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MOLECULAR TAXONOMIC ANALYSIS OF *MONOTROPA HYPOPITYS* AND ITS RELATIONSHIP WITH *PYROLACEAE*

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ABSTRACT
The relations between Monotropa hypopitys and four members of Pyrolaceae collected in Bulgaria were investigated using five ISSR markers. The genetic differences given by each used primer were illustrated graphically. The selective ability of each primer was discussed according to the obtained results. The phylogenetic relationships were illustrated using a resulting dendrogram constructed with the data from all polymorphic variants. Monotropa was separated in a single clade only by one marker and displays highest genetic similarity with Orthilia secunda. Our results suggested that Monotropoideae may belong to Pyrolaceae, together with Pyroloideae. Both groups represent two mycoheterotropic lines dependent on the nutrition, which determines their distribution. Monotropa is the terminal result of the gradual passage from authotrophy to mycorrhizal parasitism. In spite of the fact that Monotropaceae is known as a paraphyletic family, it showed a close similarity to Orthilia. The data supported the presumption that Monotropa hypopitys has to be included in the boundaries of Pyrolaceae. The obtained results could serve as a starting point to reconsider the existing classification based on morphological observations.

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Keywords: *Monotropa*, *Pyrolaceae*, ISSR markers, phylogeny

Introduction
According to the old Cronquist system *Pyrolaceae* and *Monotropaceae* are two small families of flowering plants that are partially or completely myco-heterotrophs. While *Pyrolaceae* consists of autotrophic, or semi-autotrophic perennial plants that possess evergreen leaves capable of photosynthesis, *Monotropaceae* representatives do not contain chlorophyll and are myco-heterotrophs parasitizing on fungi. These fungi form a mycorrhiza with nearby tree species.

Many hypotheses explaining the relationships between the four genera (*Chimaphila*, *Moneses*, *Orthilia*, and *Pyrola*) in the small subfamily *Pyroloideae*, position it as a part of *Ericaceae* (9). Genus *Monotropa* is represented in Europe by only one species: *Monotropa hypopitys* and is often considered in the floristic literature as a separate family. Some endemic species of *Monotropa* were found in Asia and America. The relations between the populations of *Monotropa hypopytis* display a high diversity and clear genetic divergence (14). Recent molecular taxonomy investigations, however, demonstrated that *Pyrolaceae* and *Monotropaceae* are most probably subfamilies (16, 17) or tribes (19) of the family *Ericaceae*.

Though many of the traditional groups remain valid, new molecular and morphological investigations diminished their rank to subfamilies (16) or tribes (19). Therefore recently both groups are usually referred as parts of *Ericaceae*, namely subfamilies *Pyroloideae* and *Monotropoideae* (15). Some authors (13) consider this classification as uncertain and discrepant to the phylogenetic relationships. The investigations between the members of *Pyrolaceae* confirm the monophyletic origin of the species. According to Liu et al. (18) *Orthilia* has a hybrid origin and displays a divergent position from the other members. The relationships between *Monoropoideae* and *Pyroloideae* are unclear and their taxonomic positions remain unclear: whether they are in *Ericaceae* s.l. (8) or are separate family/ies. In spite of the numerous molecular investigations, there is no universal taxonomic scheme of the discussed taxa. The purpose for investigating these groups is the lack of molecular data for the European representatives of *Monotropaceae* and *Pyrolaceae*. In this study we tested the hypothesis about the phylogenetic neighborhood of *Pyrolaceae* and *Monotropa* (15).

The interest towards both these groups in Europe is also provoked by their conservational status. The species are sensitive to the environment and especially to the mycorrhizal associations, which determine the population structures of the groups. This is the reason why the members of *Monotropoideae* and *Pyroloideae* are considered endangered to lose habitats and genetic diversity (2, 10, 12).

Most of the genera are morphologically discernible by habit, size of the reproductive stem, number of flowers in the inflorescence, odor of the flower, pollen and seed morphology, fruit morphology etc. (4, 5, 6, 7, 17, 22, 23). In the regional floristic sources both taxa (*Monotropaceae* and *Pyrolaceae*) are considered to have family status.

