Taxonomic relationships of selected Bulgarian species from *Rumex* subg. *Rumex* (*Polygonaceae*) based on ISSR markers

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Abstract. The high level of morphological similarities among the Bulgarian species of *Rumex* subg. *Rumex* makes their distinguishing difficult. ISSRs methods have proven that they ensure detection of intraspecific variation without any need of prior information about the existing sequences. Therefore, they were used in the present study.

Twelve accessions of *R. patientia*, *R. cristatus*, *R. confertus*, and *R. alpinus* were sampled in this study, with *R. pulcher* (five accessions) as a referent species. ISSR-PCR reactions of 10 primers were used for the distinction of the four studied species and the assessment of their genetic diversity. The ISSR markers exhibited significant polymorphism in the studied taxa. The genetic similarity among them served for the construction of a dendrogram. The combination of the results from the used primers classifies the studied species into two major groups: Group 1 with *R. patinetia*, *R. cristatus* and *R. confertus* is genetically similar and of similar ploidy level; Group 2 includes the diploid *R. alpinus* and the referent species *R. pulcher*.

The achieved high percentage of accuracy has shown how promising the application of this approach is for other *Rumex* species.

Key words: Bulgaria, identification, ISSR markers, subgenus *Rumex*, taxonomy

Introduction

Genus *Rumex* in Europe comprises species that belong to four subgenera: *Acetosella* (Meisn.) Rech. f., *Acetosa* (Mill.) Rech. f., *Rumex* (=*Lapathum* (Campd.) Rech. f.), and *Platypodium* (Willk.) Rech. f. (Rechinger & Akeroyd 1993). Subgenus *Rumex* is richest in species: 150 species across the world (Datta 1952), 14 of which occur in Bulgaria (including such invasive plants as *R. confertus*). This subgenus encompasses some of the most widely spread invasive weeds in the world that are highly adaptable to different environmental conditions (e.g. *R. crispus*, *R. conglomeratus*). Their successful ecological adaptation and development in new places are due to their fertility, seed dispersal adaptations, ability to sprout over a long period of time, and high germination rate.

Classical taxonomy of the subgenus *Rumex* is based mainly on morphological and anatomical features and the chromosome numbers (Lousley & Williams 1975; Himi & al. 2000). However, the widely spread natural hybridization (Rechinger 1949b) results in polyploidy (Jaretzky 1928; Ono 1930) and introgression in subgenus *Rumex*. Hence, in evolutionary terms, the existing variability leads to convergence and parallelism in the morphological traits of some species groups (Rechinger 1990). Lack of a generally accepted model for species identification in the subgenus explains the existence of

a large number of determination keys that reflect different taxonomic concepts (Rechinger 1937, 1964; Losinskaja 1936; Prodan 1952; Borodina 1979b; Pestova 1998). Therefore, classification based on morphological characteristics is often unreliable and may not describe the existing relationships among the different taxa.

Molecular markers have been used in taxonomic and phylogenetic studies in *Polygonaceae* species from Asia (Yasmin & al. 2010). Still very little attention has been given to the analysis of interspecific and intergeneric variation of the European representatives of subgenus *Rumex*.

Application of molecular markers can resolve many taxonomic problems in the subgenus. The choice of molecular technique depends on its reproducibility and simplicity. The Inter-Simple Sequence Repeat (ISSR) is a highly reliable molecular-marker technique developed by Zietkiewics & al. (1994). The ISSR analysis involves PCR amplification of regions between adjacent, inversely oriented microsatellites using single simple sequence repeat (SSR)-containing primers. The technique can be applied to any species that contains a sufficient number of SSR motifs. Its advantage is that genomic sequence data are not required (Gupta & al. 1994; Goodwin & al. 1997). The primers are 16–18 bp long, composed of any di-, tri-, tetra, or pentanucleotide SSR motifs found in the microsatellite loci, and could be flanked at the 3' or 5' end by 2–4 arbitrary nucleotides (anchored primers), giving a wide array of possible amplification products (Zietkiewicz & al. 1994; Blair & al. 1999).

