Two rare *Oxytropis* species (*Fabaceae*) endemic to the Pirin Mts, Bulgaria

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Abstract. Using morphological characters and molecular techniques, including RAPDs, ITS and *trn*L sequences, we compared *O. urumovii* and *O. kozhuharovii,* two alpine *Oxytropis* endemics to the North Bulgarian Pirin marble, with neighbouring populations of the widespread *O. campestris, O. halleri* and *O. dinarica*. *Oxytropis urumovii* is a very distinct diploid species which might be ancestral to this group and could be regarded as a palaeoendemic. The tetraploid *O. kozhuharovii* is most closely related to *O. prenja* from the Dinaric Alps, Bosnia-Herzegovina, but is a larger plant with a different facies and indumentum. It is possible that it has evolved as an allotetraploid derivative of *O. urumovii* and *O. halleri*. It is also possible that the circumpolar hexaploid *O. campestris* has evolved as an allohexaploid derivative of the diploid *O. urumovii* and a tetraploid from the Balkans, such as *O. kozhuharovii.* We present a key to summarise the differences between the Balkan *Oxytropis* species. Although about thousand mature individuals of *O. kozhuharovii* survive in the wild, the population occupies less than 200 m^2, so this species is considered to be Critically Endangered.

Key words: ITS sequence, *Oxytropis kozhuharovii, O. urumovii,* phylogeny, RAPD

Introduction

Although isolated by low ground from the Rila Mts to the north and the Central Rhodope Mts to the south, the Pirin Mts of Southwest Bulgaria nevertheless lie within a region of considerable topographical and geological complexity, known as the Rhodopean Supergroup. Even within the Pirin Mts, five distinct anticlines running roughly south-west to north-east can be identified, culminating in the highest peak, Vihren, which at 2914 m is the third highest mountain in the Balkans. The Pirin Mts result from recent Neogenic-Quaternary uplifts, although the rocks involved are extremely complex, dating from a wide variety of geological periods, and have mostly been metamorphically modified. Much of the northern Pirin Mts (Razlozhki and Sinanitsa anticlines) is marble (Zagorchev 1995). Velchev & Kenderova (1994) differentiated three glaciations of the mountain on the basis of thermoluminescence analyses of correlative deposits from the western foot of Pirin: Mindell, Riss and Wurm.

Perhaps as a result of this recent origin and glacial history, as well as the complex geology and topography, the Pirin Mts host more than 20 species endemic to the Pirin marble, according to Velčev & al. (1992). This diversity of local endemics is comparable with that of the better known Greek mountain Olimbos which lies 200 km to the south and is of very similar bulk and altitude. Amongst these Pirin endemics are two alpine species of *Oxytropis: O. urumovii* Jav. and *O. kozhuharovii* Pavlova, Dimitrov & Nikolova. A third alpine species, *O. campestris* (L.) DC. subsp. *campestris*, also occurs in these mountains (Fig. 1).

The fourth Bulgarian *Oxytropis, O. pilosa* (L.) DC. is a subalpine species which is widespread in East Europe but occurs only in a few low-altitude mountains in West and East Bulgaria.

The genus *Oxytropis* has about 300 species distributed through temperate, montane and boreal regions of the northern hemisphere. It is closely related to an even larger and more widespread genus, *Astragalus* (2500 species), from which it is distinguished only by a mucronate tooth on the abaxial side of the keel. It seems possible that *Oxytropis* is an artificial grouping; for instance, some species in both genera are 'locoweeds', selenium accumulators which are harmful to stock. However, Wojciechowski & al. (1993) and Sanderson & Wojciechowski (1996) show that three species of *Oxytropis,* including *O. campestris,* are sisters to a much larger sample of *Astragalus* which forms a single clade, but studies involving a larger number of *Oxytropis* species are awaited.

An interesting hypothesis on circumpolar speciation in the genus *Oxytropis* is offered by Yurtsev (1999). Only three genera of legumes (*Oxytropis*, *Astragalus, Hedysarum*) are typical of the Arctic, and only *Oxytropis* has undergone intense speciation there. Only a few subdivisions of the genus *Oxytropis* have contributed to this Arctic florogenesis and speciation. In the *O. campestris* "superaggregate" the lowest ploidy level in Eurasian species and races is $2n = 48$, whereas in western North America and Far-East Asia there are taxa with both $2n =$