The intersimple sequence repeats (ISSR) are semiarbitrary markers amplified by PCR using a primer complementary to a target microsatellite. The primers are 16-18 bp long; they are composed of a repeated sequence and could be flanked at the 3'- or 5'-end by 2-4 arbitrary nucleotides – anchored

primers (24). The advantages of this type of amplification lie in the fact that it does not require sequence information and yields multilocus and highly polymorphous patterns (21, 24). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. The ISST method is highly informative and combines the speed of RAPD and the reliability of the SSR. The high reliability and simplicity of the method, compared to RFLP and RAPD, make it preferred in the field of molecular taxonomy. These features, together with the higher productivity, make the method attractive in the investigations of genetic variation with closely related species (21), and in investigations of subspecies and populations (3).

The aim of the present study was to analyze the level of the genetic differentiation of *Monotropa hypopitys* and its genetic relations with the *Pyrolaceae* s.l. by ISSR markers

Materials and Methods

Plant material and vouchers

The plant materials used for this study were collected from natural populations in different floristic regions of Bulgaria, during the 2010th vegetative season. Voucher specimens of the five different species were deposed at the herbarium of Agricultural University – Plovdiv, Bulgaria – SOA (**Table 1**). Taxonomical delimitation of samples was determined by existing floristic sources and comparative materials in the herbarium collections from Agricultural University of Plovdiv (SOA), Sofia University, Department of Botany (SO) and Institute of Botany, Bulgarian Academy of Sciences (SOM). Fresh samples from each specimen were used for molecular biology studies in the Laboratory of Molecular Markers at the Department of Plant Physiology and Molecular Biology, University of Plovdiv.

DNA preparation

Fresh leaves from the collected plants were frozen in a mortar pre-cooled with liquid nitrogen and were ground with a pestle to fine powder, of which 100 mg was transferred immediately into a pre-cooled microcentrifuge tube for DNA extraction by DNeasy plant mini kit (Qiagen) following the original protocol.

The absorption at 260 nm was used to determine the concentrations of the isolated DNA samples, while the ratios A260/A280 and A260/A230, to determine the presence of contaminations like proteins, polyphenolic compounds, sugars and lipids. The average amounts of isolated DNA were 250-300 ng and the above contaminations were present in negligible amounts.

Primers

Five ISSR primers (**Table 2**) from the primer Set #9 (University of British Columbia, Nucleic Acid-Protein Service Unit, NAPS Unit. http://www.michaelsmith.ubc.ca/services/NAPS/ Primer_Sets/) were used for this study. Because the production of Primer Set #9 was discontinued by UBC –NAPS Unit, the primers were ordered from Metabion International

TABLE 1

List of voucher specimens – location (floristic regions, MGRS position, nearest toponym and altitude), collector, collection dates, sample and voucher specimen number*.

***Monotropa hypopitys* L.**
Rila: 34TGM05, Semkovo, 1664 m (Ts. Raycheva) 4.08.2010, SOA s/n #MNT10-23
Rhodopes (West): 34TGM36, Yundola, 1641 m (K. Stoyanov and Ts. Raycheva) 3.06.2010, SOA s/n #10MNT001
***Pyrola chlorantha* Sw.**
Rila: 34TGM18, Borovets, 1455 m (K. Stoyanov and Ts. Raycheva) 16.07.2010, SOA s/n #10PYR009
Rhodopes (West): 34TGM05, Belitsa, 1449 m (Ts. Raycheva) 9.08.2010, SOA s/n #PYR10-27
Rhodopes (Middle): 35TLG00, Pamporovo, 1400 m (Ts. Raycheva) 4.08.2010, SOA s/n #PYR10-22
***Pyrola minor* L.**
Balkan Mountains (Middle): 35TLH13, Chouchoul Hut, 1355 m (K. Stoyanov) 10.08.2010, SOA s/n #10PYR003
Rhodopes (Middle): 35TLG13, Pavelsko, 1358 m (K. Stoyanov and Ts. Raycheva) 4.06.2010, SOA s/n #10PYR002
***Orthilia secunda* (L.) House**
Balkan Mountains (West): 34TGN44, Etropole, 572 m (K. Stoyanov and Ts. Raycheva) 8.07.2010, SOA s/n #19ORS005
Stara Planina (Middle): 35TLH13, Shouchoul Hut, 1355 m (K. Stoyanov) SOA s/n 10.08.2010, #10ORS019
Rila: 34TGM18, Borovets, 1502 m (Ts. Raycheva and K. Stoyanov) 16.07.2010, SOA s/n #10ORS004
Rhodopes (West): 35TGM35, Yundola, 1314 m (Ts. Raycheva) 8.08.2010, SOA s/n #ORT10-28; 35TGM36, Starina (Yundola), 1618 m (K. Stoyanov and Ts. Raycheva) 3.06.2010, SOA s/n #10ORS031
Rhodopes (Middle): 35TLG01, Pamporovo, 1372 m (Ts. Raycheva) 4.08.2010, SOA s/n #ORT10-21
***Moneses uniflora* A. Gray**
Rhodopes (West): 34TGM36, Yundola, 1596 m (K. Stoyanov and Ts. Raycheva) 3.06.2010, SOA s/n # 10MON001
Rhodopes (Middle): 35TLG13, Pavelsko, 1367 m (K. Stoyanov and Ts. Raycheva) 4.06.2010, SOA s/n #10MON002

