Onobrychis pindicola and *O. montana* (Fabaceae) in the Pirin and Slavyanka Mts (SW Bulgaria): can we distinguish between them?

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Abstract. It has been considered that there are two perennial members of *Onobrychis* Mill. (Sect. *Onobrychis*), namely *Onobrychis pindicola* subsp. *urumovii* Degen & Dren. and *Onobrychis montana* subsp. *scardica* (Griseb.) P. W. Ball, distributed in the Pirin Mts, SW Bulgaria. They are not easy to distinguish following the identification key. We tested two alternative statements by measuring plant samples of several *Onobrychis* populations growing in the Pirin and Slavyanka Mts, and using both morphological and molecular approaches: 1) statement one – there are two, more or less defined, separate taxa *Onobrychis pindicola* and *O. montana* in the Pirin and Slavyanka Mts and their individuals/ramets are: 1a) separated and located at particular distinct sites 1b) individuals/ramets of both taxa are mingled sympatrically and bloom simultaneously; or 2) statement two – there is only one polymorphic species in the Pirin and Slavyanka Mts. A representative sample of flowers was dissected into components and 13 flower features were measured. Three characters of the leaves were also measured on representative plant samples. Principal components analysis (PCA) was applied to detect relationships (groups of similarity) between various plant samples and other statistical tests. Also RAPD, ITS and *Trn*L sequences techniques, were used for analyses and interpretations. The results suggest that there is no clear pattern of differentiation among populations, which does not allow the definition of subgroups. We have concluded that there is only one polymorphic species in the Pirin and Slavyanka Mts., for which the prior combination is *Onobrychis pindicola* subsp. *urumovii* Degen & Dren.

Key words: molecular analysis, morphological analysis, *Onobrychis pindicola, O. montana*

Introduction

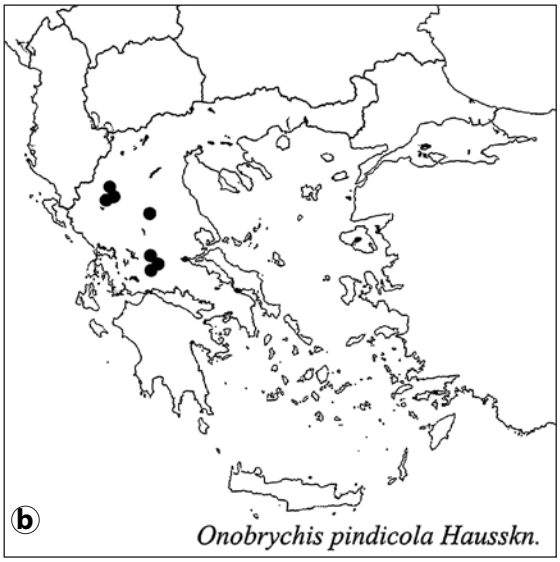
Genus *Onobrychis*, consists of more than 130 species. The interspecies relationship is still a subject of discussion and debate (Kar & al. 2014). New endemic species are still described (Ranjbar 2009; Ranjbar & al. 2010).

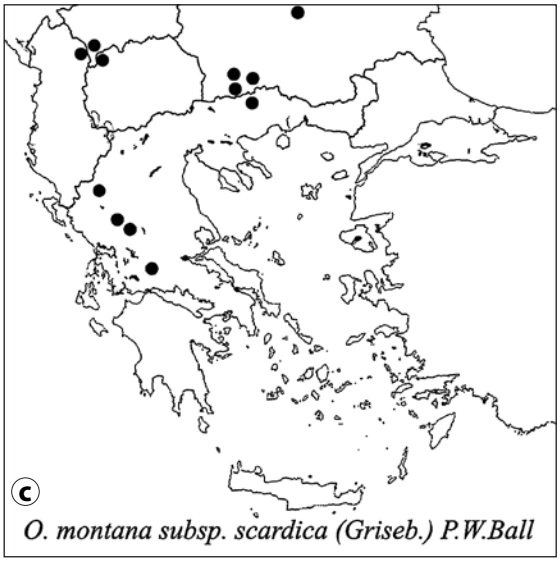
Onobrychis pindicola subsp. *urumovii* Degen & Dren. is a local endemic on the marbles of Pirin and Slavyanka Mts, SW Bulgaria (Velchev 1992; Euro+Med PlantBase 2011). It has been considered that on the marbles of these two mountains occur both *Onobrychis pindicola* subsp. *urumovii* and *Onobrychis montana* subsp. *scardica* (Griseb.) P. W. Ball (Kozuharov 1976;