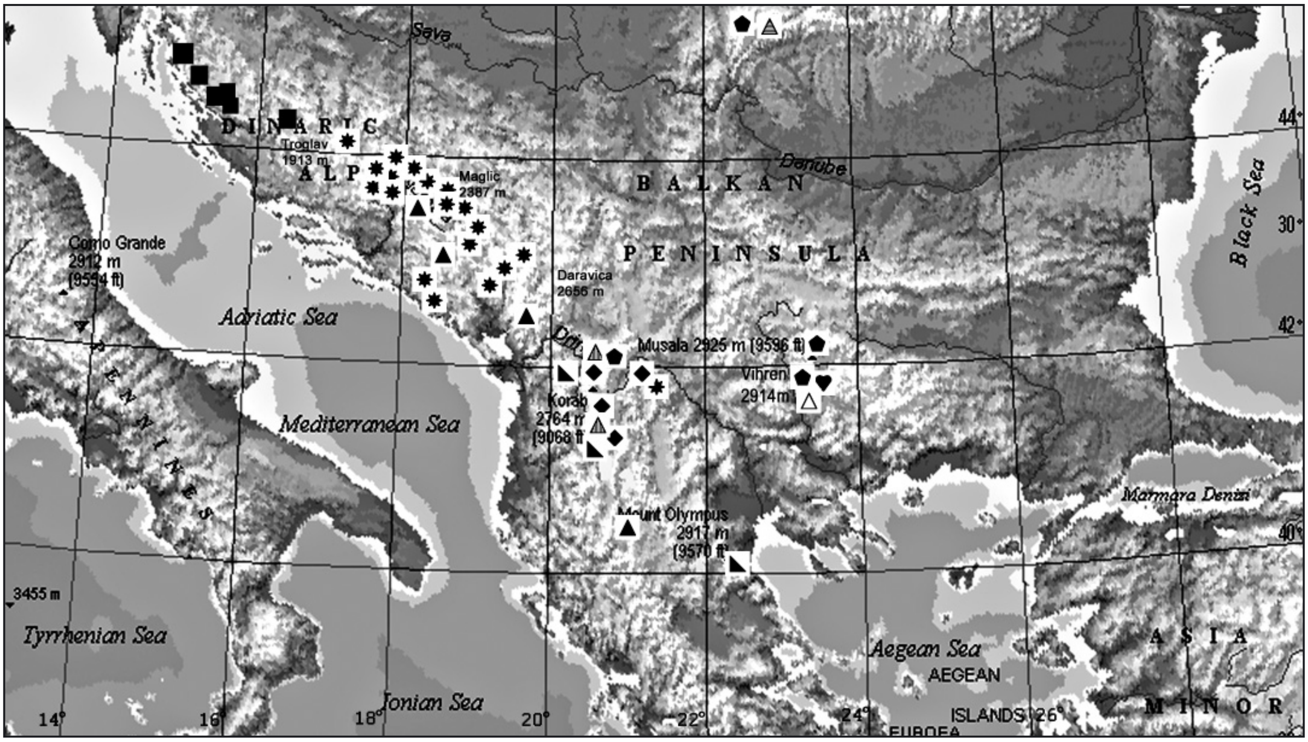

Fig. 1. Distribution of the acaulescent *Oxytropis* species in the Balkans (compilation after Hayek 1927; Leins & Merxmüller 1968; Diklich 1972; Kožuharov 1976; Chrtek & Chrtkova 1983; Strid 1986).

Yellow flowers

■ *O. dinarica* (Murb.) Wettst. subsp. *velebitica* Chrtek & Chrtkova**:** Mt Velebit, 1400–1600 m;

✸ *O. dinarica* subsp. *dinarica*: Mt Velez ,1600–1800–2300 m;

◆ *O. dinarica* subsp. *weberi* Chrtek & Chrtkova: Mt Korab, 2000 m, Popova Šapka, Šar Planina, NW Macedonia ($2n = 16$);

♥ *O. urumovii* Jáv**.** ($2n = 16$)**,** Pirin marbles, above 2500 m;

⬟ *O. campestris* (L.) DC. subsp. *campestris* ($2n = 48$), Pirin and Rila marbles, at about 2500 m, Prizren (Kosovo), Carpathians.

Purple flowers

△ *O. kozhuharovii* Pavlova, Dimitrov & Nikolova ($2n = 32$), Pirin marbles, above 2500 m;

▲ *O. prenja* (Beck) Beck, 2350 m shist, Gramos and mountains of the W part of the Balkan Peninsula;

◬ *O. halleri* subsp. *korabensis* (Kümmerle & Jav.) Chrtek & Chrtková, Mt Korab, Prizren (Kosovo);

⧋ *O. halleri* Bunge ex Koch, Carpathians and Slovenia ($2n = 32$);

◣ *O. purpurea* (Bald.) Markgr. ($2n = 16$)**,** limestone ridges 1900–2800, Olimbos, Albania, Macedonia

16 and 32, which are not always morphologically distinct: e.g. *O. gorodkovii* in the easternmost Chukotka Peninsula, (Yurtsev 1986, 1988, 1994, 1997, 1999; Yurtsev & al. 1978, 1994). At the same time, morphometric analyses reveal significant differences between some North American taxa – the tetraploid *O. campestris* var. *chartacea*, and the hexaploid *O. campestris* var. *johannensis* (Chung & al. 2004). In other cases, polyploidy has accompanied and consolidated evolutionary adaptation to contrasting cold treeless environments (as in *O. sverdrupii* $2n = 48$, *O. wrangelii* $2n = 64$; or *O. viscida* $2n = 16$ and 32, *O. middendorffii* $2n = 48$) (Yurtsev 1986, 1988, 1994, 1997, 1999; Yurtsev & al. 1978, 1994). In contrast, neither molecular nor morphometric data support the differentiation of the Eastern Alpine endemic *O. campestris* subsp. *tiroliensis* from the widespread *O. c.* subsp. *campestris* (Schönswetter & al. 2004). *Onobrychis campestris* s.str. is a glacial relict on the Balkan peninsula connected to the central European mountains and especially to the Alps (Andreev 1989). The evolution of the whole series *Sordidae* is based on auto- and aloploidy (Küpfer 1974; Andreev 1989). As a whole the taxonomy of *O. campestris* s. latiss. remains unsolved.

There are several species of *Oxytropis* in the Balkans (Fig. 1, Hayek 1927; Leins & Merxmüller 1968; Diklich 1972; Kožuharov 1976; Chrtek & Chrtkova 1983; Strid 1986). In this study we focus on the acaulescent species with semi-bilocular and almost completely bilocular legumes.

The aims of the present study were to use morphological and molecular evidence to investigate the distinctness and add "a brick in the wall" in the efforts to elucidate the possible evolutionary origins of *O. urumovii* and *O. kozhuharovii*. Both taxa are strongly localised in the wild, and if their specific rank can be justified, their conservation becomes a matter of some urgency. Unfortunately, it has not been possible to include *O. prenja* (Beck) Beck in the study.

Materials and methods

In late July 2001, the authors visited 10 sites with *Oxytropis* in the northern Pirin Mts: six with *O. campestris*, four with *O. urumovii* and the only known site for *O. kozhuharovii.* Those sites used in the analysis are listed in Table 1. At each site, samples of leaves, flowers and fruits were taken from a minimum of five individuals for morphological examination, and leaf material was dried with silica gel for later DNA extraction. In August 2002, material of *O. halleri* subsp. *halleri* was collected from five individuals at each of the two sites on the northern coast of Scotland, and in July 2003, material of *O. dinarica* subsp. *weberi* was collected from Northwest Macedonia (Table 1).