*For clarity the sample numbers listed here were used in all other schemes and figures.

AG, Martinsried, Germany and upon arrival were dissolved in DNase-free water to 100 mmol final concentration.

TABLE 2

Sequences of the ISSR primers used in this study

Primer	Sequence
p7	$(AC)_8GA$
p811	$(GA)_8C$
p817	$(CA)_8A$
p826	$(AC)_8C$
p836	$(AG)_8Y^*A$

*Y: (C,T)

TABLE 3

Distribution of the polymorphic ISSR products in groups by species and primers

Species\Primers	p7	p811	p817	p826	p836
Monotropa hypopithys	12	15	17	11	10
Moneses uniflora	5	2	9	6	6
Orthilia secunda	8	11	11	10	12
Pyrola chlorantha	5	9	10	7	8
Pyrola minor	5	14	13	9	9

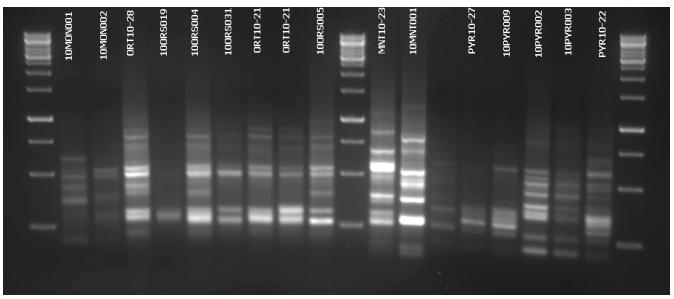

Fig. 1. ISSR products amplified by primer 817. Samples: *Monotropa hypopitys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002; 1 kb DNA ladder.

ISSR-PCR reaction conditions

Approximately 150 ng DNA template was taken from each sample and mixed in 200 µL PCR tube with 1 µL primer (100 mmol·L^{-1} concentration), 25 µL PCR master mix (Fermentas) and 22 µL DNase-free water (supplied with the PCR kit). The PCR tubes were placed in a TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried out by using the following program: initial DNA denaturation at 94 °C – 5 min; next 35 cycles of 94 °C – 1 min; 55 °C – 1 min 30 s; 72 °C – 2 min 30 s, and final extension at 72°C for 6 min. The PCR products were mixed with 7.5 mL of loading dye (Fermentas), loaded onto 1.5% agarose gel containing 0.5 mg·mL^{-1} ethidium bromide (final concentration) covered with 1X TAE buffer and separated by applying 7 volts per cm electrical currency. The size of the products was determined by comparison with a 1 kb DNA ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light.

Data analysis

The gel images were captured by BIO-VISION+3026.WL system (Vilber Lourmat) using four different exposition times and processed by the accompanying software. The amplified unambiguous bands were scored by molecular masses with GelPro Analyzer. Next, they were manually allocated into classes of molecular weights for completion of Boolean matrices for a presence/absence (0/1) of bands with the results of each primer.

The binary data were used to construct rectangular matrices using the PAST ver. 1.89 software (11) based on the data of each gel exposition. The distances obtained from all images were recalculated to average distances for each primer. All the average matrices were summarized to a consequent distance matrix. The results based on genetic distances of the studied species were used to construct a resulting unrooted tree by the T-Rex 3.0a1 software (Vladimir Makarenkov, University of Quebec in Montreal), using the unweighted neighbor-joining method (20). The dendrogram was plotted by the software TreeView 1.6.6. ver. 2001 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html)

Results and Discussion

Five ISSR markers were used to evaluate the genetic relations between *Monotropa* and *Pyrolaceae*. Data about the primers and the amplified polymorphic products are given in **Table 3**. The PCR products are grouped by size and the ISSR marker used. The obtained results demonstrated significant genetic polymorphism between the five investigated species, as well as a wide genetic variation between the samples of the separated populations (**Fig. 2**, **Fig. 3**, **Fig. 4**, **Fig. 5**, **Fig. 6**). A relatively high number of ISSR products were obtained from the samples of *Monotropa hypopitys* and *Orthilia secunda*, while the lowest number of fragments were obtained from the samples of *Moneses uniflora* (**Table 3**).

