

New insights regarding the taxonomy and phylogeography of *Onobrychis montana* DC. subsp. *transsilvanica* (Fabaceae)

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Abstract

Onobrychis montana DC. Subsp. *transsilvanica* (Simonk.) Jáv. (Fabaceae) is a plant taxon, endemic to the South-Eastern Carpathians, whose taxonomic status is treated inconsistently by the classic taxonomy based on morphological traits. In a previous study, by sequencing several chloroplast DNA regions and AFLP genotyping, we tried to elucidate the controversy concerning its taxonomic status in relation to the more widespread *O. montana* DC., and determined its phylogeographic structure within the Carpathians. The present study, based on DNA fingerprinting by seven SSR markers, brings more insights on this problematic taxon by conferring higher resolution and sub-clustering previously identified phylogeographic groups. However, the newly SSR genetic data are in agreement with molecular evidence revealed by AFLP and chloroplastic SNPs and do not support *O. montana* DC. Subsp. *transsilvanica* (Simonk.) Jáv. As a distinct species from *O. montana* DC.

Keywords: Carpathians; microsatellites; *Onobrychis*; phylogeographic groups; taxonomy

Introduction

As a large mountain chain extending across Central Europe, the Carpathians are one of the main constituents of the European Alpine System (Ozenda, 1985). Their geographical position, extent, isolation, landscape heterogeneity, well-preserved environment, and relatively low impact of Quaternary glaciations mark them as important targets for studies on European biodiversity and biogeography (Mráz and Ronikier, 2016). The Carpathians are seen as a biodiversity hotspot due to species richness (Tasenkevich, 1998), plant

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communities' diversity (Chytrý *et al.*, 2015), endemism (Hurdu *et al.*, 2016; Mráz *et al.*, 2016), and area of taxa speciation (Cieślak *et al.*, 2000; Španiel *et al.*, 2011; Mitka *et al.*, 2016; Sutkowska *et al.*, 2017). However, the current knowledge on taxonomic validity, genetic diversity, and accurate distribution of a significant number of Carpathian endemic and endangered non-endemic vascular plant species is still fragmentary and incomplete (Ronikier, 2011; Španiel *et al.*, 2011; Kolář *et al.*, 2016).

In this light, our previous study (Băcilă *et al.*, 2015) represented the first attempt based on molecular markers (AFLP and cpDNA markers) to provide both phylogeographic and phylogenetic insights for the SE Carpathian endemic taxon, *O. montana* DC. Subsp. *transilvanica* (Simonk.) Jáv. The taxon shares close, yet controversial, taxonomic relationships and a strong morphological resemblance with the Alpine allopatric species *Onobrychis montana* DC. However, the genetic structure inferred from AFLP and cpDNA data did not support *O. montana* DC. Subsp. *transilvanica* (Simonk.) Jáv. As a distinct species from *O. montana* DC.

Microsatellites (simple sequence repeats or SSRs) have been the most widely used markers for genotyping plants over the past 20 years because they are highly informative, codominant, multi-allele genetic markers that are experimentally reproducible and transferable among related species (Mason, 2015). These markers are enormously useful in studies of population structure, genetic mapping, and evolutionary processes. In plants species from the spontaneous flora, SSRs are particularly used for: genetic diversity studies; gene flow and crossing over rates estimations; and, above all, to infer infraspecific genetic relations (Vieira *et al.*, 2016). Therefore, the present study turned to SSRs markers for in-depth tackling of taxonomic boundaries between the *O. Montana* DC. Subsp. *transilvanica* (Simonk.) Jáv. And *O. montana* DC.

The aim of this study was to confirm (or not) the taxonomic rank of *O. montana* DC. Subsp. *transilvanica* (Simonk.) Jáv. As a subspecies of *O. montana* DC. And to refine its pre-existent phylogenetic pattern.

Materials and Methods

Sampling

Sixteen populations of *Onobrychis montana* DC. And *O. montana* DC. Subsp. *transilvanica* (Simonk.) Jáv. were sampled from the Alps and the Carpathians Mountains (Table 1) along with one population of *Hedysarum hedysaroides* Schinz & Thell., chosen as outgroup. More details about the sampling strategy can be found in Băcilă *et al.*, 2015.

DNA extraction

Total DNA was extracted using DNeasy 96 Plant Mini Kit (Qiagen) according to the manufacturer's protocol. More details can be found in Băcilă *et al.* (2015).

