH and superoxide dismutase SOD, extracted from *P. leucophylla* and *P. elatior* specimens from the Romanian Carpathians.

Materials and methods

Plant material, consisting of young leaves, was harvested from the wild and preserved at -80°C until electrophoretic analyses were performed. For each taxon two populations, consisting of 15 individuals, were collected from different locations: *P. leucophylla* from the Ciucas Mountains and Ceahlau Massif and *P. elatior* from Piatra Craiului and Stana de Vale (Fig. 3).

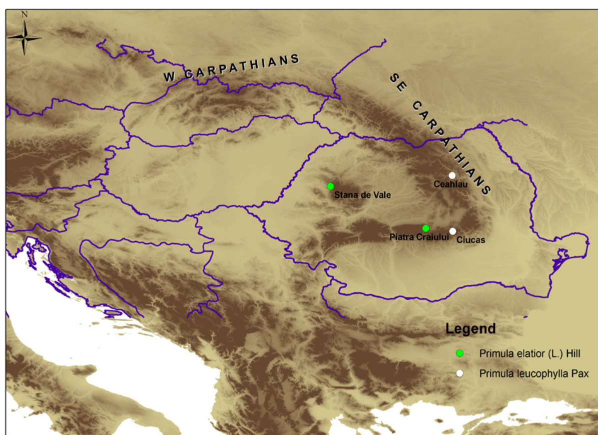


Fig. 3. Sampling localities

A day before electrophoresis the samples were prepared after the following protocol: 60 mg of plant material was weighed for each sample and transfered into 1,5 ml test tubes which were immediately introduced in liquid nitrogen. Then, the plant material was macerated with the aid of glass powder in grinding mortars, that were previously cooled down in the deep freezer. The grinded material was then transfered into the extraction buffer in a proportion of one part plant material to two parts isoenzyme extraction buffer.

The isoenzyme extraction buffer was prepared following Kato (1987) with some modifications according to Singliarova *et al.* (2008): 0.1 M Tris-HCl (pH 8), 70 mM mercaptoethanol, 26 mM sodium metabisulfite, 11 mM L-ascorbic acid, 4% soluble PVP, the pH being adjusted after the addition of the ascorbate. Crude homogenates were centrifuged at 14000 rpm for 10 minutes at 4°C. Supernatants were stored at -80°C or immediately loaded onto gels. Electrophoresis (PAGE) was carried out using 8.16% separation polyacrylamid gel with the buffer 1.82 M Tris-HCl, pH 8.9; 4% stacking gel with the buffer (0.069 M Tris-HCl, pH 6.9) and electrode buffer (0.02 M Tris, 0.24 M glycine, pH 8.3).

The staining procedures followed Konnert and Werner (2004), Krahulec *et al.* (2004) and Peckert *et al.* (2005). For esterases the staining procedures followed a combination of different procedures used in our laboratory (Utter, 1985; Pasteur *et al.*, 1987; Murphy *et al.*, 1990; Acquaah, 1992).

PAGE was peformed on four isoenzyme systems (abbreviation of the system is given at the end of the system name and its EC number is given within parantheses): esterase Est (EC 3.1.1.1), 6-phosphogluconate dehydrogenase 6-PGDH (EC 1.1.1. 44), shikimate dehydrogenase SKDH (EC 1.1.1.25) and superoxide dismutase SOD (EC 1.15.1.1).

The enzymes surveyed in the present paper were classified according to the Nomeclature Committee of the International Union of Biochemistry and Molecular Biology (2006).

Results and discussion

Polyacrylamid gel electrophoresis patterns of the enzymes Est, 6-PGDH, SKDH and SOD, were consistently resolved and analyzed for fifteen individuals in each population, which revealed electrophoretic bands of activity presumably controlled by 9 monomorphic loci (Tab. 1.).

Tab. 1. The same alleles detected at 9 fixed isozyme loci in two species *P. leucophylla* and *P. elatior* from the Romanian Carpathians

Locus and allele	*P. elatior*	*P. leucophylla*
$Est\text{-}1^1$	1.00	1.00
$Est\text{-}2^1$	1.00	1.00
$Est\text{-}3^1$	1.00	1.00
$Est\text{-}4^1$	1.00	1.00
$Pgdh\text{-}1^1$	1.00	1.00
$Pgdh\text{-}2^1$	1.00	1.00
$Pgdh\text{-}3^1$	1.00	1.00
$Skdh\text{-}1^1$	1.00	1.00
$Sod\text{-}1^1$	1.00	1.00

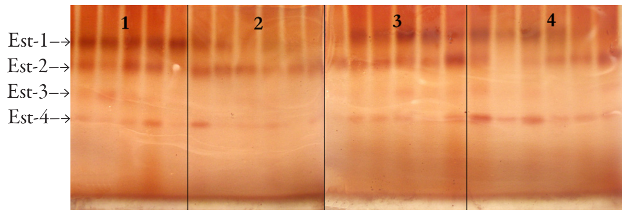


Fig. 4. Zymogram of esterase showing the monomorphic loci fixed for the same alleles Est-1^1, Est-2^1, Est-3^1 and Est-4^1(*P. leucophylla*: 1. Ciucas Mountains and 2. Ceahlau Massif; *P. elatior*: 3. Piatra Craiului and 4. Stana de Vale). Intercalibration zymograms representing 6 individuals from each population

The isoenzyme loci and alleles were identified and classified numerically according to their decreasing electrophoretic mobilities towards the anode. The zymogram description for each enzyme examined is presented below.