The cluster analysis showed a clear division into three basic genetically neighboring groups (**Fig. 2**, **Fig. 3**, **Fig. 4**, **Fig. 5**, **Fig. 6**). We did not observe significant differences between the samples by their geographic origin, which could also be due to the limiter number of samples per population collected.

The best results were achieved by the using two primers: p.7 (**Fig. 2**) and p817 (**Fig. 4**). They amplified polymorphic products whose grouping coincides with the known taxonomic distribution of the investigated samples by species. When p7 was used, both samples of *Monotropa hypopitys* were positioned in separate clusters taking divergent position from all representatives of *Pyrolaceae* s.s.

The samples from different species of *Pyrolaceae* s.str. were grouped in overlapping groups. Two basic clusters are visible: *Moneses uniflora*/*Pyrola chlorantha* and *P. minor*/*Orthilia secunda*. This division by p7 is close to the viewpoint for the position of *Monotropa* out of *Pyrolaceae* s.str.

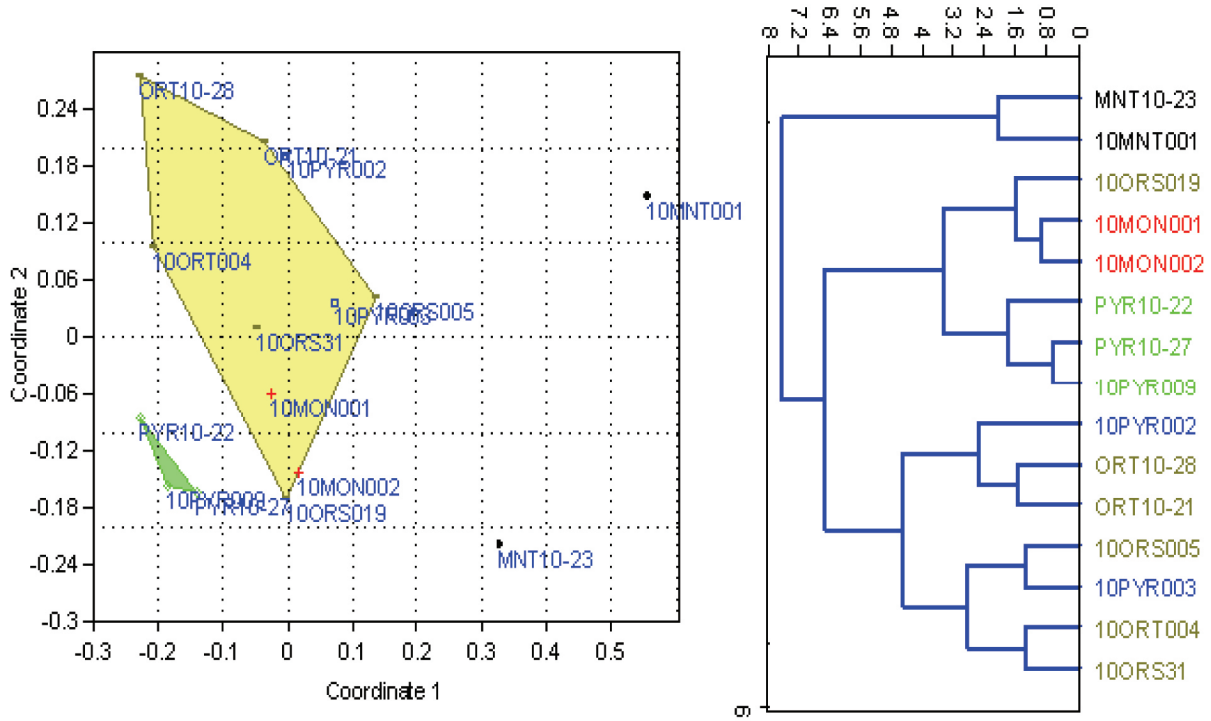

Fig. 2. Cladogram based on the distribution of the ISSR products amplified by primer p7. Samples: *Monotropa hypopitys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002 (**Table 1**).