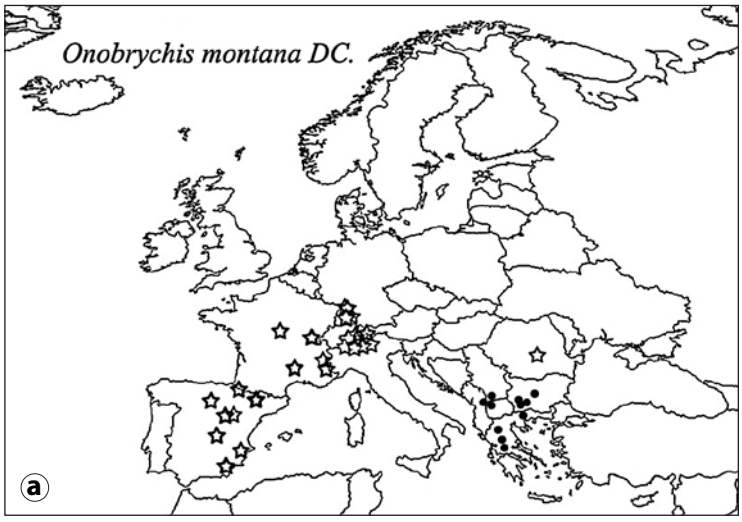
Euro+Med PlantBase 2011). The range of *O. m.* subsp. *scardica* is wider and extends to the Central Stara Planina, (Fig. 1, Kozuharov 1976). Pavlova and Manova (2000) distinguish *Onobrychis pindicola* Hausskn. subsp. *urumovii* Deg. & Dren from *O. montana* DC. in the Pirin Mts. Aneva and coauthors have listed the flora of Mt Slavyanka and identified *Onobrychis pindicola,* but do not mention *O. montana* (Aneva & al. 2015). These two taxa are perennial members of *Onobrychis* Mill. (Sect. *Onobrychis*). They are distributed in the subalpine habitats, as well as partially in openings of the spruce forest belt in Bulgarian mountains (Kozuharov 1976).

We have previously studied the breeding systems and pollination ecology of *Onobrychis pindicola* growing on Pirin marbles and the plants were found to be self-incompatible and obligatorily dependent upon bumblebees for pollen transport (Kozuharova 1999). In the course of this research we have noticed that the diagnostic feature used to distinguish the two taxa which are considered to grow in the Pirin Mts, namely *O. p.* subsp. *urumovii* and *O. m.* subsp. *scardica*, slightly overlapped. This key character refers to whether the standard is equal to the keel of the flower or is slightly shorter (*O. p.* subsp. *urumovii*); or whether the standard is 1–2 mm shorter than the keel (*O. m.* subsp. *scardica)* (Kozuharov 1976).

Both taxa differ from other *Onobrychis* by the lack of a stem and the densely tufted habit (Ball 1968; Kozuharov 1976). *O. p.* subsp. *urumovii* is endemic to the Pirin and Slavyanka Mts in Bulgaria, grows in stony and grassy habitats on carbonate rocks in the coniferous and subalpine belts (Fig. 1), and was considered relatively rare across Bulgaria (Velchev & al. 1984), but currently its IUCN status is evaluated as of Least Concern (Dimitrov 2009). The typical *O. pindicola* Hausskn.







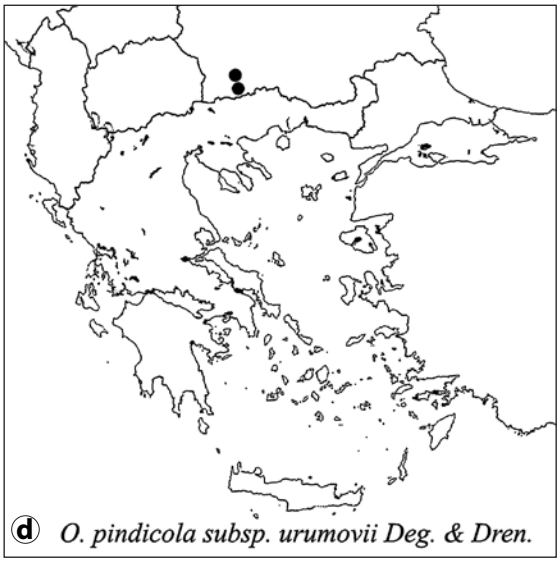
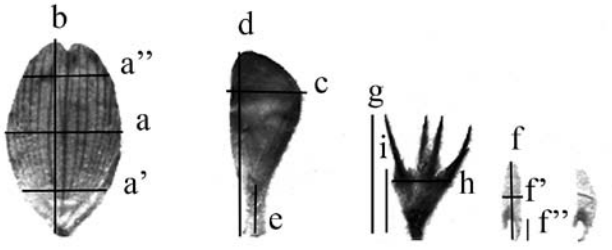


Fig. 1. Distribution of *O. montana* and *O. pindicola* – Compilation of information from Kozuharov 1976, Strid 1986 and Euro+Med PlantBase 2011; **1a –** *O. m. montana* (☆), and *O. m. scardica* (●); **1b –** *O. m. scardica* (●); **1c –** *O. pindicola* (black dot); **1d –** *O. p. urumpvii* (●).