SSR fingerprinting

SSR fingerprinting was performed using seven microsatellites, which we previously tested for amplification, polymorphism and replicability in *O. montana* DC. subsp. *transilvanica* (Simonk.) Jáv. and *O. montana* DC. Primer sequences and PCR conditions are also amply presented in our previous study.

Data analysis

Alleles scoring was performed with GeneMapper v.4.0 software (Applied Biosystems, Thermo Fisher Scientific, USA). Nei's gene diversity (Nei, 1987) and the frequency downweighted marker values (DW; Schönswetter and Tribsch, 2005) were estimated using the AFLPdat R-script (Ehrich, 2006). A frequency matrix was generated and subsequently used within SplitsTree v.4.10 (Huson and Bryant, 2006) and a

neighbor-joining network was constructed using the Neighbor-net method. Bootstrap values were calculated from 1000 replicates. The relationships among individuals were analysed using Principal Coordinates Analysis (PCoA) based on the between-individual Jaccard similarities (Jaccard, 1901) computed with PAST v.4.13 software (Hammer *et al.*, 2001). Spatial analysis of molecular variance was performed using the program SAMOVA v.1.0 (Dupanloup *et al.*, 2002), the parameters being setup according to Băcilă *et al.* (2015).

STRUCTURE v.2.3.4 software (Pritchard *et al.*, 2000) was used to detect genetic groups and to inference the population structure, as described in Băcilă *et al.* (2015).

Table 1. Sampled populations of *Onobrychis montana* DC. and *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv.: numbering, country (Ro - Romania; Fr - France; Po - Poland; Sk - Slovakia; Mne - Montenegro), mountain range (SW: South-Western Carpathians; SE: South-Eastern and Eastern Carpathians; W: Western Carpathians), population code (OT - *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv.; OM - *Onobrychis montana* DC., followed by the abbreviation of the mountain range from where the populations were collected). (Table partially reproduced from Băcilă *et al.*, 2015)

No	Country	Mountain range	Code
<i>O. montana</i> DC. subsp. <i>transsilvanica</i> (Simonk.) Jáv.			
1	Ro	SW Carpathians, Retezat Mts.	OTRM
2	Ro	SW Carpathians, Retezat Mts.	OTR
3	Ro	SE Carpathians, Maramureşului Mts.	OTM
4	Ro	SE Carpathians, Ceahlău Mts.	OTCh
5	Ro	SE Carpathians, Giurgeu-Hăşmaş Mts.	OTGH
6	Ro	SE Carpathians, Ciucaş Mts.	OTC
7	Ro	SE Carpathians, Pietra Craiului Mts.	OTPC
8	Ro	SE Carpathians, Bucegi Mts.	OTB
9	Ro	SE Carpathians, Bârsei Mts.	OTBV
10	Ro	SE Carpathians, Făgăraş Mts.	OTF
<i>O. montana</i> DC.			
11	Fr	Alps, Cottian Alps	OMAC
12	Fr	Alps, Dauphiné Alps	OMA
13	Fr	Alps, Jura	OMJ
14	Mne	Dinaric Alps, Durmitor Mts.	OMAD
15	Po	W Carpathians, High Tatras	OMTW
16	Sk	W Carpathians, Belianske Tatras	OMBT

Results

The gene diversity varied from 0.121 to 0.845 (Table 2), the highest values being exhibited by the two populations of *O. montana* DC. from the Western Carpathians (OMBT and OMTW). The rarity estimated by the frequency down-weighted marker value (DW) also showed higher values for the two populations of *O. montana* DC. from the Tatra Mountains.

The Neighbor-net diagram (Figure 1) revealed a geographic division well supported by bootstrap values between nine clusters. The SE Carpathians populations of *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. were split into three clusters: one consisting of OTC, OTGH, OTCh, and OTM populations, second composed by OTB, OTBV, and OTPC populations, and a third represented by OTF population. The populations of *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. from the SW Carpathians (OTR and OTRM) represented a separate cluster. Each population of *O. montana* DC. from the Alps was separated from

the others. The last cluster of the diagram was composed by the two populations of *O. montana* DC. from the Western Carpathians (OMBT and OMTW).