Esterases

The isozyme pattern of esterase presumably encoded by the monomorphic loci Est-1, Est-2, Est-3 and Est-4 which were fixed for the same alleles Est-1^1, Est-2^1, Est-3^1 and Est-4^1, in all specimens studied (Tab. 1, Fig. 4).

Phosphogluconate dehydrogenase

The phosphogluconate dehydrogenase enzyme was represented by a three-band pattern presumably encoded by the monomorphic loci Pgdh-1, Pgdh-2 and Pgdh-3. These loci were fixed for the same alleles Pgdh-1^1, Pgdh2^1 and Pgdh-3^1 in all (Tab. 1, Fig. 5).

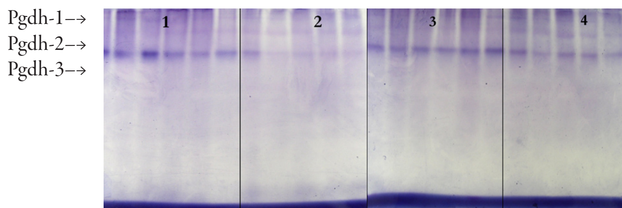


Fig. 5. Zymogram of phosphogluconate dehydrogenase showing the monomorphic loci fixed for the same alleles Pgdh-1^1, Pgdh-2^1 and Pgdh-3^1(*P. leucophylla*: 1. Ciucas Mountains and 2. Ceahlau Massif; *P. elatior*: 3. Piatra Craiului and 4. Stana de Vale). Intercalibration zymograms representing 6 individuals from each population

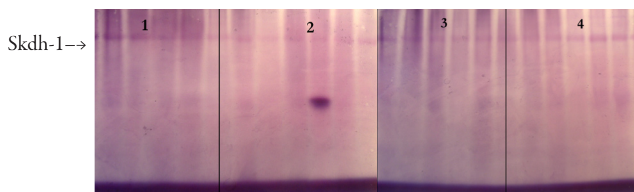


Fig. 6. Zymogram of shikimate dehydrogenase showing the monomorphic locus fixed for the same allele Skdh-1^1 (*P. leucophylla*: 1. Ciucas Mountains and 2. Ceahlau Massif; *P. elatior*: 3. Piatra Craiului and 4. Stana de Vale). Intercalibration zymograms representing 6 individuals from each population

Shikimate dehydrogenase

The enzyme shikimate dehydrogenase showed one monomorphic loci Skdh-1 which was fixed for the same allele Skdh-1^1 in all specimens (Tab. 1, Fig. 6). There was also another locus noticeable in the intermediate region of the gel. This locus was very clearly visualized in only one individual and therefore it was not used for the genetic interpretation.

Superoxide dismutase

Superoxide dismutase showed a monomorphic locus Sod-1, which was fixed for the same allele Sod-1^1 in all plant specimens (Tab. 1, Fig. 7).

Considering that the taxonomic status of *P. leucophylla* still remains complex, polemic, intriguing and at the same time very attractive, we believe that the information generated by molecular markers (isoenzymes, sequencing and AFLP) complemented with morphometric data should significantly contribute to a better understanding of this matter and additionally provide basic support for the present plant genetic fund management and future conservation. In this context, the application of isoenzymes as auxiliary molecular tools must pursue a continued search for discovering significant polymorphic loci in an attempt to clarify the taxonomic status of *P. leucophylla*.

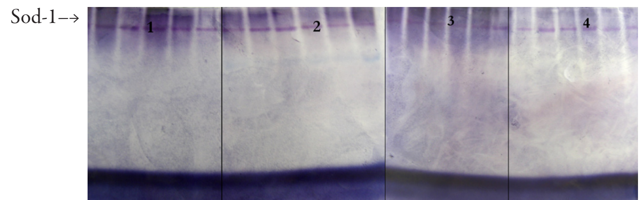


Fig. 7. Zymogram of superoxide dismutase showing the monomorphic locus fixed for the same allele Sod-1^1 (*P. leucophylla*: 1. Ciucas Mountains and 2. Ceahlau Massif; *P. elatior*: 3. Piatra Craiului and 4. Stana de Vale). Intercalibration zymograms representing 6 individuals from each population

Conclusions

The isoenzymatic marker systems analyzed detected no genetic intra- and inter-specific variability within the analyzed populations of *P. leucophylla* and *P. elatior*.

Nine monomorphic loci were identified through the isoenzymatic markers analyzed. Therefore, these markers are not applicable as a diagnostic taxonomic markers for possible identification and delimitation of *P. leucophylla* from *P. elatior*.

Auxiliary isoenzyme markers and/or DNA markers might be used for deciphering polymorphic loci to identify the taxonomic status of *P. leucophylla*.

Acknowledgments

The authors are thankful to the Ministry of Education, Research and Innovation, which supported the present

study through the programme IDEAS (NP-II-ID-PCE-2007-1), as a part of the National Research, Development and Innovation Plan for the period 2007-2013, refered to as the National Plan II (NPII). The number and the title of the project are: 405/1.10.2007/"Clarifying the taxonomical position of certain Carpathians-Balkan plant species through morpho-ecological and molecular biology methods".

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