is endemic to the Balkans (Fig. 1, Table 1), but it does not occur in Bulgaria. (Ball 1968; Strid 1986; Euro+Med PlantBase 2011). *O. p.* subsp. *urumovii* is a perennial plant that forms dense tufts, has an almost vertical reddish-brown rhizome, and its stems are short or lacking. Its leaves are pinnately compound and normally bear four to seven pairs of lanceolate, hairy leaflets and a similar terminal leaflet, and its numerous purple flowers are borne on dense racemes. The legume is round and dentate (Kozuharov 1976); it is a tetraploid (Andreev 1981). *Onobrychis montana* DC. is a mountain plant of Europe and Asia Minor. The typical subspecies occurs across the mountains of Central and South Europe, while *O. m.* subsp. *cadmea* (Boiss.) P. W. Ball occurs in Asia Minor – Turkey and Israel, as well as on Peloponnesus (Fig. 1, Ball 1968; Strid 1986; Euro+Med PlantBase 2011). *O. m.* subsp. *scardica* (Griseb.) P. W. Ball is a Balkan endemic (Ball 1968; Strid 1986; Euro+Med PlantBase 2011). *O. m.* subsp. *scardica* grows in stony and grassy habitats on carbonate rocks, in the coniferous and subalpine belts of the Pirin and Slavyanka Mts, Stara Planina and the Rhodopes (Fig. 1, Kozuharov 1976). Like *O. pindicola subsp. urumovii*, *O. m.* subsp. *scardica* is a perennial plant that forms dense tufts, stems are short or missing, its leaves are pinnately compound and normally bear four to seven pairs of lanceolate, hairy leaflets and a similar terminal leaflet. The numerous purple flowers are borne on dense racemes, the legume is round and dentate (Kozuharov 1976), and the taxon is a tetraploid (Andreev 1991). A statistical approach to biometric data is vital, if taxonomical problems and evolutionary processes are to be analysed objectively (Frederiksen and Petersen 1997; Eddie & Ingrouille 1999; Klimko & al. 2007; Rakić & al. 2012; Iamonico 2012; Egan 2015). Molecular markers are useful to solve phylogenetical problems in Fabaceae (Duan & al. 2016).

The aim of the present study was to test two alternative statements by measuring plant samples of several *Onobrychis* populations growing in the Pirin and Slavyanka Mts and using morphological and molecular approaches: 1) statement one – there are two taxa in the Pirin and Slavyanka Mts and their individuals/ramets are: 1a) separated and located at particular distinct sites, or 1b) individuals/ramets of both taxa are mingled sympatrically and bloom simultaneously; or 2) statement two – there is only one polymorphic species in the Pirin and Slavyanka Mts.

Table 2. Flower dissected into components and features measured and calculated in morphological analysis.



Symbol	Features
g	Maximum length of calyx, cm
i	Maximum length of calyx tube, cm
h	Maximum width of calyx, cm
k=g-i	Maximum length of calyx teeth, cm
b	Maximum length of standard petal, cm
a	Maximum width of standard petal cm
a'	Width of the standard petal close to the top, cm
a"	Width of the standard petal close to the beak, cm
d	Length of keel petals, cm
c	Maximum width of keel petals, cm
e	Length of beak to keel, cm
f	Maximum length of wing petal, cm
f'	Maximum width of wing petal, cm
f"	Length of beak to wing, cm
	Length of leaf rachis, cm
	Total number of leaflets/leaf (leaflets per side = [n/2] – 1)
	Length/width median leaflet on a leaf

Material and methods

Sampling

We sampled 11 locations (and recorded GPS coordinates for the waypoints) of *Onobrychis pindicola/montana* in the Northern Pirin Mts (Fig. 2). At each location, samples of leaves, and flowers (if available) were taken from a minimum of five individuals for morphological examination, and leaf material was dried with silica gel for later DNA extraction. Vouchers were collected and preserved in the Herbarium of the Faculty of Pharmacy, MU-Sofia, and are available for revision if requested.

Morphological analysis

In Table 1, we compiled the morphological and karyological data from literature, in order to decide which morphological features to analyse. We did not use the length of the bract in the calculations, because as we made measurements we have noticed that within the

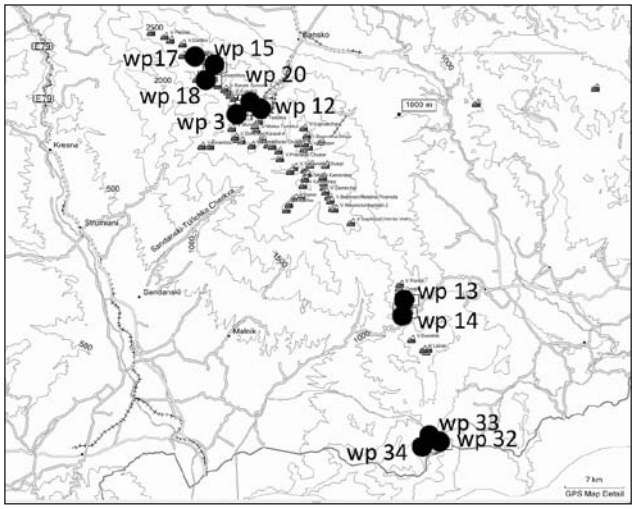


Fig. 2. Sampling locations (waypoints) of *Onobrychis* on the map of Pirin and Slavyanka Mts.