A minimum number of two flowers per plant and a minimum of five plants per site were rehydrated in warm dilute alcohol, dissected into components, dehydrated and mounted on a sheet. The sheets were digitalized (images scanned at 1:1). A total of 14 characters were measured on flowers and a further three measurements were made of leaves (Table 2, Fig. 5). The flower characters were measured digitally using Adobe Photoshop 5.0. Measurements of the leaves were made

Table 1. Sites from which *Oxytropis* were collected in the wild. Datum WGS 1984, UTM projection.

No	Species	Site	Altitude m	Longitude E (W)	Latitude N	collection date
3	*O. campestris*	Vihren chalet to Kabata	2410	23°24´10.3″	41°45´38.1″	08.07.2001
5	*O. campestris*	Kabata	2640	23°23´50.6″	41°45´38.5″	08.07.2001
7	*O. campestris*	Kazan I, Vihren	2605	23°24´11.3″	41°46´17.2″	08.7.2001
8	*O. urumovii*	Kazan I, Vihren	2605	23°24´10.8″	41°46´17.3″	08.07.2001
16	*O. kozhuharovii*	Yellow rocks, Okadenski cirque	2170	23°20´45.5″	41°49´12.3″	28.07.2001
19	*O. campestris*	Razlozhki Souhodol	2570	23°21´41.6″	41°47´52.7″	29.07.2001
20	*O. urumovii*	Razlozhki Souhodol	2570	23°21´41.6″	41°47´52.7″	29.07.2001
21	*O. urumovii*	Kazan II, Vihren	2251	23°24´50.9″	41°46´18.2″	31.07.2001
22	*O. campestris*	Vihren chalet to Kabata	2200	23°24´54.3″	41°45´26.8″	01.08.2001
	O. halleri	Invernaver, UK	5	04°13´40″W	58°31´48″	08.08.2002
	O. halleri	Strathy, UK	25	03°59´55″W	58°34´20″	08.08.2002
	O. dinarica subsp. *weberi*	Meadows, Popova Šapka, Šar Planina, NW Macedonia				06.1996 (A. Novotna)

Table 2. Characters recorded in morphological analysis.

	Length of bract, cm
g	Maximum length of calyx, cm
i	Maximum length of calyx tube, cm
h	Maximum width of calyx, cm
g^{-i}	Maximum length of calyx teeth, cm
b	Maximum length of standard petal, cm
b′	Length of beak of the 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 (the narrower base to the keel)
e′	Length of keel tooth, cm
f	Maximum length of wing petal, cm
f′	Maximum width of wing petal, cm
f″	Length of beak to wing, cm
	Length of rhachis to leaf, cm
	Total number of leaflets/leaf (leaflets per side = [n/2] – 1)
	Length/width median leaflet on a leaf

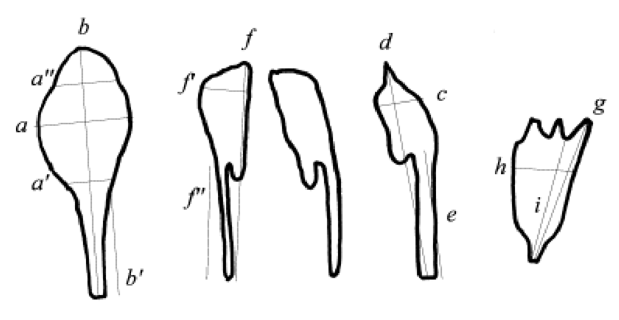

Fig. 5. Characters recorded in morphological analysis. The legend is in Table 2.

using a pocket micrometer. Descriptive statistics were calculated for individuals within populations, and using this data, for populations for each species. To analyse morphological differences between taxa, average readings per population were used for *O. campestris*, but measurements were only taken for single populations of *O. urumovii* and *O. kozhuharovii*, and for these species mean readings per individual were computed.

Inspection showed that six floral characters were diffferentiated between at least two of the species examined, and these were used in a principal components analysis (PCA), using a covariance matrix. Two of the three vegetative characters examined differentiated at least two of the taxa.

DNA was extracted using a CTAB with chloroform method (Weising & al. 1995) from five individuals per sampling location. We amplified a total of 46 polymorphic RAPD fragments using four primers (Operon Technologies): OPA8 (12 polymorphic bands), OPA12 (15 polymorphic bands), OPA6 (11 polymorphic bands), and OPA4 (8 polymorphic bands) in 25µl reactions. Each reaction consisted of 1 x *Taq* buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01 % Tween-20), 2.0 mM $MgCl_2$, 0.08 mM each dNTP, 0.2 µM primer, 1.0U *Taq* (Bioline) and 0.5 µl template DNA. The reaction cycle was 94 °C for 3 min, then 45 cycles of 94 °C for 30 sec, annealing temperature for 30 sec, 72 °C for 1.5 min, with a final extension of 72 °C for 4 min. Annealing temperatures were 40 °C for OPA8 and OPA12, 36 °C for OPA6 and OPA4. All reactions were performed in a PTC-100^{TM} thermocycler (MJ research). PCR products were visualised on 1.4 % agarose gels stained with ethidium bromide. We regarded the presence/absence state of RAPD bands, comparable between species and individuals, as a data matrix of bi-state characters varying between each individual. . We viewed the relationships between these as a single linkage dendrogram, based on a simple euclidian distance matrix, using cluster analysis available within the Principle Components Analysis program in the MINITAB 14 statistical package.