The technique is more reliable than RAPD and generates a larger number of polymorphisms per primer because variable regions in the genome are targeted (Hantula & al. 1996). The potential use of ISSR markers depends on the variety and frequency of microsatellites, which differ within species and with the targeted SSR motifs (Morgante & Olivieri 1993). Furthermore, the number of bands produced by an ISSR primer with a given microsatellite repeat should reflect the relative frequency of that motif in the genome and would provide an estimate of the motif abundance as an alternative for library hybridization (Blair & al. 1999). The high reproducibility makes the ISSR method suitable for studies of genetic variation in closely related species (Fang & Rosse 1997; Nagaoka & Ogihara 1997).

The aim of this study is: 1. to test the applicability of ISSR molecular markers as a tool for assessing genetic differentiation of the species of subgenus *Rumex*; 2. to explore phylogenetic relationships of four Bulgarian representatives of this group, by combining classical morphological and molecular taxonomic approaches.

To our best knowledge, this is the first study of the taxonomic relationships between the species of the subgenus *Rumex* based on ISSR techniques. Such study will provide extra criteria and data for objective differentiation of the species within the group. This work is part of a comprehensive biosystematic investigation of the Bulgarian species of genus *Rumex* and is aimed at updating the taxonomic scheme of the group.

Material and methods

Plant material and vouchers

The plant material used for this study was collected by the authors from different floristic regions in Bulgaria, during the vegetative seasons of years 2003–2007 (Table 1). Voucher specimens of the five species are deposited at the herbarium of the Agricultural University, Plovdiv, Bulgaria (SOA) and the Institute of Biodiversity and Ecosystem Research – BAS (SOM) (Table 1). The samples were identified with the help of the existing floristic sources and comparative herbarium materials in SOA, SO and SOM. Taxonomy and phylogenetic relationships among the Bulgarian species of subgenus *Rumex* were studied by means of the comparative morphology of vegetative and generative organs. For determination of species, the greatest significance was attributed to the valve features in mature, fully developed plants (Rechinger 1932; Snogerup 1991; Mosyakin 2005). Our observations have confirmed that most of the taxonomically reliable morphological features for subgenus *Rumex* can be found in the valves of mature plants (length/width of valves, tubercle, nuts and fruit stalk length) (Table 2).

The floristic regions and subregions are given as accepted in the multivolume edition of *Flora RP Bulgaricae,* and the localities are mapped according to Kozhuharov & al. (1983). Information on the floristic regions in Table 1 is numbered as follows: Black Sea Coast (1.1 *Northern*; 1.2 *Southern*), NE Bulgaria (2), Danube Plain (3), Znepole Region (7), Vitosha Region (8), Rila Mts (15), Rhodopi Mts (17.1 *Western*; 17.3 *Eastern*), Tracian lowland (18), and Toundzha Hilly Country (19).

The molecular taxonomic studies were conducted in the Laboratory of Molecular markers at the Department of Plant Physiology and Molecular Biology, University of Plovdiv.

Table 1. List of accessions to the Bulgarian species of subgenus *Rumex* included in this study

No of accession, floristic region, UTM data, altitude, locality, date of collecting, number of voucher specimen

***R. patientia* L.**
700 (17.3) LF-69, 452 m a.s.l., Momchilgrad town, 14.07.2005, SOA 057075;
701 (7) FN-43, 916 m a.s.l., Paramun village, 05.07.2006, SOM 163984;
702 (19) MH-20, 126 m , near Nova Zagora town, 10.08.2005, SOA 059482;
703 (3) FP-55, 70 m a.s.l., along Danube River: village Archar, Vidin district, 23.07.2006, SOA 057063.

***R. cristatus* DC.**
300 (1.1) NJ-90, 10 m a.s.l., near Balchik town, 18.06.2004, SOA 56939;
302 (17.3) LF-99, 272 m a.s.l., Rogach village, Krumovgrad district, 14.07.2005, SOA 56942.

***R. alpinus* L.**
400 (17.1) GM-36, 1350 m a.s.l., in grassy places, near Zdravets village, 31.08.2005, SOM 163927;
401 (8) FN-81, 1810 m a.s.l., Mt Vitosha: around Aleko mountain chalet, 19.08.2003, SOA 056386;
402 (15) GM-27, 1580 m a.s.l., around Belmeken dam, 14.07.2003, SOM 163928.