same plant variability was 1–2 mm and thus this feature could not be considered discrete. According to Ball (1968) and Kozuharov (1976), there are considerable differences between the species in stipule length, flower length, and fruit diameter. However, when we measured the sampled plants we have noticed that the stipule length varied within a single individual within a range that was suggested for both species and thus was not discrete enough (e.g. stipules of the base leaves of an individual plant were 7 mm and those of stem leaves were 3 mm). The fruit diameter depended on the stage of the fruit maturity. Therefore, we did not use these characters in our morphological analysis. The flower length is reflected by the flag/keel length and was not analyzed separately.

Minimum two flowers per plant and minimum five plants per location were rehydrated in warm dilute (40 %) alcohol, dissected into components, dehydrated and mounted on a sheet. The sheets were digitized (images scanned at 1:1). A total of 13 features were measured on flowers (Table 2). The flower features were measured digitally using Adobe Photoshop 5.0 (Kozuharova and Richards 2006). We have checked the features considered important for the identification key (Table 1, Ball 1968, Kozhuharov 1976) to test how they differ among 1) the individuals within the sampled populations, and 2) among the sampled populations. Therefore, we tested the ratio standard (flag) petal length against keel length (Table 2), considering the fact that this is a leading character for identification – standard equal to keel or slightly shorter (*O. p. urumovii*), versus standard 1–2 mm shorter than keel

Table 1. Comparison between morphological features of *Onobrychis pindicola* **and** *Onobrychis montana,* **according to the full description of the** *Flora on PR Bulgaria* **according to Ball 1968, Kozuharov 1976. Legend: * not found in Bulgaria, Ca – calyx, Co – corolla.**

Characters	*Onobrychis pindicola*		*Onobrychis montana* 2n=4x=28 (Favarger 1997) and one habitat in W. Tatra Mts (Przywara1980) 2n=4x=28 *Onobrychis montana* subsp. *cadmea* (Boiss.) P.W. Ball (Papanicolaou 1984)	
	**O. p. pindicola*	***O. p. urumovii***	***O. m. scardica***	**O. m. montana*
		2n=4x=28 (Andreev 1981)	2n=4x=28 (Andreev 1991)	
Wings	Wings equal to Ca or slightly shorter		Wings obviously to 4 times shorter than Ca	
	equal to slightly shorter	equal to slightly shorter	3–4 times shorter	almost equal
Standard (Flag, banner) towards keel	Standard equal to keel or longer sometimes slightly shorter		Standard 1–2 mm shorter than keel [1]	
	Standard longer than keel	Standard equal to keel or slightly shorter		
Bract	Bract reaches the base of the calyx tube or reaches their tips		Bract shorter then calyx tube	
Ca teeth towards Ca tube	Ca teeth 1.5–2.5 times longer than Ca tube Co violet	Ca teeth 2.5–4.5 times longer than Ca tube Co purple	Ca teeth 0.5–2 mm Ca teeth not more than 3 times as long as tube	Ca teeth 3–5 mm Ca teeth 3–4 times as long as tube
Leaflets Number Length Width	4–7 (8) pairs (5) 7–10 (11) mm (2) 3–4 (5) mm		5–8 pairs 4–18 (20) mm (1.5) 2–5 (7) mm	
Stipules length	3–5 mm		6–8 mm	
			(5) 10–20 mm long (2) 3–5 mm wide	4–7 (10) mm long 2–4 mm wide
Flowers	Numerous, 10–12 mm long		(15) 20–30 (40), 8–10 mm long	
Fruit	2–4 mm diameter with 5–6 teeth		6–12 mm diameter	

(*O. m. scardica*). We also tested the calyx length against the wing length, and a third parameter, the ratio calyx length towards calyx teeth length (Tables 1 and 2).

Principal components analysis

PCA reduces dimensionality of the space of variables in direction of the highest variance of the system, new variables being linear combinations of the previous variables, replacing the old coordinates of the factor space. The new coordinates are called latent factors or principal components. The interpretation of the new factors is the main goal since they deliver useful information about latent relationships within the data set. The results are indicated by two sets: factor scores giving the new coordinates of the factor space with the location of the objects, and factor loadings informing of the relationship between the variables.

Only statistically significant loadings (> 0:70) are important for the modeling procedure. The new principal components (latent factors) explain a substantial part of the total variance of the system for an adequate statistical model. Usually, the first principal component (PC1) explains the largest part of the system variation and each additional PC has a respective contribution to the variance explanation but with less significance. A reliable model normally requires a number of PCs, so that over 75 % of the total variation is explained (Massart and Kaufman 1983; Massart & al. 1997).