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 amplified for 24 individuals (9 *O. campestris*, 8 *O. urumovii*, and 7 *O. kozhuharovii*) using primers *Trn*L-c and *Trn*L-d (Taberlet & al. 1991) in 25 µl reactions containing 1x *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, 1.0U *Taq* (Bioline), and 0.5 µl template DNA. The reaction cycle was 35 cycles of 93 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min. The internal transcribed spacer region (ITS) was also amplified for each of these 24 individuals using the primers ITS4 and ITS5 (White & al. 1990) and the same PCR component concentrations as for the *Trn*L intron amplification. The reaction cycle for ITS was 94 °C for 5 min followed by 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 2 min, with a final extension of 72 °C for 4 min.

PCR products were sequenced directly. All PCR products were purified using QIAquick® PCR Purification Kits (Qiagen). Purified PCR products were then sequenced using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems) following manufacturer's recommended conditions, and sequences detected on an ABI 310 Prism® automated sequencer (Applied Biosystems). Sequences were aligned and edited using ProSequence (Filatov 2002).

Most species in this study had been characterised cytologically previously, but the chromosome number of *O. dinarica* was unknown. Material was collected by A. Novotna from the meadows of Popova Šapka, Šar Planina, NW Macedonia in July 1996 (voucher kept in personal collection in the Faculty of Pharmacy, Sofia). The haploid chromosome number in the pollen grains was studied by the acetocarmine method suggested by Heywood (1967) and Taschetto & Pagliarini (2004) on seven buds at different stage and two flowers. As a control we used 10 flowers and buds of *O. campestris* from the Pirin and Rila Mts, with a known chromosome number (*n* = 24).

Results

Morphological analysis

Using pairs of flower characters, the only combination which completely separated the species was calyx teeth length and keel length (Fig. 6), although *O. urumovii* also has longer bracts and a longer calyx than the other species (Fig. 7), and is undoubtedly the most distinct of the four species studied. Generally, the yellow-flowered *O. dinarica* falls closest to the purple-flowered *O. kozhuharovii* morphologically (Fig. 8). *Oxytropis kozhuharovii* has narrower and less numerous leaflets than most *O. campestris* (Fig. 4). In general, the hexaploid *O. campestris* tends to fall between the diploid *O. urumovii* and the tetraploid *O. kozhuharovii*, morphologically.

The haploid chromosome number in the pollen grains of *Oxytropis dinarica* was counted as *n* = 8.

Molecular analysis

Neither ITS nor *Trn*L were particularly variable. For both markers, only two polymorphic sites were detected out of 452 base pairs sequenced (ITS) and 454 base pairs (*Trn*L). A neighbour-joining tree of the *Trn*L polymorphisms (two insertion/deletions) differentiated *O. urumovii* from the other species, but did not differentiate *O. kozhuharovii* from *O. campestris*. The ITS polymorphisms (two base substitutions) did not differentiate any of the species. Only the RAPD data provided enough resolution to adequately examine variation between the sampling locations.

The results from the neighbour-joining unrooted tree based on RAPD presence/absence data (Fig. 9) and from the principal component dendrogram analysis of the RAPD band matrix (Fig. 10) are very similar. In both analyses, the four species examined remained distinct, with good bootstrap support in the neighbour joining analysis of Nei's genetic distance.

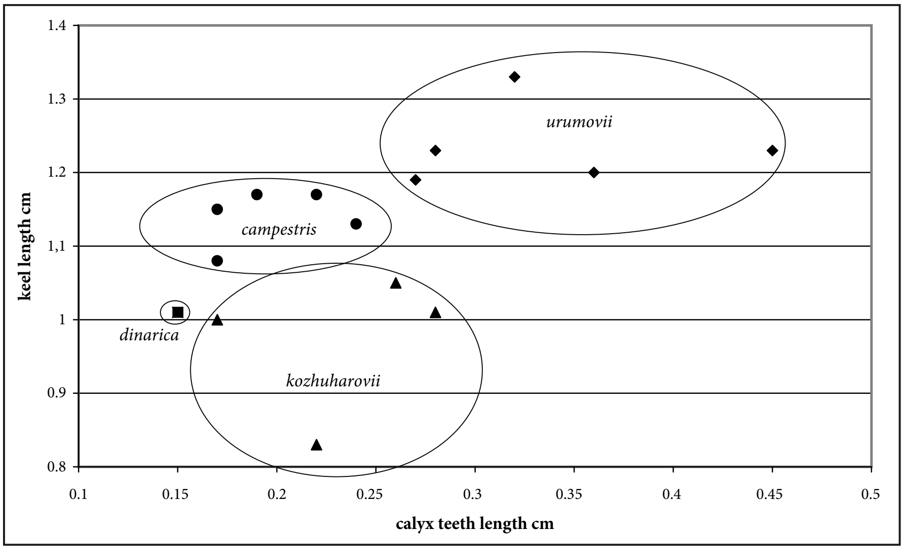

Fig. 6. Mean values in cm of calyx tooth length and keel length for four species of *Oxytropis*. Readings for *O. urumovii* and *O. kozhuharovii* are means of individuals from single populations. Readings for *O. campestris* and *O. dinarica* are means of five individuals for a population.