***R. confertus* Willd.**
900 (2) MJ-52, 210 m a.s.l., in a sunflower field, near Razgrad town, 16.08.2005; SOA 056932;
901 (2) MH-99, 241 m a.s.l., between Shumen town and Struino village, 16.08.2005, SOA 056934;
902 (7) FN-34, 790 m a.s.l., near Trun town, 05.06.2006, SOA 059624.

***R. pulcher* L.**
200 (1.1) NG-67, 20 m a.s.l., Kraymorie village, Burgas district, 23.06.2003, SOA 59238;
203 (18) KG-99, 300 m a.s.l., around Pyasuchnic dam, 22.06.2003, SOA 56407;
202 (17.3) MF-28, 78 m a.s.l., Odrintsi village, Ivailovgrad district, 15.07.2005, SOA 163906, SOM 163906;
205 (1.1) NG-67, 20 m a.s.l., Kiten village, 06.06.2006, SOM 163887;
208 (1.1) NG-59, 25 m a.s.l., Sozopol town, 03.07.2004, SOA 59239.

Table 2. Morphological parameters used to determine the studied species of genus *Rumex*.

Characteristic	Taxon				
	R. patientia x±Sx (mm)	*R. confertus* x±Sx (mm)	*R. cristatus* x±Sx (mm)	*R. alpinus* x±Sx (mm)	*R. pulcher* x±Sx (mm)
Valve length	8.09±0.02	7.07±0.03	6.34±0.03	4.89±0.03	4.73±0.02
Valve width	6.95±0.03	6.49±0.03	6.01±0.02	4.73±0.03	3.02±0.03
Tubercle length	1.63±0.03	2.48±0.02	2.57±0.03	–	2.38±0.03
Tubercle width	0.81±0.01	1.36±0.01	1.61±0.02	–	1.18±0.01
Nut length	3.49±0.03	3.57±0.02	2.91±0.02	2.50±0.03	2.31±0.03
Nut width	1.82±0.02	1.80±0.01	1.80±0.01	1.46±0.01	1.45±0.02
Fruit stalk length	10.26±0.1	8.19±0.05	7.62±0.07	8.86±0.09	4.21±0.02

DNA isolation

Nine-day-old seedlings of representative samples were frozen in mortar and pestle pre-cooled with liquid nitrogen and grinded to fine powder. Of each sample, 100 mg were transferred immediately into a pre-cooled microcentrifuge tube for DNA extraction with DNeasy plant mini kit (Qiagen cat. No 69104), applying 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 were applied to determine the presence of such contaminations like proteins, polyphenolic compounds, sugars, and lipids. The average amounts of the isolated DNA were 250–300 ng and the above counted contaminations were present in negligible amounts.

Primers

One hundred ISSR primers 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 tested in this study. Owing to the fact that the production of Primer Set #9 was discontinued by UBC–NAPS Unit, the primers were ordered from Metabion International AG, Martinsried, Germany, and upon arrival were dissolved in DNase-free water to 100 mmol final concentration.

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, Cat No K0171) and 22 μL DNase-free water (supplied with the master mix kit). The PCR tubes were placed in TC-512 THERMAL CYCLER (Techne) PCR apparatus and PCR amplification was carried out by using the following program: initial DNA melting at 94 °C – 5 min; next 35 cycles at 94 °C – 1 min; 43/58 °C – 1 min 30 s (the chosen annealing temperatures were 2 °C below the melting temperatures of primers); 72 °C – 2 min 30 s, and a final extension at 72 °C for 6 min. The PCR products were mixed with 7.5 mL of loading dye (Fermentas #R0611), loaded onto 1.5 % agarose gel containing 0.5 mg/ml ethidium bromide (final con-

centration) covered with 1X TAE buffer and separated by applying 3.5 volts per cm of electrical current. The size of the products was determined by comparison with a 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 accompanying software. The amplified unambiguous bands were scored by molecular masses using the program GelPro Analyzer. Next, they were manually allocated to classes of molecular weights for completion of the Boolean matrices for a presence/absence (0/1) of bands with the results of each selected primer.

The binary data were employed to construct rectangular matrices by the PAST ver. 1.89 computer program (Hammer & al. 2001), using Ward's hierarchical method (Ward 1963) based on the unweighted pair group method with an arithmetic mean to generate a dendrogram and describe relationships among the genotypes.