In our study, PCA was performed on three flower morphological characters (*FK_1* meaning the ratio between "flag length to keel", *diff* and *Cal_W_1*; meaning, respectively, the ratio "calyx tube depth to calyx teeth" and the ratio calyx to wings). Measurements of the leaves were made using a pocket micrometer (Table 2). Three leaf features (length/width of 1st and 4th leaflet, and length of rachis) were used in PCA.

Molecular analysis

DNA was extracted using a CTAB with chloroform method (Weising & al. 1995) from five individuals per sampling location (namely wps 3, 12, 13, 14, 15, 17, 18, 20, 33, 32, 34, and for comparison a sample of *O. montana* from Rhonda, Pyrenees Fig. 1a). All individuals sampled for DNA analyses were also measured for morphological analysis as described above (except those from 32 and 34). We also measured additional individuals from the same sites for morphological analysis. We amplified a total of 29 polymorphic RAPD fragments using two primers (Operon Technologies): OPA2 (14 polymorphic bands) and OPA11 (15 polymorphic bands) in 25 µl reactions (Kozuharova & al. 2007).

Nei's genetic distance between sampling locations was calculated from the RAPD presence/absence data using RAPDDIST 1.0 (Black 1995), with 1000 bootstrap replications. A consensus neighbour joining tree was calculated from the bootstrap replicate Nei's distance matrices using PHYLIP 3.57c (Felsenstein 1993), and was visualised using TREEVIEW 1.6.1 (Page 1996).

The chloroplast *Trn*L intron was sequenced for 16 individuals of *Onobrychis* (two individuals at each of the eight study locations: 3, 12, 13, 14, 15, 17, 18, 20A) using primers *Trn*L-c and *Trn*L-d (19 Taberlet & al. 1991). The nuclear internal transcribed spacer region (ITS) was also sequenced for each of these 16 individuals using the primers ITS4 and ITS5 (White & al. 1990). Details of the amplification and sequencing protocols are presented in another paper (Kozuharova & al. 2007).

Results

Morphological analysis

We observed variability in the morphology of the dissected flowers. Differences in the shape of the flag were noticeable among individuals at one and the same site (Fig. 3). The PCA revealed that two subgroups based on geographical principle concerning the Pirin and Slavyanka Mts cannot be divided with certainty (Fig. 4). Two latent factors were determined as responsible for the morphological data set structure – PC1, explaining over 40 % of the total variance and conditionally named "ratio flag length to keel" factor (statistically significant factor loading for FK_1; FK_1 meaning the ratio between "flag length to keel"), and PC2 explaining over 30 % of the total variance conditionally named "difference" factor (statistically significant factor loadings for *diff* and Cal_W_1; "diff" meaning the ratio "calyx tube depth to calyx teeth" and Cal_W-1 meaning the ratio calyx to wings). Fig. 4 represents the clustering of the plant samples on PC1 vs. PC2 factor score plot. In general, no significant separation of the plant samples could be detected. Five different subgroups could be found and their separation is obviously due to geographical (altitudinal) factors. In order to check the cluster homogeneity

and the statement concerning the presence of one or two taxa, the Wilcoxon – Mann – Whitney test for homogeneity was applied. It demonstrated that no statistically significant difference could be found between the five identified clusters, or between the plant samples belonging to each of the clusters. Therefore, two separate taxa located at particular distinct sites cannot be distinguished (Figs. 2 and 4). The analyses also revealed that individuals/ramets of two distinguishable taxa are not mingled sympatrically and do not bloom simultaneously at the same locations. The PCA revealed that there is only one polymorphic species in the Pirin and Slavyanka Mts (Fig. 4).

The principal components analysis (PCA) of the leaf features (Fig. 5) corresponds with altitude and spatial separation of the populations in the Pirin Mts. Populations that are distant in space but situated at approximately the same altitude have similar leaf features, e.g. waypoints 1, 2, 3 and 18, as well as waypoints 15 and 12 (Fig. 2). This could be explained by phenotypic expressions of the leaf features resulting from environmental pressures.