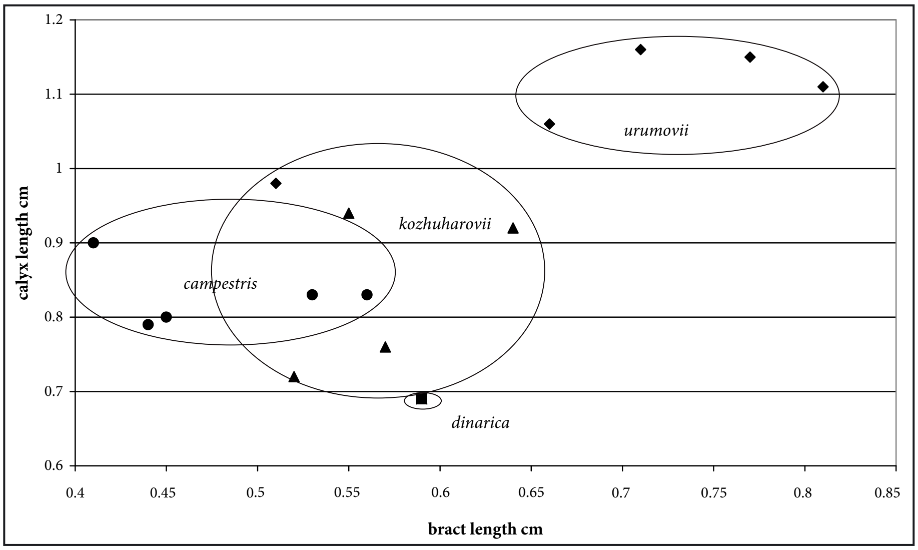

Fig. 7. Mean values in cm of bract length and calyx length for four species of *Oxytropis*. Readings for *O. urumovii* and *O. kozhuharovii* are means of individuals from single populations. Readings for *O. campestris* and *O. dinarica* are means of five individuals for a population.

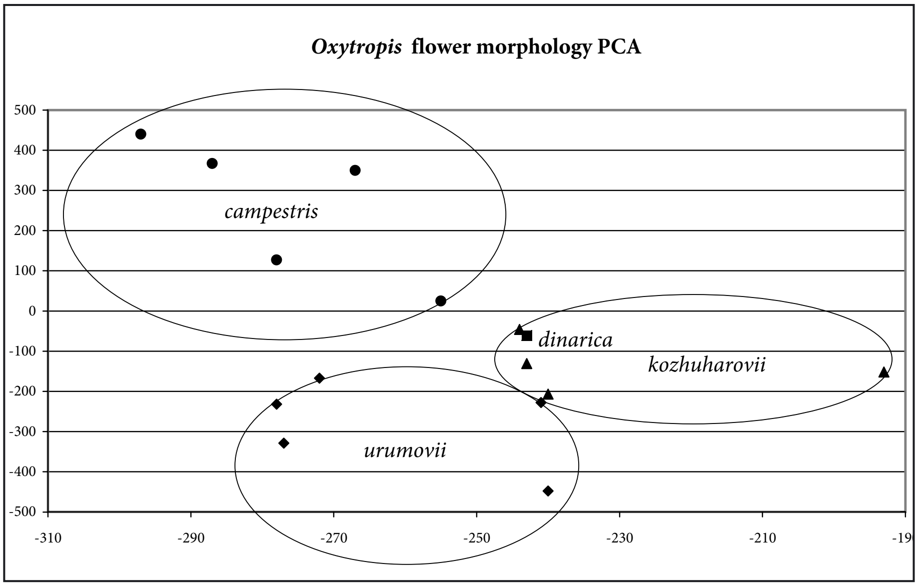

Fig. 8. Principal components analysis first and second vectors after multivariate analysis for six floral characters in four species of *Oxytropis*. Readings for *O. urumovii* and *O. kozhuharovii* are means of individuals from single populations. Readings for *O. campestris* and *O. dinarica* are means of five individuals for a population.

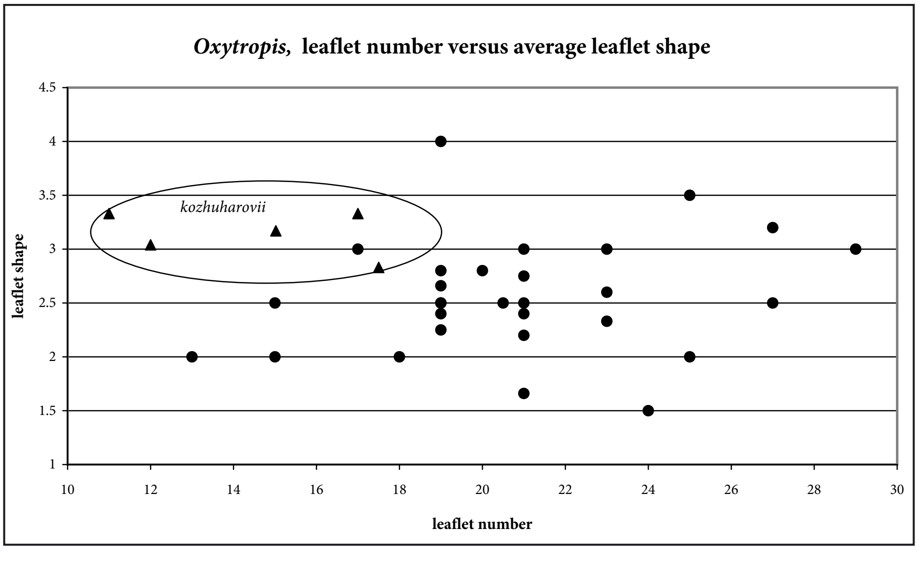

Fig. 4. Mean values for five leaves of leaflet shape (length/width) and total leaflet number per leaf for six individuals of *O. kozhuharovii* and 27 individuals of *O. campestris.*

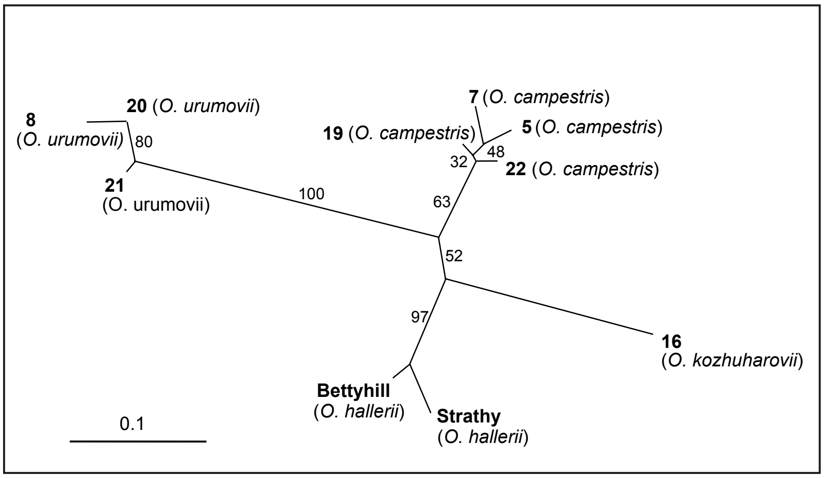

Fig. 9. Nei's genetic distance between sampling locations calculated from the RAPD presence/absence data with 1000 bootstrap replications. A consensus neighbour-joining tree was calculated from the bootstrap replicate Nei's distance matrices and visualised using TREEVIEW.