Table 2. Genetic parameters: Nei's gene diversity (Div) and DW values (M_{DW}) for *Onobrychis montana* DC. and *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. N= number of individuals included in the analysis. Populations codes as in Table 1

No	Code	N	Div	M_{DW}
<i>Onobrychis montana</i> DC. subsp. <i>transsilvanica</i> (Simonk.) Jáv.				
1	OTM	5	0.372	0.913
2	OTCh	5	0.304	0.9139
3	OTGH	5	0.275	0.9139
4	OTC	5	0.149	0.9139
5	OTBV	5	0.121	0.8370
6	OTB	5	0.227	0.8370
7	OTPC	5	0.299	0.837
8	OTF	5	0.423	0.958
9	OTRM	5	0.432	0.923
10	OTR	5	0.543	0.923
Mean			0.3145	0.8972
SD			0.12	0.0436
<i>Onobrychis montana</i> DC.				
11	OMBT	5	0.793	1.1917
12	OMTW	5	0.845	1.1917
13	OMAD	5	0.215	0.5331
14	OMA	5	0.195	0.8341
15	OMAC	5	0.239	0.5905
16	OMJ	4	0.351	0.6857
Mean			0.439	0.8378
SD			0.312	0.252

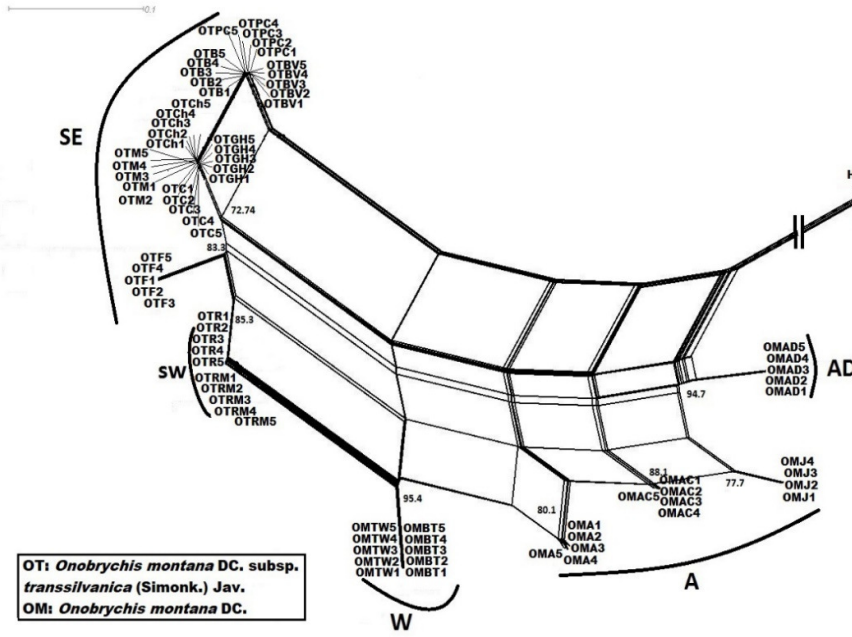


Figure 1. The Neighbor-net diagram of the *Onobrychis montana* DC. and *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations. Bootstrap values above 50% are shown on major branches. Populations codes as in Table 1. A: Alps and Jura; AD: Dinaric Alps; SW: South-Western Carpathians; SE: South-Eastern and Eastern Carpathians; W: Western Carpathians; H. hed: *Hedysarum hedysaroides*

The pattern revealed by the Neighbor-net was basically confirmed by the SAMOVA analysis (Figure 2), with the sole difference that OMAC and OMJ populations of *O. montana* DC. from the Alps were grouped together.

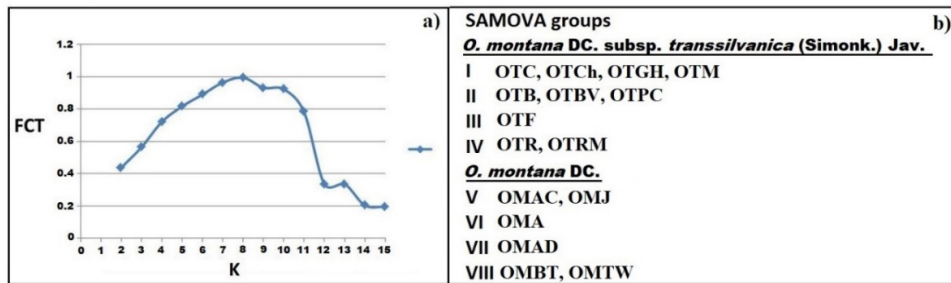


Figure 2. a) Best K = 8 based on FCT values. b) SAMOVA grouping of *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. (groups I-IV) and *O. montana* DC. (groups V-VIII). Populations codes as in Table 1

The STRUCTURE analysis (outgroup excluded) indicated that the major split in the data set was obtained at K = 2, separating the Alps and the Carpathians. The highest mean likelihood was obtained for K = 6 (Figure 3 b) and only a few samples were admixed. The six clusters included the populations from the Alps (all the populations together), Western Carpathians, SW Carpathians, and three separate groups for the SE Carpathians (Figure 3 a).