The distances obtained for each of the selected primers were introduced in a new diagonal matrix. All average matrices were summarized to a consequent distance matrix. The results based on genetic distances of the studied species were used to construct a consequent unrooted tree by the T-Rex 3.0a1 software (Makarenkov 2000, University of Quebec in Monreal), using Unweighted Neighbor Joining method. The dendrogram was plotted by the PhyloDraw software ver. 0.82.

Results

Morphological characterization

The identification key for the five studied taxa is based on seven morphological features of completely mature fruits (valves, tubercles, fruits stalk, and nut) in freshly collected specimens (Table 2, Fig. 1).

Identification key of the studied *Rumex* species:

1. Valves without tubercles ..... ***R. alpinus*** L.

1*. Valves with 1 or 3 tubercles ..... **2** (incl. *R. crispus*, *R. kerneri*, *R. conglomeratus*, *R. sanguineus*, *R. hydrolapathum*)

2. Valves entire or with small, irregular marginal teeth up to 0.5–0.8 mm long ..... **3** (incl. *R. stenophyllus*)

2*. Valves clearly dentate, teeth longer than 1 mm ..... ***R. pulcher*** L. (incl. *R. palustris*, *R. maririmus*, *R. obtusifolius*)

3. Fruit stalks longer than 10 mm. Valves with 1 small fusiform tubercle (occasionally absent), width up to 1 mm; mature valves light-brown ..... ***R. patientia*** L.

3*. Fruit stalks mostly shorter than 10 mm. Valves with 1–3 ovoid tubercles, width of the largest one up to 1 mm, mature valves reddish-brown **4**

4. Plants without creeping rhizome. Basal leaves noncordate, without trichomes, after ripening of fruits disappear. Valves with 3 uneven tubercles .... ***R. cristatus*** DC.

4*. Plants with creeping rhizome. Basal leaves deeply cordate, with short trichomes, persistent after fruit ripening. Valves with 1 developed tubercle, often the remaining 2 tubercles smaller ..... ***R. confertus*** Willd.

Selection of ISSR primers

Initially, we tested the ability of each ISSR primer to produce polymorphic bands suitable for distinguishing sections and subsections within the subgenus *Rumex*. For this purpose we studied the whole #9 set of primers with all DNA templates isolated from the five species. The successful amplification of polymorphic PCR products was achieved with ten of the primers at annealing temperature of 55 °C (Fig. 2). The primers that successfully amplified microsatellite regions in the genome of the *Rumex* species are presented in Table 3. They produced between 15 and 26 polymorphic bands ranging between 735 bp and 9750 bp in size (Fig. 2, Table 3).

Table 3. ISSR primers amplified the unambiguous polymorphic PCR products and were used in data analysis. The ranks of significance were obtained after cluster-analysis.

Primer	Primer sequence	Number of polymorphic bands	Fragments, kB (min-max)
p2	**(AC)8AG**	17	1523–5714
p7	**(AC)8GA**	23	1412–9750
p810	**(GA)8T**	23	757–4571
p811	**(GA)8C**	26	1514–5571
p817	**(CA)8A**	15	1350–3929
p826	**(AC)8C**	20	929–326
p836	**(AG)8YA**	17	848–3350
p841	**(GA)8YC**	20	1597 -5250
p857	**(AC)8YG**	18	735–3350
p891	**HVH(TG)7**	20	1589–5188