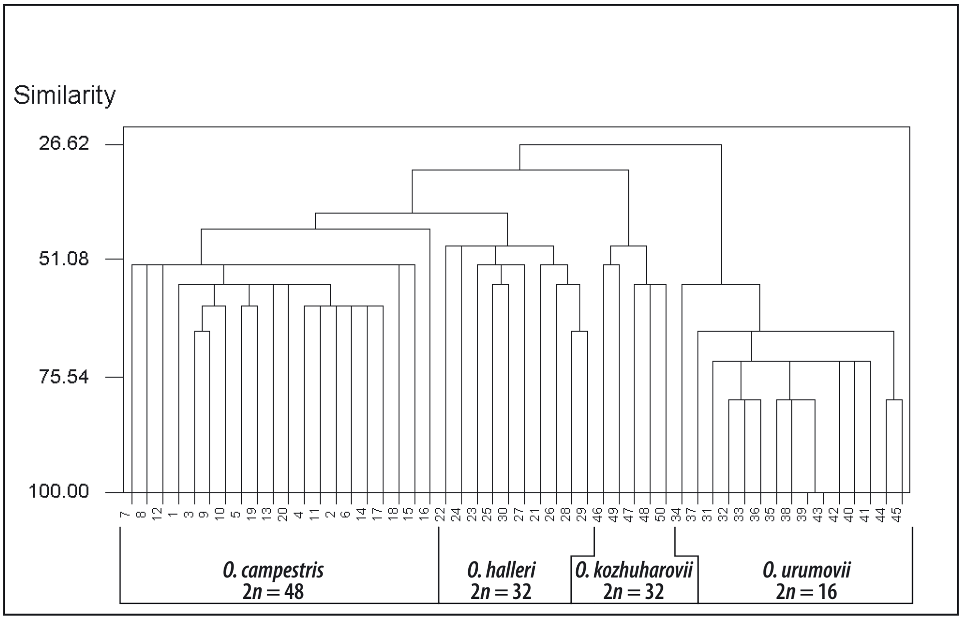

Fig. 10. Dendrogram of cluster analysis in ten populations of *Oxytropis* classified in four species, based on presence or absence of 46 RAPD bands.

Discussion

Oxytropis urumovii is a distinctive species compared on first sight to the other Balkan *Oxytropis* acaulescent species (with a semi-bilocular or almost completely bilocular legume, Appendix 1, Figs. 1 & 2), The whole plant, including the fruit, is rather densely covered with long (2–3 mm) patent whitish hairs, which contrast with the scape and calyx, the green colour of which is often suffused with black. Amongst the European species of *Oxytropis*, *O. urumovii* is diagnosed by being acaulescent, having leaves with about eight pairs of leaflets, nearly free stipules (adnate to the petiole for no more than one quarter), calyx-teeth which are much shorter than the tube, yellowish flowers and an ovoid semi-bilocular legume (Fig. 2, Leins & Merxmüller 1968; Kožuharov 1976). It is diploid with $2n = 16$ (Kruscheva 1986; Pavlova 1996). The karyotype is symmetrical, consisting of $2n = 4m + 12sm = 16$ small and medium size chromosomes. (Pavlova 1996). In addition, the author notes that endopolyploidy ($2n = 48$) is also observed and that is how she interprets the previous report of a hexaploid number (Andreev 1981). It is immediately distinguished from O. *campestris* by the much longer, denser and generally patent indumentum (usually sparse and semi-appressed in *O. campestris*), flowers that turn reddish rather than blackish at the tip, nearly free stipules (in *O. campestris* stipules are adnate to the petiole for one-third to three-quarters of their length), and a smaller legume (see also Leins & Merxmüller 1968; Kožuharov 1976). In general, it has a dwarfer

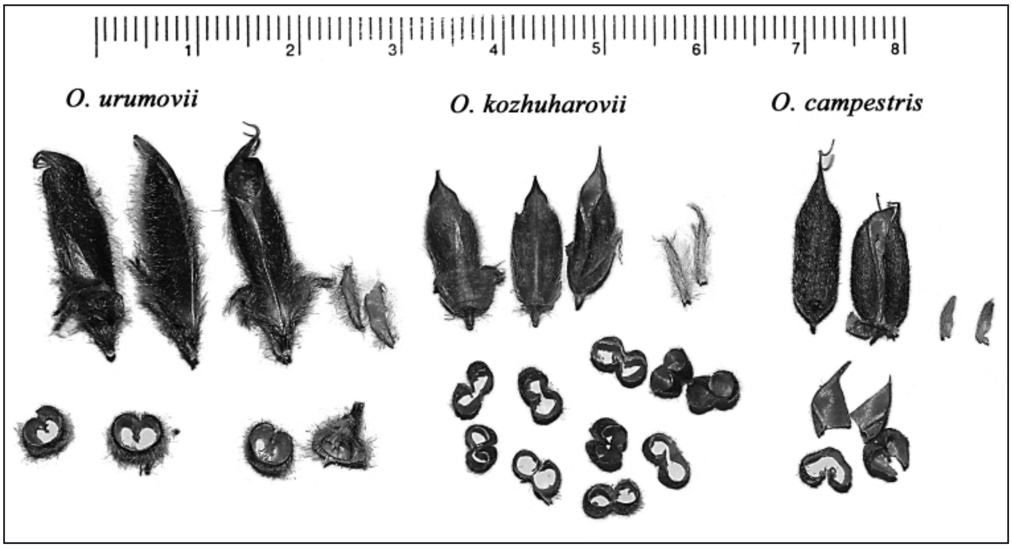

Fig. 2. Legumes of *O. urumovii, O. kozhuharovii* and *O. campestris* – views of the central vein, accretion, and (below) transverse sections.