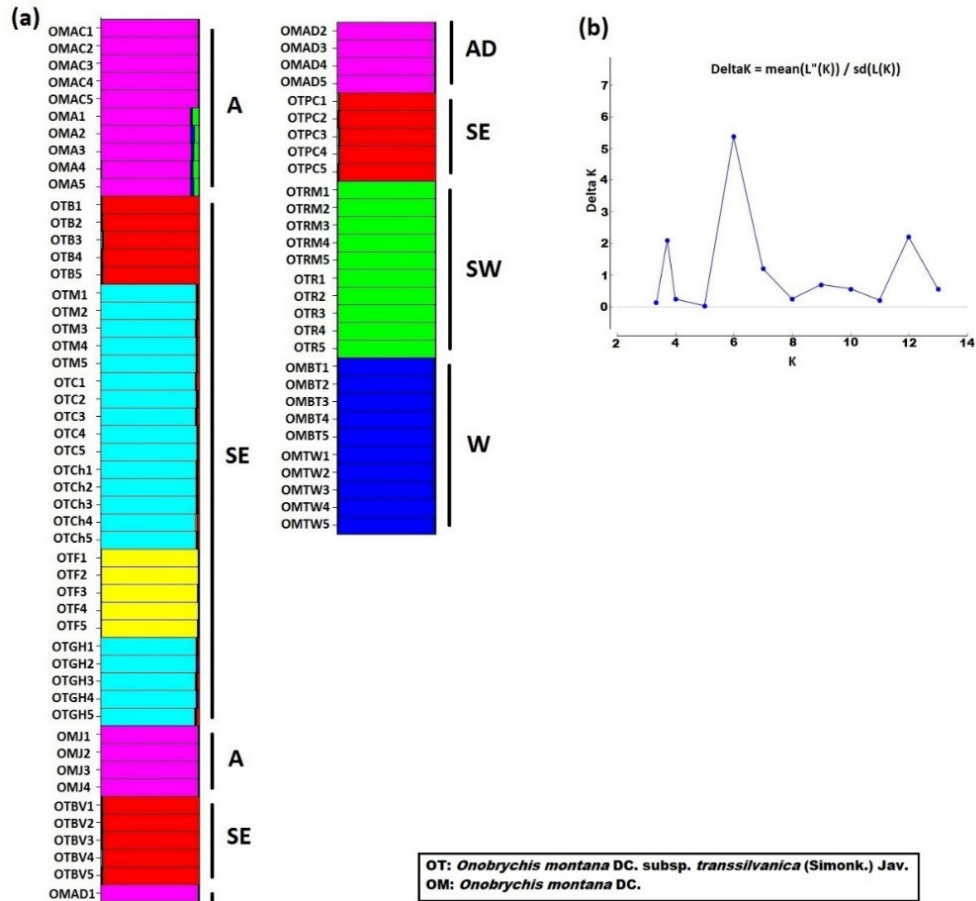


Figure 3. (a) Results of STRUCTURE analysis performed on all individuals of *Onobrychis montana* DC. and *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. sampled populations. Populations codes as in Table 1. A: Alps and Jura; AD: Dinaric Alps; SW: South-Western Carpathians; SE: South-Eastern and Eastern Carpathians; W: Western Carpathians; (b) Inference of best K - based on the output data from STRUCTURE and the ad hoc criterion proposed by Evanno *et al.* (2005)

The Principal Coordinates Analysis (Figure 4) separated the populations according to the main biogeographic groups, the major split being the same as in case of STRUCTURE analysis, respectively the separation between the Alps and the Carpathians.