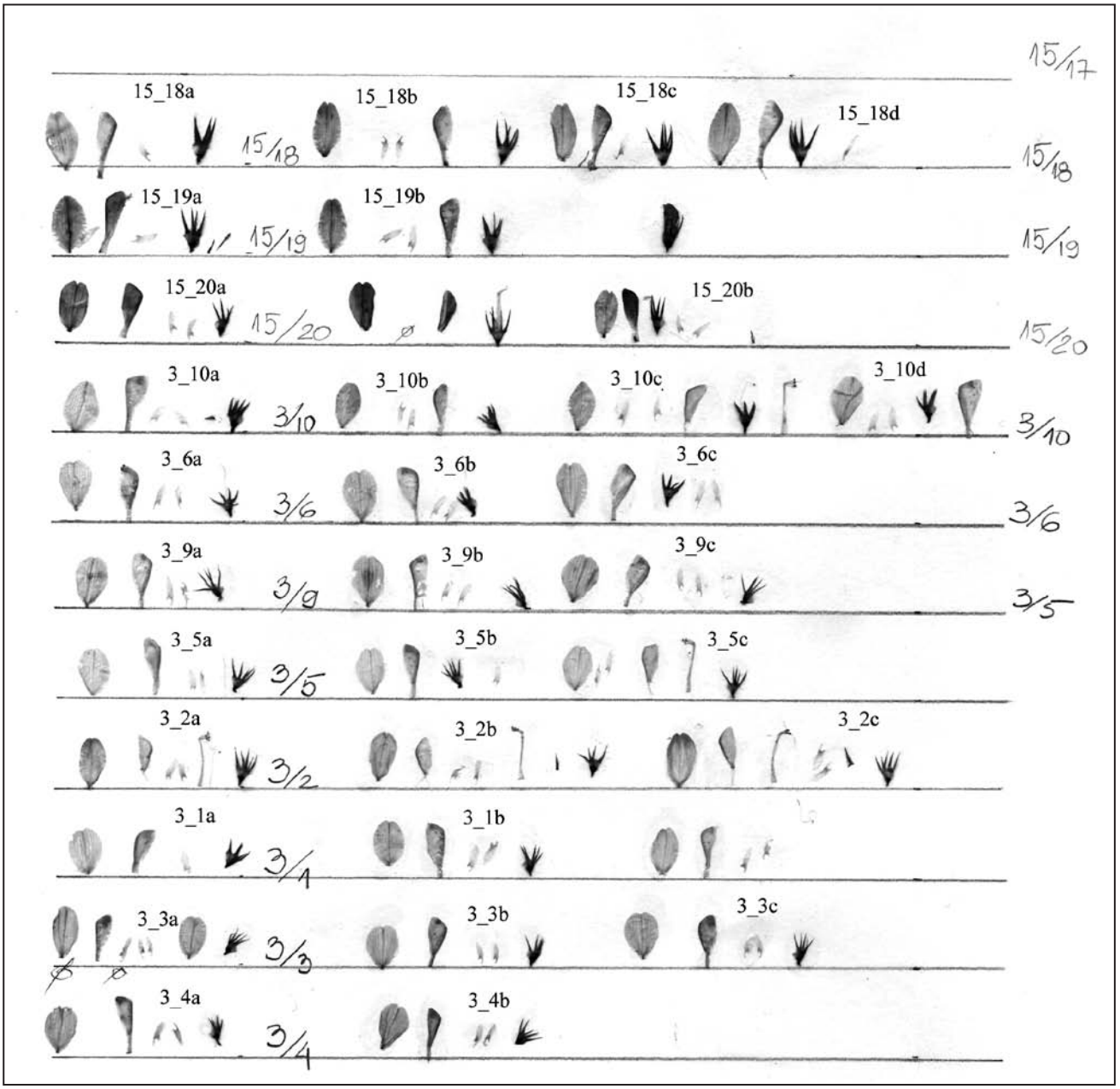


Fig. 3. Flower dissected into components – examples from site 3 and site 15 (individuals/ramets marked with numbers and flowers marked with letters).