Appendix 1***

Key to Balkan species of *Oxytropis* with semi-bilocular or almost completely bilocular legume.

1. Plant caulescent, stem >20 cm; calyx teeth >calyx tube; flowers yellow (widespread subalpine) .... **O. pilosa**

1*. Acaulescent alpine; calyx teeth <half length of tube; flowers yellow or purple. **2**

2. Calyx teeth <25 % length of calyx tube; hairs on fruit 2–3 mm, dense; flowers yellow (Croatia to Albania and Macedonia, three subspecies) .... **O. dinarica**

2*. Calyx teeth >25 % length of calyx tube; hairs on fruit usually less than 1 mm (except occasionally *O. urumovii*); flowers yellow or purple **3**

3. Fruit bilocular, with a septum in the dorsal valve; stipules with (1-) 2 or more veins; leaf indumentum velutinous (the hairs would be less than 0.5 mm long, although it is their soft dense quality that is important); flowers purple (widespread, but only in Mt Korab in the Balkans, e.g. widespread subsp. *halleri* $2n = 32$, subsp. *velutina* – $2n = 16$ restricted to the Alps, but in Mt Korab subsp. *korabensis* .... **O. halleri**

3*. Fruit semi-bilocular, lacking a septum in the dorsal valve; stipules one-veined; leaf indumentum usually pilose or sericeous but not velutinous; flowers yellow or purple **4**

4. Stipules free; calyx <1 cm; indumentum dense; inflorescence peduncle somewhat exceed the subtending leaf; raceme 4–12-flowered, flowers cherry-red; peduncle and legume with long, patent hairs (N Greece, Albania, SW Macedonia) .... **O. purpurea**

4*. Stipules adnate to petiole **5**

5. Stipules adnate to petiole for 25 % or less; calyx >1 cm and keel >1.2 cm in length; flowers yellow and indumentum dense; inflorescence peduncle about as long as subtending leaf – shorter or equal, seldom longer; throughout (N Pirin) .... **O. urumovii**

5. Stipules adnate to petiole for half length or more; calyx <1 cm, keel <1.2 cm; flowers yellow or purple; indumentum sparse except occasionally on calyx **6**

6. Hairs on calyx long and dense, especially at apex, exceeding calyx teeth in length; inflorescences exceeding leaves; flowers purple (one site in N Pirin .... **O. kozhuharovii**

6*. Calyx with short sparse indumentum, or nearly glabrous; inflorescences not exceeding leaves (peduncle about as long as subtending leaf) flowers yellow or purple **7**

7. Leaflets usually >9 pairs; flowers yellow (widespread alpine .... **O. campestris**

7*. Leaflets 6–8 pairs; flowers purple; raceme 1–4-flowered, peduncle and legume with short, appressed hairs (Dinaric Alps, Bosnia-Herzegovina .... **O. prenja**

and stiffer habit than the rather lax and sprawling *O. campestris*. According to Pavlova (1996), the karyotype of the population *O. campestris* in the Pirin Mts consists of $2n = 24m + 20sm + 4st = 48$, and the chromosomes are of medium size.

Leins & Merxmüller (1968) included *O. campestris* subsp. *dinarica* Murb. within *O. urumovii*, but Chrtek & Chrtkova (1983) reinstated the combination of Wettstein (1892) – *O. dinarica* (Murb.) Wettst., and described two new subspecies for this taxon, which occurs from Croatia (subsp. *velebitica*) to Bosnia and Montenegro (subsp. *dinarica*) and to Macedonia and Albania (subsp. *weberi*) (Fig. 1). In general, *O. dinarica* lies between *O. campestris* and *O. urumovii*, not least in general habit. It resembles the former species by the stipules which are adnate to the petiole for half their length, but the indumentum resembles more *O. urumovii*, except on the fruit on which the short (<1 mm) hairs are similar to those of *O. campestris*. The calyx of *O. dinarica* is diagnostic, for the calyx teeth are only about one-fifth the length of the calyx tube, but in the other two species they are more than one-quarter the length of the tube. In all three of these species the flowers are yellowish. *O. dinarica* is reported as diploid ($2n = 16$) for the first time in this paper. It is clearly a distinct species, so that *O. urumovii s. s.* can be regarded as being endemic to the Bulgarian Pirin Mts.

Unlike the species mentioned previously, the other Pirin endemic *Oxytropis*, *O. kozhuharovii* is a tetraploid with $2n = 32$ (Pavlova & al. 1999). It is only known from a single locality from the northernmost part of the Pirin, the Yavorov anticline, where it was discovered and described very recently (Pavlova & al. 1999). *Oxytropis kozhuharovii* is the only Bulgarian *Oxytropis* with blue or purple flowers. However, at least one and possibly two purple-flowered species occur in the region around the border between Macedonia and Albania, some 220 km to the west, notably Mt Korab (Fig. 1). Here an endemic subspecies of the widespread species *O. halleri* Bunge ex Koch, *O. h.* subsp. *korabensis* (Kummerle & Jav.) Chrtek & Chrtkova, occurs (Fig. 1). As discussed by Pavlova & al. (1999), *O. kozhuharovii* closely resembles members of the *O. halleri* complex, but differs in the structure of the legume which, like that of *O. campestris* and *O. urumovii,* lacks a septum in the dorsal valve (semi-bilocular). All subspecies of *O. halleri* have a septum in the dorsal valve, although that in subsp. *korabensis* is narrow and poorly developed. Stipules are another feature in which *O. kozhuharovii* resembles more the *O. campestris* complex than *O. halleri*. Most forms of *O. halleri* have stipules with several longitudinal veins, whereas those of *O. kozhuharovii* are single-veined. However, the reduced stipules of *O. halleri* subsp. *korabensis* have only 1–2 veins. We would add that the indumentum of the *O. halleri* complex tends to be velutinous, but that in *O. kozhuharovii* is longer and patent.