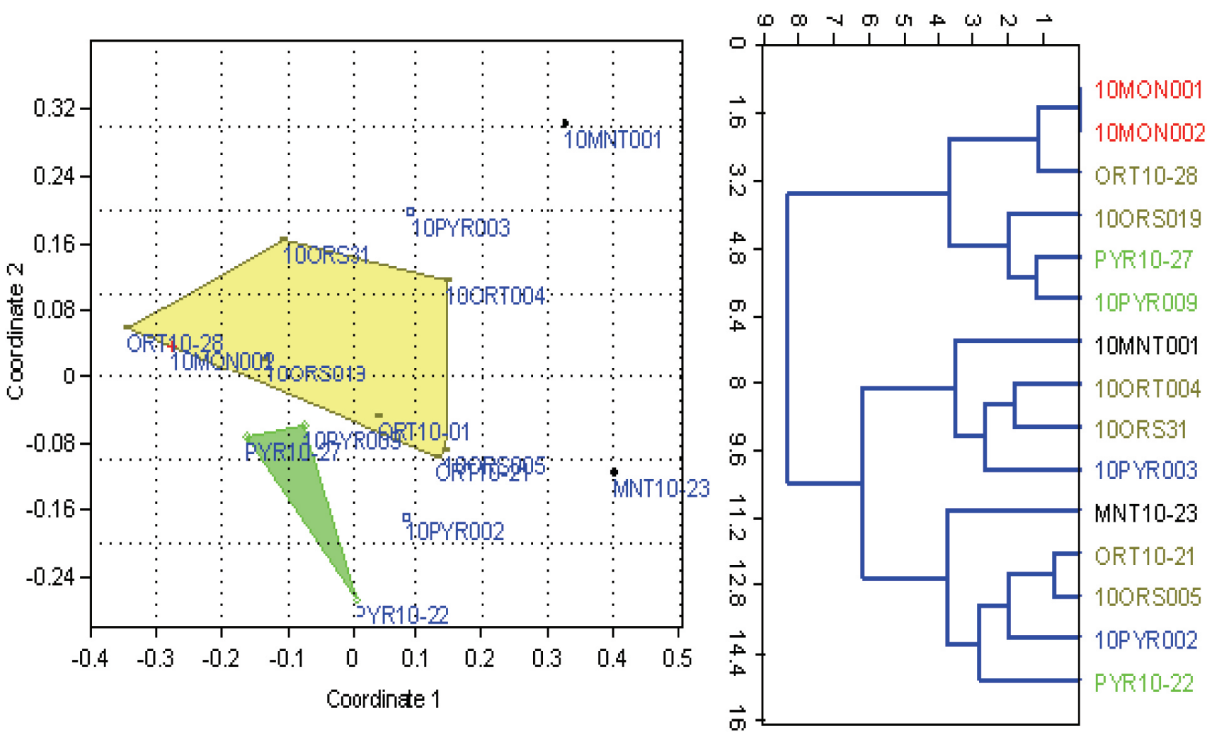

Fig. 3. Cladogram based on the distribution of the ISSR products amplified by primer p811. Samples: *Monotropa hypopitys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002 (**Table 1**).