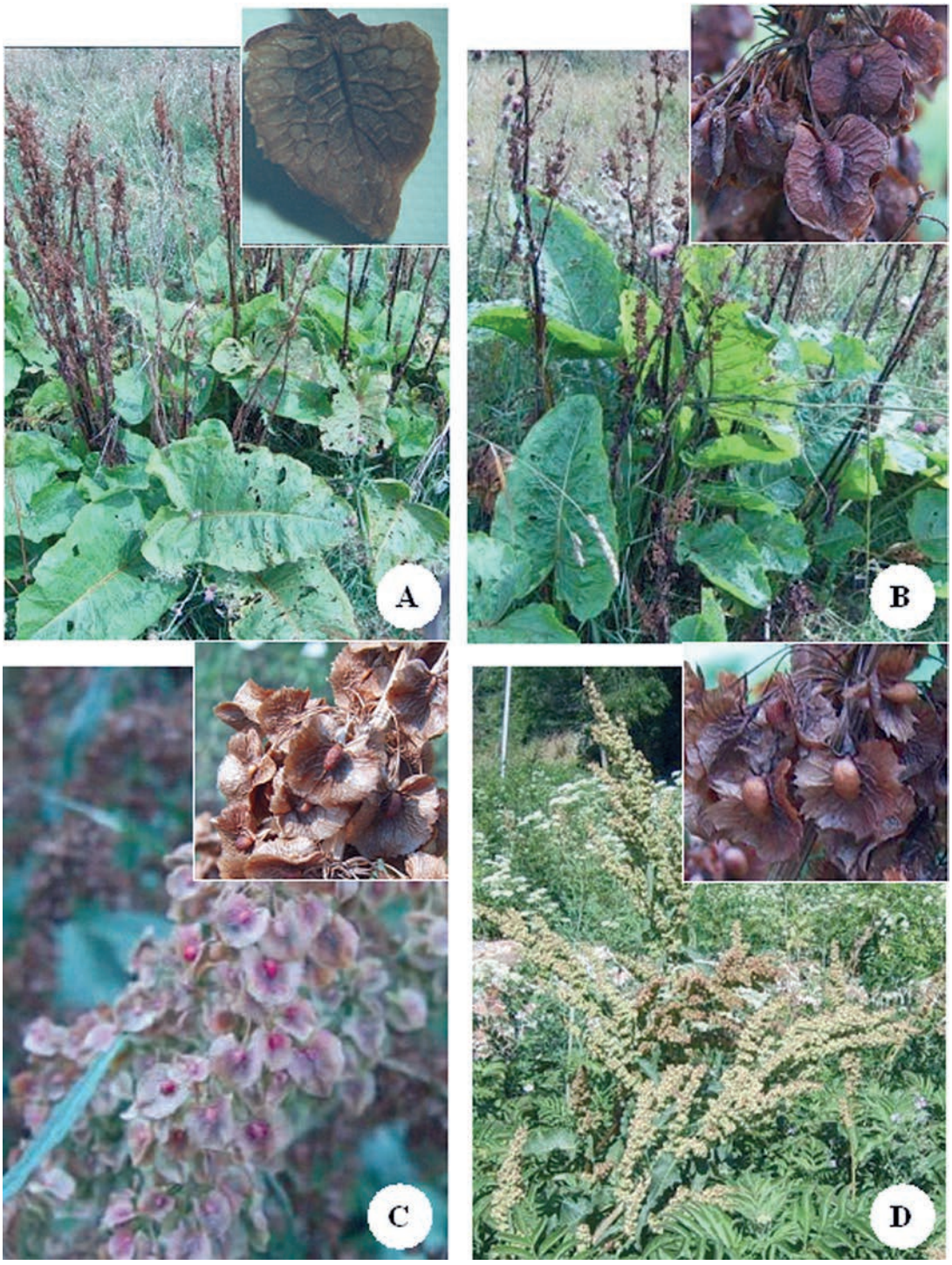

Fig. 1. Habit and mature valves of four morphologically similar Bulgarian species studied with ISSR markers: **A** – *R. alpinus*; **B** – *R. confertus*; C – *R. patientia*; **D** – *R. cristatus*. (Photographs by Raycheva).