In our opinion, the most distinctive feature of *O. kozhuharovii* is the indumentum of the calyx, particularly the apex, which is very densely covered with long white hairs, which equal or exceed the calyx teeth (Fig. 3). This contrasts strikingly with the rest of the plant in which the patent indumentum is long but sparse and inconspicuous.

Although it resembles *O. halleri* superficially, it seems likely that *O. kozhuharovii* is in fact more closely related to *O. prenja* which also has semi-bilocular legumes. From an examination of the herbarium material at **K** and **E**, it is clear that the latter is a dwarfer plant in which the scape exceeds the rather prostrate foliage. Decisively, it is scarcely hairy

Fig. 3. *O. kozhuharovii,* illustrating the narrow leaflets and the diagnostic long hairs at the apex of the calyx.

even on the calyx. It is also said to have fewer pairs of leaflets per leaf (usually 6–7 rather than 10–11 for *O. kozhuharovii*), but this disagrees with our findings for the latter species (Fig. 4). Also, according to Leins & Merxmüller (1968), *O. prenja* is said to have a longer (15–19 mm) standard which is emarginate at the apex, and a shorter keel tooth ('beak') of only 0.5 mm. However, although Fig. 4 in Pavlova & al. (1999) appears to show an emarginate standard in *O. prenja,* the apex of the standard in this species is said in Table 1 to be 'convex', and the standard illustrated does not appear to differ in length from that of *O. kozhuharovii* at about 1.3 mm. Also, although Pavlova & al. (1999) state that the length of the keel tooth in the latter species is 'circa 1 mm' (diagnosis) or '1–1.5 mm' (final line), the present study found that it varied between 0.3 and 0.8 mm, and did not differ from that of *O. prenja* in this regard.

Oxytropis prenja was ignored by Chrtek & Chrtkova (1983) and seems to be little known outside its native area. The description in Leins & Merxmüller (1968) would not distinguish it from *O. kozhuharovii*. However, according to a recent visitor, its general appearence is unlike that of the latter species (fid. Anonymous reviewer). Its image has twice appeared on postage stamps for Bosnia-Herzegovina, as a painting (1997) and a photograph (2003). Accompanying website texts claim it as an endemic of the Dinaric Mts, Prenj Planina, Čvrsnica, and Vran, where it occurs in limestone crevices at altitudes of 1900–2228 m. This taxon has also been said to occur in Albania and Macedonia (Greuter 1989), where it may have been confused with *O. halleri* subsp. *korabensis*.

We conclude that each of the localised Balkan endemic *Oxytropis* are distinct species. In our view, they are best differentiated as shown in the key (Appendix 1).

The molecular analysis revealed the following: *O. urumovii* proved to be the most distinct species of the four. As it is diploid, it could be argued that *O. urumovii* is ancestral to this group and a palaeoendemic element of the flora of the Pirin Mts.

Compared to Bulgarian *O. campestris* and Scottish *O. halleri, O. kozhuharovii* is about equally related to each. Both analyses would be consistent with the hypothesis that *O. campestris* could have arisen from the allohexaploid union between *O. urumovii* and *O. kozhuharovii,* thus agreeing with the morphological data (unfortunately, material of *O. dinarica* was received too late to be included in this analysis).

In addition, the molecular evidence suggests that *O. kozhuharovii* could be the allotetraploid derivative of the sympatric diploid endemic *O. urumovii* and the widespread diploid (and tetraploid) *O. halleri,* the nearest populations of which occur today in Mt Korab, some 220 km away. Today, this disjunction seems significant, but during the Late Glacial stadials mountain steppe vegetation (*Artemisia – Chenopodiaceae – Poaceae*) in which they occur dominated at high and mid-altitudes (Wijmstra 1969; Florschütz & al. 1971; Bottema 1974, 1979; Lang 1994; Tzedakis 1994, 1999; Bozilova 1996; Bozilova & al. 1989; Bozilova & Tonkov 2000; Tonkov & al. 2002, 2006; etc). At that time species that now occur at a higher altitude were more widely distributed at lower altitudes.

Conclusion

Conservation

Of the local endemic *Oxytropis* in the Balkans, we have no information on the status of the subspecies of O. *dinarica, O. halleri* subsp. *korabensis* or *O. prenja,* although a website classifies the latter species as *Vulnerable*. *Oxytropis urumovii* is localised to the northern marble part of the Bulgarian Pirin Mts, above 2000 m. Although it is not locally frequent, the population occupies a large territory, part of it within a protected region, and in our judgement its status is not currently under threat. Its IUCN category is *Vulnerable* according to the *Red Data Lists of Vascular Plants and Fungi in Bulgaria* (unpubl.).

The only known locality for *O. kozhuharovii* lies in the northernmost part of the Bulgarian Pirin Mts, known as the Yavorov anticline. Plants were limited to an area of steep rough grassland on marble. A total of about three thousand mature plants were discovered after extensive searches but they occupy a territory of only about 200 m^2. Some were of considerable size and thought to be many decades old. Only a few seedlings or juvenile plants could be found. Although large amounts of seed are apparently set, regeneration appears to be poor. The locality is very remote, and for the moment rarely visited by man, and not heavily grazed by domesticated animals, but might be threatened by land-slips or avalanches. Its

IUCN category is *Critically Endangered* (unpubl.). The real threat is the current active development of this mountain as a huge ski resort. *O. kozhuharovii* is a distinctive species which must be regarded as amongst the most threatened plants in Europe. There is an urgent need to promote its regeneration, perhaps by raising seed in cultivation and planting out seedlings in exclosures to protect them from grazing by wild animals.

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