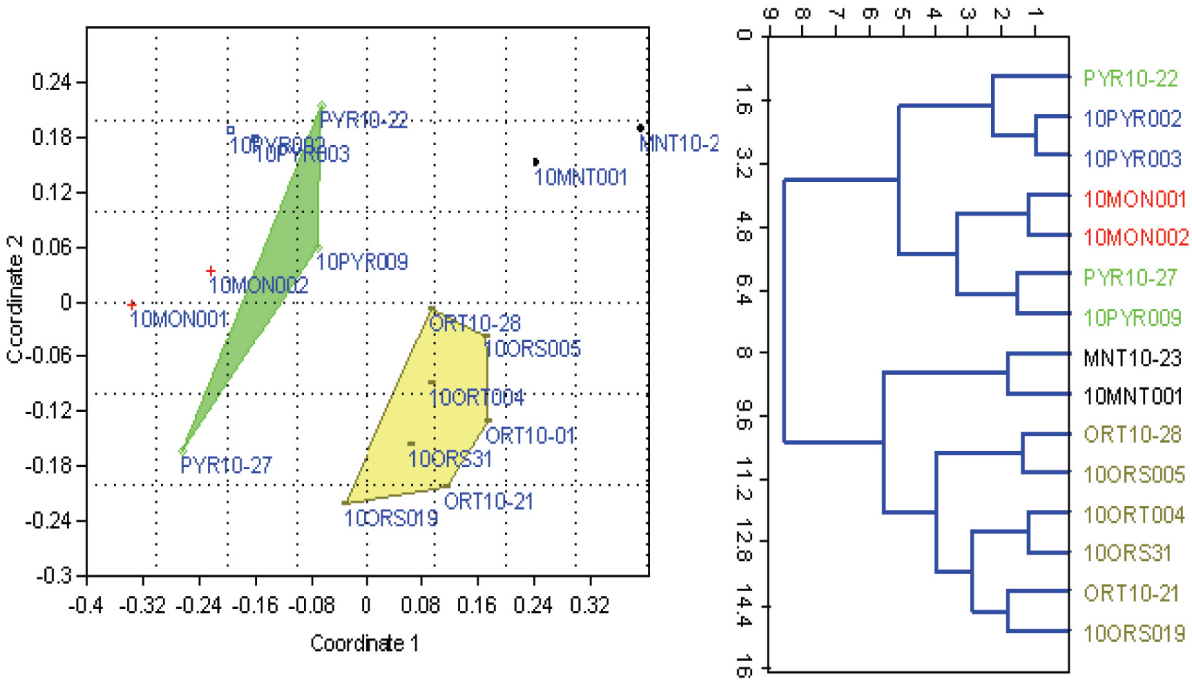

Fig. 4. Cladogram based on the distribution of the ISSR products amplified by primer p817. Samples: *Monotropa hypopithys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002 (**Table 1**).

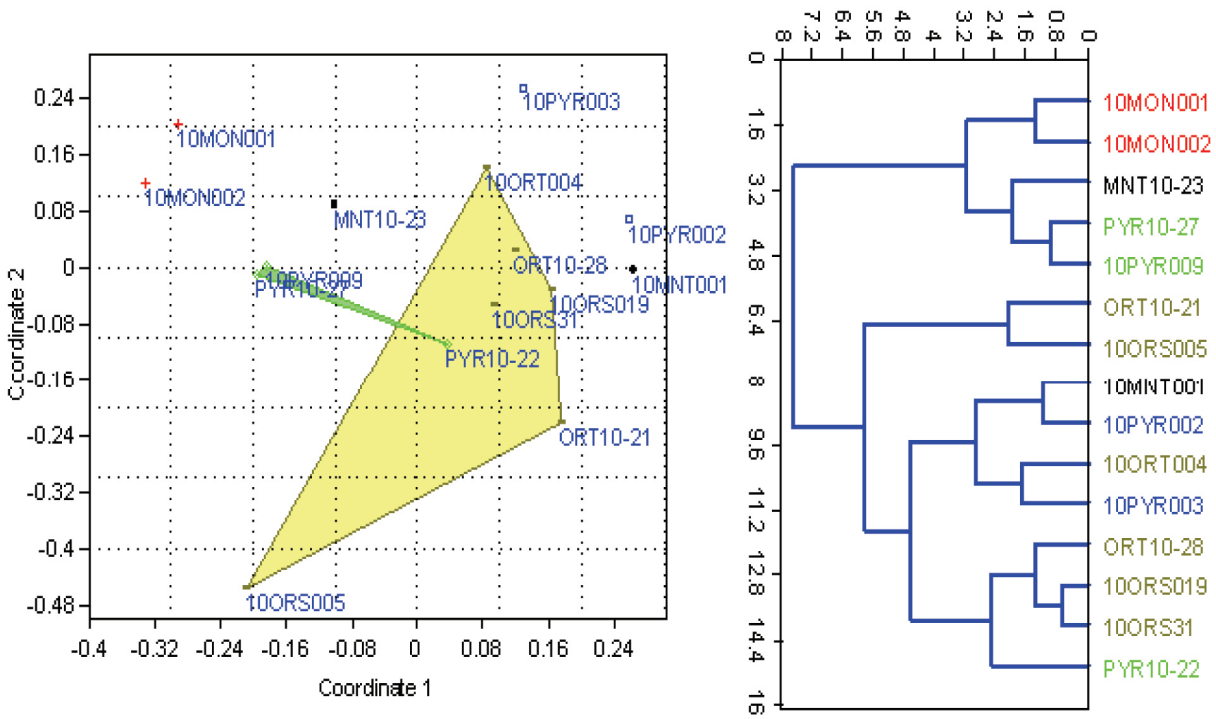

Fig. 5. Cladogram based on the distribution of the ISSR products from amplified by primer p826. Samples: *Monotropa hypopithys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002 (**Table 1**).