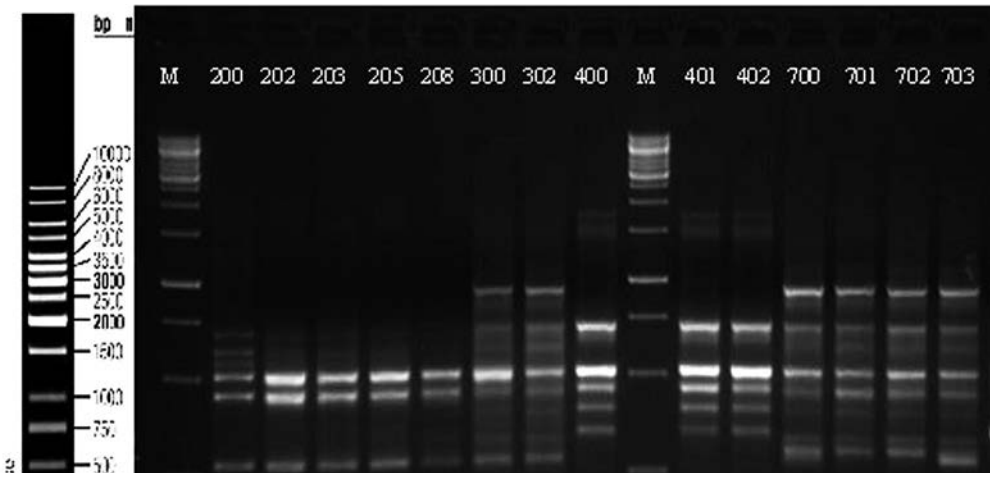

Fig. 2. Amplified polymorphic ISSR bands of 17 samples from five species, generated by using primer p836 – left marker (M). The numbers represent different *Rumex* genotypes, according to Table 1.

Data analysis

Amplified unambiguous bands were scored to compile a presence/absence matrix that was processed with PAST software, in order to build clusters (Fig. 3). Some of the primers like p7, p810 and p811 have not demonstrated significant taxonomic grouping, but were not completely excluded from the consequent analysis because they produced few unique species-specific fragments (Fig. 3 b, c, d).

The polymorphic bands produced by each of the ten primers were scored and employed to construct a consequent diagram that displayed a clear separation of the studied species into two clades (Fig. 4). The first clade clustered together all samples from *R. pulcher* (200, 202, 203, 208, 205) and *R. alpinus* (400, 401, 402). The second clade consisted of samples of *R. patientia* (700, 701, 702, 703), *R. confertus* (900, 901, 902) and *R. cristatus* (300, 302). The samples of *R. patientia* and *R. confertus* were clustered in one subclade, probably because they are genetically closer, while the *R. cristatus* samples were in a separate subclade (Fig. 4).

Discussion

Taxonomists are still interested in the subgenus *Rumex*, especially when the number and rank of taxa are concerned. So far taxonomic literature has accepted the subsectional classification suggested by Rechinger (1932, 1937, 1949a, b, 1964). After his monographic works, the genus *Rumex* has not been subject to comprehensive taxonomic studies.

Some taxonomists (Borodina 1979 a, b) include the subsections *Conferti* Rech. f.; *Aquatici* Rech. f.; *Alpini* Rech. f.; *Crispi* Rech. f.; *Stenophyli* Rech. f. and *Longifolii* Rech. f. into the subgenus *Rumex* (= *Patientiae* Rech.f.).

In the present study, *R. patientia* is always in one cluster whit *R. cristatus* and *R. confertus,* indicating a closer relation with them than with the remaining studied species.

Morphological similarity of the four species is often the reason for their taxonomic misidentification, while ISSR analysis shows that the four species have well-defined genetic profiles.

The natural character of the species group of *R. patientia*, *R. confertus* and *R. cristatus* is supported by phenotypic data. All three taxa have a similar habit and the mature valves are entire, with one developed tubercle (Table 2, Fig. 1). *Rumex confertus* has been treated by some earlier authors as a subspecies of *R. alpinus* (Boissier 1879), due to the shape of basal leaves and the fact that they are preserved throughout the growing season (Fig. 1, A and B). Along with this, the main morphological features that distinguish *R. confertus* from its close relatives are: presence of trichomes on the lower leaf surface; well developed one, seldom three tubercules on the fruit valves (Table 2). Specimens from *R. cristatus* have been wrongly identified as *R. patientia* in some of the deposited collections (sub *R. patientia*, Achtarov, SOM 17794, N. Stoyanov, SOM 92344 and Achtarov, SOM 100165). The reasons for this are the similar morphological and numerical features of the valves and the fruits (Table 2), similar habit and ruderal habitats occupied by the two taxa.

A study of the numerical strength of the populations of *R. confertus* in Central Europe describes the species as an invasive and aggressive weed (Jehlik & al. 2001, Stosik 2006). Its distribution in Bulgaria has been recently confirmed by Raycheva & Dimitrova