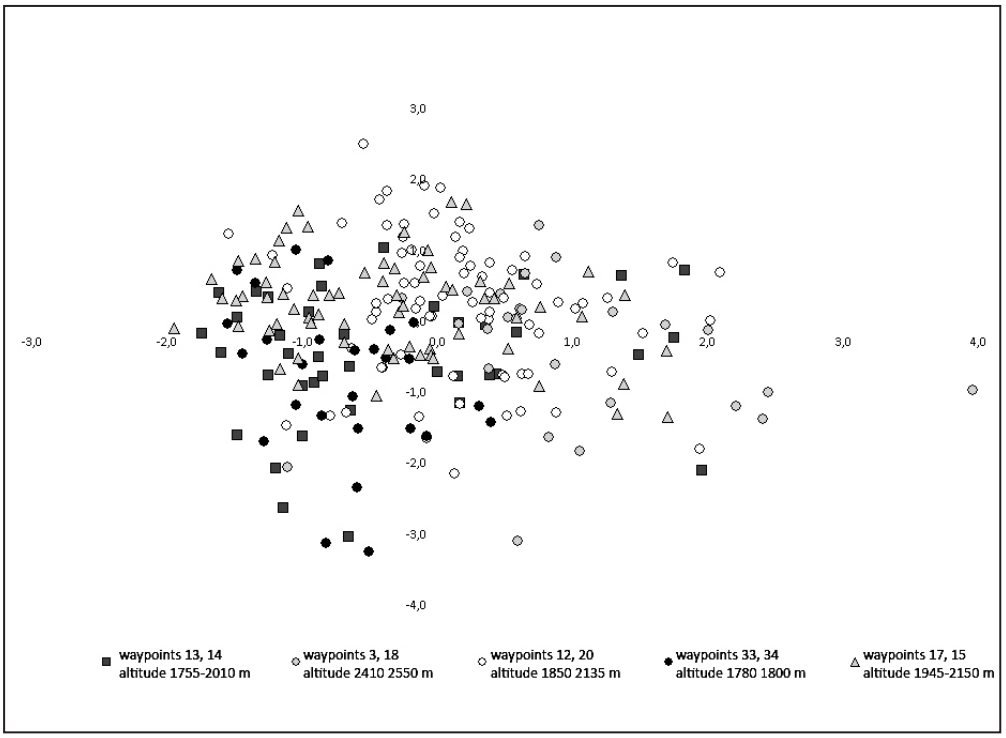


Fig. 4. PCA of three flower characters: ratio standard (flag) petal length towards keel length, calyx length towards the wing length, and ratio calyx length towards calyx teeth; Legend: Sampling locations (waypoints) 3, 12, 13, 14, 15, 17, 18, 20, 33, 34.

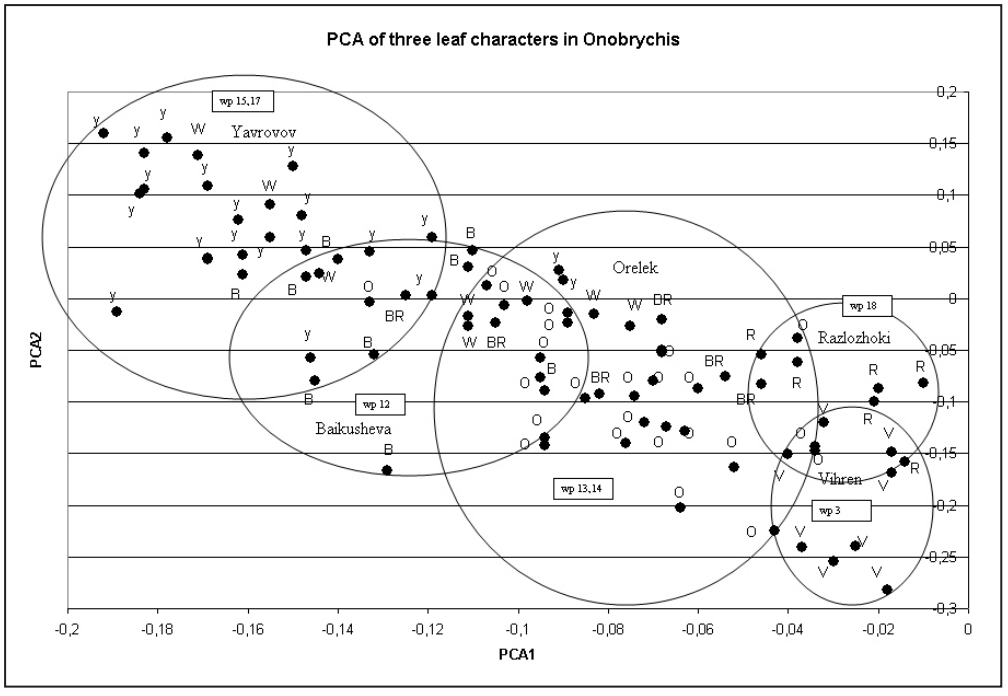


Fig. 5. PCA of three leaf characters: rachis length of the compound leaf, ratio length width towards length of top leaflet, ratio length width towards length of fourth leaflet.

Molecular analysis

Neither the ITS nor the *Trn*L sequences were variable enough to assess whether the samples collected were from two distinct groups. There was only one variable site out of the 457 base pairs sequenced for ITS, and 4 variable sites out of the 431 base pairs sequenced for *Trn*L. The RAPD markers were more variable, but the neighbour joining tree of Nei's genetic distances based on the RAPD data was poorly resolved (bootstrap values were ≤ 53) and did not show any clear separation of the samples into two groups (Fig. 6, *Onobrychis* Nei's tree). Therefore, defining subgroups, namely two separate taxa, is not possible on the basis of molecular data.

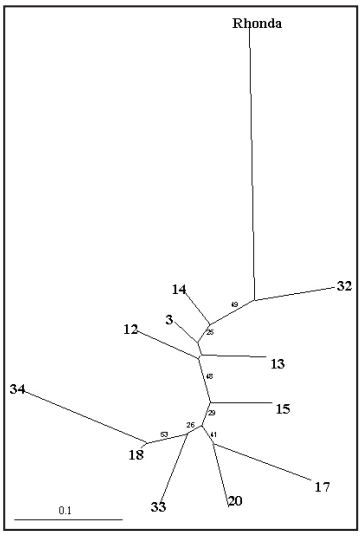


Fig. 6. *Onobrychis* consensus neighbor-joining tree of Nei's genetic distance between sampling locations calculated from the RAPD presence/absence data using RAPDDIST 1.0, with 1000 bootstrap replications and visualised using TREEVIEW 1.6.1. l; Legend: Sampling locations (waypoints) 3, 12, 13, 14, 15, 17, 18, 20, 33, 32, 34 and Rhonda.