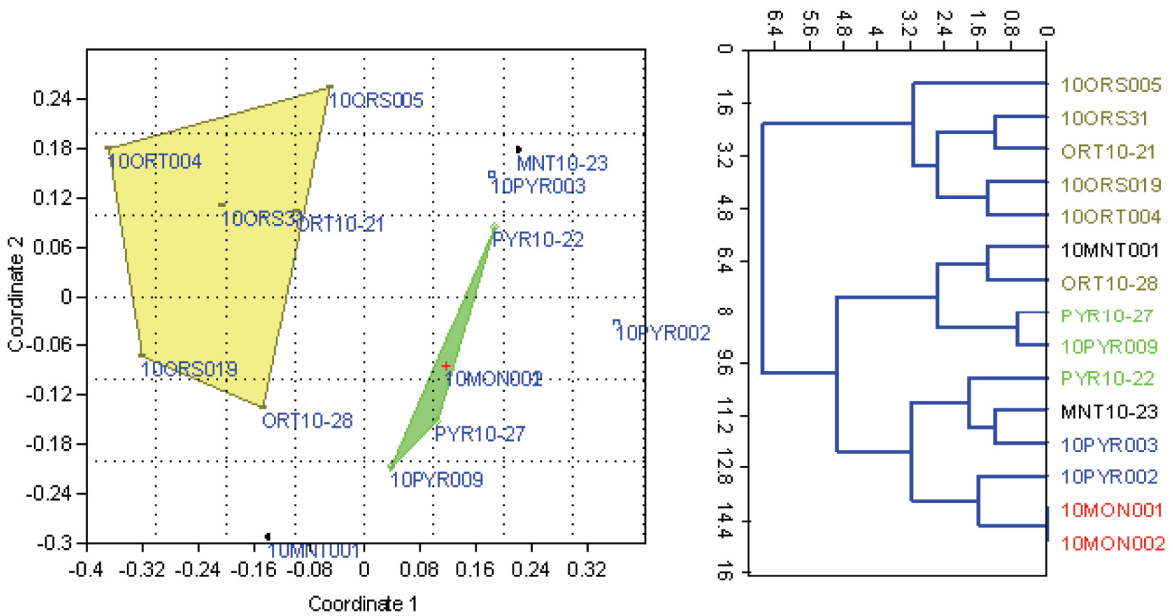

Fig. 6. Cladogram based on the distribution of the ISSR products amplified by primer p836. Samples: *Monotropa hypopithys* L. - MNT10-23, 10MNT001; *Pyrola chlorantha* Sw. - 10PYR009, PYR10-27, PYR10-22; *Pyrola minor* L. - 10PYR003, 10PYR002 ;*Orthilia secunda* (L.) House - 19ORS005, 10ORS019, 10ORS004, ORT10-28, 10ORS031, ORT10-21; *Moneses uniflora* A. Gray - 10MON001, 10MON002 (**Table 1**).