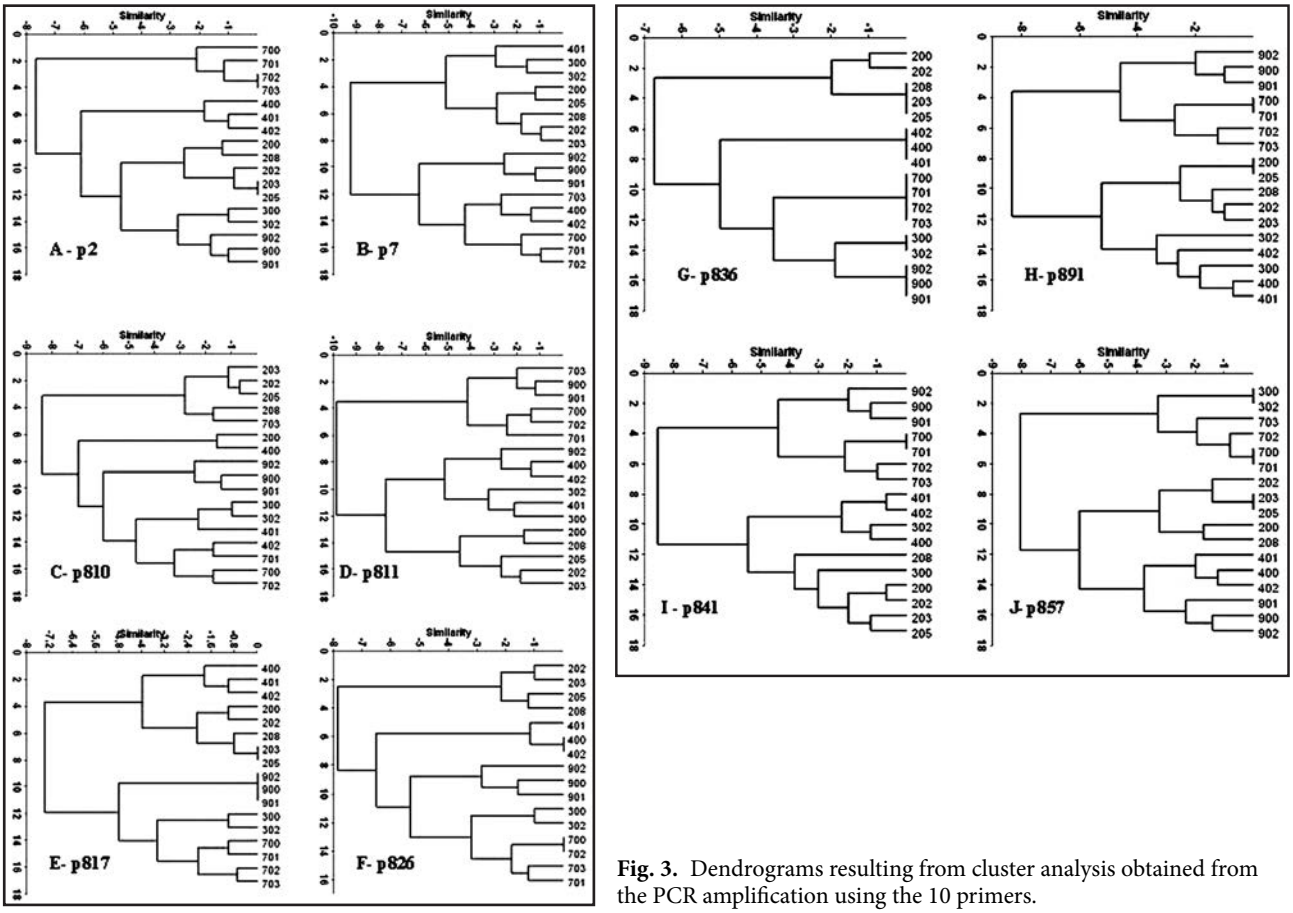

Fig. 3. Dendrograms resulting from cluster analysis obtained from the PCR amplification using the 10 primers.