Discussion

The morphological analysis (PCA) has revealed that two subgroups located at particular distinct sites are not detectable. The analyses also revealed that individuals/ramets of two distinguishable taxa are not mingled sympatrically and do not bloom simultaneously at the same locations. There is a vast morphological polymorphism. In the field, we observed obvious habit (general constitution) differences between the plants growing at lower altitude (locations marked as waypoints 12, 20, 15, 17), and those growing at higher altitude (marked as waypoints 18, 3). Even to the naked eye it was obvious that the plants growing at lower altitude in the high grass were bigger and taller, while the plants from the stony places with almost no grass at higher altitude were short and tiny. Presumably, this would group together the plants from locations 18 and 3 separately from the other locations; however, the PCA of the flower features did not confirm that (Fig. 4). This corresponds to the results of Fabbro and Körner (2004). They tested whether increased investment in pollination attraction by alpine plants compensated for assumed pollinator scarcity at high altitude and revealed that although shoot mass is massively reduced at high altitudes, display area and biomass of individual flowers were remarkably similar at low and high altitudes. The strategy of alpine plants for better pollination success is towards maintaining their flowers longer (Fabbro and Körner 2004). Also, altitude and snow cover duration gradient influence significantly the alpine vegetation (When & al. 2014). Multiple factors integrated together are responsible for the plant distribution (Carlson & al. 2013). It is not surprising to observe morphological variability in a plant taxon distributed both in subalpine and alpine belts of the Pirin and Slavyanka marbles.

Pavlova & Manova (2000) distinguish between *Onobrychis pindicola* Hausskn. subsp. *urumovii* Deg. & Dren (Bulgaria, Pirin Mts, peak Vihren SO 96788) and *O. montana* DC. (Bulgaria, Pirin Mts, Yavorov touristic chalet SOM 12943) by the pollen morphology – polar equatorial size ratio and sexzine versus nexine, although the authors put them together in the same group as the differences are small. However, there is no statistical difference in pollen size between *O. montana* and *O. pindicola* on any of the first three measurements they made. The means are slightly different but the standard errors relatively large (Table 2 in Pavlova & Manova). Therefore, their results do not contradict our findings of a large polymorphism that cannot be divided into two defined separate taxa.

Our results suggest that a systematic structure cannot be determined with certainty, which does not allow definition of subgroups, attributable to two separate taxa. Phylogeographic studies frequently reveal multiple morphologically cryptic lineages within species. What is not yet clear is whether such lineages represent nascent species, or evolutionary ephemera (Singhal & Moritz 2013). At this stage of investigation, we can state that only *Onobrychis pindicola* subsp. *urumovii* occurs in the Pirin and Slavyanka Mts.

The status of the endemic Carpathian taxon *Onobrychis transsilvanica* Simonk. in relation to the more widespread *Onobrychis montana* DC. was investigated using two molecular marker systems. It was shown that *O. transsilvanica* was a result of either a recent postglacial speciation with incomplete lineage sorting, or genetic divergence followed by subsequent continuous gene flow during the glacial period. The genetic structure of the complex does not support *O. transsilvanica* as a distinct species from *O. montana*. Within the Carpathians, the extant populations of *O. transsilvanica* comprise two major allopatric lineages, which have been isolated from each other for a long period of time. Unexpectedly, the major genetic break was not in line with a classical biogeographical boundary in the Carpathians, but rather separated a group from the southwestern edge of the mountains (Bãcilã & al 2015). As our results show similar situation in the Pirin and Slavyanka Mts, it seems possible that in the mountains of the Balkans, *O. montana* had gone

through recent postglacial local speciation processes with more or less incomplete lineage sorting.

Conclusion

The statement that there are two taxa namely *Onobrychis pindicola* and *O. montana* in the Pirin and Slavyanka Mts was rejected, because the morphological analysis demonstrated that two subgroups cannot be divided with certainty. Also, based on our molecular data, it is impossible to define subgroups, namely two separate taxa. The PCA revealed that there is only one polymorphic species in the Pirin and Slavyanka Mts. The diagnostic characters of the plants that we have measured, although rather variable, were close to those given for *Onobrychis pindicola* subsp. *urumovii*: the standard is equal to keel or slightly shorter and sometimes slightly longer, the calyx teeth are 2.5–4.5 times longer than calyx tube, and the wings are slightly shorter than calyx, but not 3–4 times shorter.

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Authors' contributions. E. Kozuharova – acquisition of data (measuring plant morphology), conception and design, N. Benbassat acquisition of data (measuring plant morphology), Vladimir Kochmarov acquisition of data (measuring plant morphology), M. Hale, acquisition of DNA data, analysis and interpretation, V. Simeonov and M. Nedyalkova – statistical analysis and interpretation of plant morphology data, K. Wolff acquisition of DNA data, analysis and interpretation, A.J. Richards – conception and design analysis and interpretation.

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