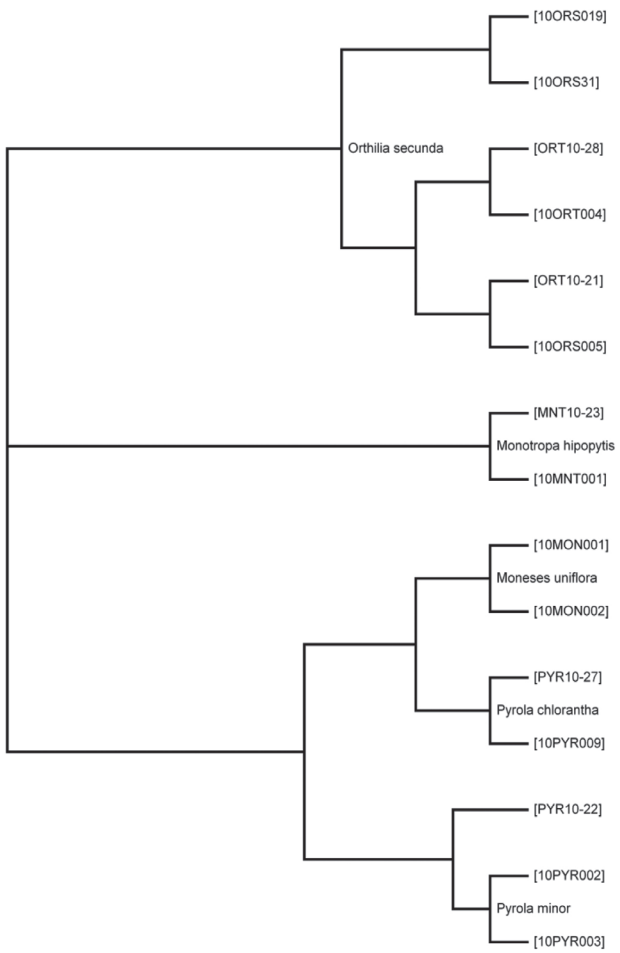

Fig. 7. Resulting cladogram based on genetic distances of the studied species using Unweighted Neighbor Joining method. The samples are described in greater detail in **Table 1**.

The application of p817 also allowed to distinguish *Monotropa hypopithys* specimens, however they were part of a subcluster within the cluster containing also representatives of *Orthilia.*

The rest of the primers used gave products showing a low genetic diversity, leading to mixed samples from different populations. The lack of geographic differentiation is probably due to the vegetative reproduction.

Similar grouping was seen in the products of p811, but with wider variability (**Fig. 3**). The cluster analysis showed most stable levels of the grouping in *Moneses uniflora* – both specimens have equal spectra.

The products of p826 were unclearly grouped (**Fig. 5**). Probably this marker is not selective enough on interspecies level.

The p836 marker separated the samples of *Orthilia secunda* from all other species. The highest similarity was observed between the samples of *Moneses uniflora* (**Fig. 6**).

In the resulting cladogram (**Fig. 7**) three equivalent clades were formed: *Orthilia*, *Monotropa* and *Pyrola*/*Moneses*. In spite of the taxonomical studies based on morphological data, the samples of *Moneses uniflora* occupied an internal clade of *Pyrola*. This result does not correspond to the idea that *Moneses* is a separate genus. The genetic similarity between *Pyrola* and *Moneses* seems much higher in comparison to *Orthilia* and *Monotropa*. The results indicate the dubious position of *Moneses uniflora* in a separate genus.

The highest diversity between the investigated plants was shown in the material of *Orthilia secunda*. The position of *Orthilia* samples in a general clade confirms the status of the

taxon and this corresponds to its floral morphology and habit. The position of *Monotropa* is on the same level as *Orthilia*. In spite of the comparatively small number of samples, they are grouped in a joint clade, near to *Orthilia*. This fact indicates that both species show higher genetic similarity than the other taxa of *Pyrola* and *Monenses.* Therefore, the used markers could not confirm the status of *Monotropaceae* as a separate family.

The comparatively high levels of genetic diversity in *Monotropa hypopitys* and *Orthilia secunda* probably were due of the fact that sexual reproduction is more widely distributed among these genera than in the other species. A reason of this hypothesis is the spatial structure and the size of the populations. Both of the species are glacial relicts. The historical phytogeographic formation of the population structure and diversity of both species (1) are reason to observe a higher genetic diversity in the North, and lower in the South. Relatively to the climatic changes, the populations of both species are decreasing in Central and Southern Europe because of the lost habitats. Generally, in the populations of *Monotropa hypopitys* a tendency for a lower genetic diversity is observed compared to that of *Orthilia secunda.* This alarms that in the future *Monotropa hypopitys* could become an endangered species as a result of the human activity in the typical habitats – forests and boreal localities.

Conclusions

We think that the genetic diversity of the investigated groups is comparatively low because the vegetative reproduction is more widely distributed than the sexual one. The primers p7 and p817 showed the highest selectivity. The results were in agreement to the viewpoint that genus *Orthilia* has a relatively separated position from the rest of the *Pyrolaceae* species (17). The independent position of genus *Moneses* could not be confirmed by the used markers.

The results are in favor of the hypothesis (15) that *Monotropoideae* is a part of *Pyrolaceae*, and *Monoropa hypopitys* is a final result of a gradual transition from autotrophy to mycorhysis.

Our results indicate a lack of clear evidence about the phylogenetic position of *Orthilia* and *Monotropa hypopithys*, as well as the taxonomical independence of *Monotropa* in a separated family. These results could serve as a basis for further investigations using additional markers and specimens.

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