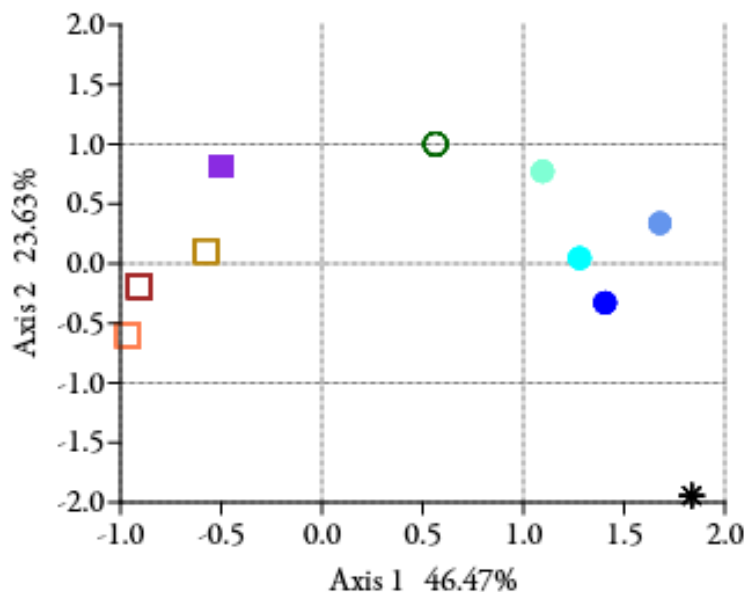


Figure 4. Principal Coordinates Analysis of *Onobrychis montana* DC. and *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. based on Jaccard's similarity coefficient. The symbols from the chart represents the following populations: brown square - OTC, OTGH, OTCh, and OTM; coral square - OTB, OTBV, and OTPC; darkgoldenrod square - OTF; blueviolet fill square - OTR and OTRM; darkgreen empty dot – OMBT and OMTW; blue dot – OMAD; aqua dot -OMAC; aquamarine dot - OMA; cornflowerblue dot – OMJ; black star - *Hedysarum hedysaroides* Schinz & Thell. Colours names are according to PAST software

Discussion

The Nei's genetic diversity values were higher in *O. montana* DC. populations compared to *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations (Table 2). However, this is not unexpected when taking into consideration the large geographical distance between the sampling points of *O. montana* DC. The overall mean value for diversity (0.376) was higher than the mean value derived from AFLP markers for the same populations (0.017) (Băcilă *et al.*, 2015).

SAMOVA analysis based on SSRs (Figure 2) revealed a refined resolution of *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. grouping comparative with AFLP markers (Băcilă *et al.*, 2015). The best grouping (K=8) separated the populations of the taxon into four groups: 1 = group formed by the two populations from Retezat Mountains (OTRM and OTR); 2 = group formed by four populations sampled from the South-Eastern and Eastern Carpathians, respectively Ciucaș (OTC), Maramureșului (OTM), Ceahlău (OTCh), and Giurgeu-Hășmaș Mountains (OTGH); 3 = group formed by three populations sampled from Bucegi (OTB), Piatra-Craiului (OTPC), and Bârsei Mountains (OTB); 4 = OTF population sampled from Făgăraș Mountains. Also, in case of *O. montana* DC. four groups were formed: 1 = group formed by two populations sampled from Cottian Alps (OMAC) and Jura Mountains (OMJ); 2 = OMA population sampled from Dauphiné Alps; 3 = OMAD population sampled from Dinaric Alps; 4 = group formed by the two populations from the Tatra Mountains (OMBT and OMTW).

The same pattern of separation for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations was revealed by the Neighbor-net diagram (Figure 1), the Bayesian clustering (Figure 3a), and PCoA (Figure 4) analysis.

The distribution of *Onobrychis* sp. genotypes was in relation with the geographic distribution of these populations. In this context, the Alpine populations of *O. montana* DC. were more distant from the rest, but different from each other. *O. montana* DC. populations from the Tatras also formed a separate group, whereas *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations were split in four groups. The fragmented genetic pattern for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations can be attributed either to their glacial survival in separate local refugia and/or to the geographic fragmentation of the Carpathians, which generated habitat insularity that is the premise of the patchy distributions of the Carpathian flora and the emergence of centers of endemism (Pawłowski, 1970). However, the isolation of the two SW *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. populations was also detected by cpDNA and AFLP markers (Băcilă *et al.*, 2015) and this distribution pattern was consistent with studies performed on other Carpathian species: *Campanula alpina* (Ronikier *et al.*, 2008, 2014); *Hieracium* sect. *Cernua* (Szeląg, 2006), *Eritrichium nanum* (Șuteu *et al.*, 2023). These results suggested the existence of a biogeographic barrier between the SW part and the rest of the Carpathians, which led to the species survival in a glacial refugium in the southwestern part of the SE Carpathians (Huntley and Birks, 1983). The other three groups of populations were constructed around massifs already renowned as centers of endemism within the Carpathians. One group included populations from Maramureșului, Ceahlău, Ciucaș, and Giurgeu-Hășmaș Mts. and it surrounded the Rodna Mountains, which according to Hurdu *et al.* (2012) exhibit the highest degree of endemism from the South-Eastern Carpathians and might have represented a possible alpine refugium. The second group comprised populations from: Piatra Craiului, Bucegi (as a major hotspot of alpine flora in Romania - Coldea *et al.*, 2009, and ranking in second place regarding the degree of endemism from the South-Eastern Carpathians - Hurdu *et al.* (2012), and Bârsei Mts. The third group was made out of Făgăraș Mts., which represent the third center of endemism from the South-Eastern Carpathians in terms of the number of species (70 endemic plant species according to Hurdu *et al.*, 2012).

Overall, in case of *Onobrychis montana* s.l. the resolution of the SSR markers was greater than in case of cpDNA and AFLPs, and more groups of populations were revealed. Nevertheless, neither these markers do support species recognition for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv., as it appeared from the Neighbor-net diagram (Figure 1) where the outgroup, *Hedysarum hedysaroides*, was included.

Congruence between the taxonomic patterns revealed by AFLPs and SSRs has been found in other comparative studies, but the level of congruence differed depending on the purpose pursued and the species analyzed. Thereby, for example, Marulanda *et al.* (2007) concluded that AFLP and SSR were complementary because their joint analysis provided additional elements to explain the complex inter relationship between wild and cultivated *Rubus* species. Gillaspie *et al.* (2005) used three AFLP primer combinations and 10 microsatellites to assess genetic diversity and relatedness between *Vigna unguiculata* subspecies, but both markers failed to successfully group the subspecies into distinct clades. Garcia *et al.* (2004) reached the conclusion that AFLP seemed to be the best-suited molecular assay for fingerprinting and assessing genetic relationships among tropical maize inbred lines. Similarly, there are other studies that have preferred AFLP markers over SSRs (Dessalegn *et al.*, 2009; Costa *et al.*, 2016).

However, there are numerous examples of studies which preferred SSRs over AFLPs and SNPs. Stodart *et al.* (2005) found that SSR markers detected a higher gene diversity and a higher value of PIC than AFLPs in case of 44 bread wheat landraces; in case of mangrove species, *Avicennia marina*, Maguire *et al.* (2002) found that SSRs detected more genetic differentiation between populations (19% vs 9%) and subspecies (35 vs 11%) than AFLPs; Rawat *et al.* (2014) found that SSR markers revealed a more distinct grouping of genotypes based on resin yield as compared to ISSR and AFLP markers in case of 53 genotypes of *Pinus roxburghii*; Sorkheh *et al.* (2017) found that the polymorphic information content (PIC) was the highest for SSR markers in a study focused on genetic diversity in *Prunus*.

However, the present SSR data were in agreement with the latest taxonomy based on morphological characteristics (Ciocârlan, 2009; Sârbu *et al.*, 2013) and with the molecular data inferred from cpDNA and AFLP markers, and did not support species recognition for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. Therefore, we concluded as appropriate its taxonomic rank as a subspecies of *O. montana* DC.

Conclusions

The SSR markers conferred a higher resolution of grouping for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv. and *O. montana* DC. compared to cpDNA and AFLP analysis that were previously published. The refined phylogeographic pattern permitted the revelation of groups formed around renowned centers of endemism and potential glacial refugia.

The newly SSR genetic data were in agreement with current morphological classification and with molecular evidence issued by other molecular markers (namely AFLP and chloroplastic SNPs) and did not support species recognition for *O. montana* DC. subsp. *transsilvanica* (Simonk.) Jáv.

Authors' Contributions

The contributions of authors to the manuscript are as follows: conceptualization: IB, GC; field work: IB, DȘ, AC, CG, ZRB; data curation: IB, DȘ; formal analysis: IB; funding acquisition: IB; investigation: IB; methodology: IB; project administration: IB; writing - original draft: IB; writing - review and editing: IB and DȘ. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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