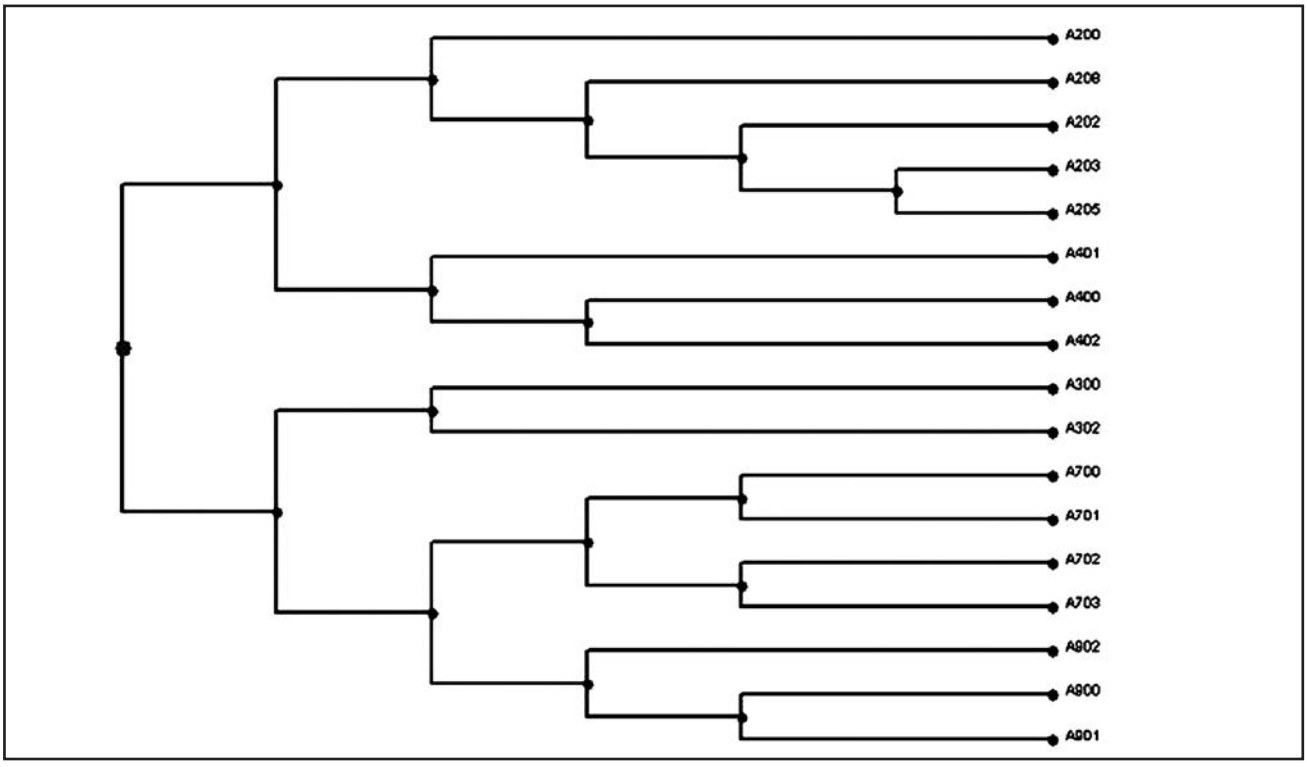

Fig. 4. Consequent diagram generated by Ward's cluster analysis calculated from ISSR bands produced by 10 primers of 17 samples, representing the genetic distance of five Bulgarian species of the subgenus *Rumex*, visualized by PhyloDraw.

(2007). According to the used primers, the applied method distinguishes the samples of *R. confertus* geographically. The two different clusters correspond to two different genotypes. The cluster from W Bulgaria includes the samples from Tran town, (902), both in the intermediate (Fig. 3) and in the final general dendrogram (Fig. 4), while the other cluster unites the two samples from Razgrad (NE Bulgaria) and Montana towns (NW Bulgaria).

Samples 400 (Rhodopi Mts), 401(Mt Vitosha) and 402 (Rila Mts) belong to *R. alpinus*. After using of the 10 different primers, the samples group together, despite their different geographical origin. The populations of *R. alpinus* has fallen into a separate clade, which is genetically more distant from the remaining species (Fig. 4). This result correlates with its ecological specialization: the species is adapted to alpine conditions. The molecular, ecological and morphological data received at that stage confirm the divergent position of *R. alpinus*, and support its classification in the monotype subsection *Alpini* Rech. f.

So far, the morphological characteristics, chromosome number and molecular data from the present study do not provide enough evidence that *R. confertus* has to be classified in a separate subsection *Conferti* (Rechinger 1949a). On the contrary, most of the used primers show that *R. confertus* belongs to one and the same clade with *R. patientia* and *R. cristatus*. (Fig. 3: p 836, 826, 817, 891). Additional statistical support to the genetic structure of the group is evident from the generalized cladogram (Fig. 4).

On the basis of the morphological features, genetic similarities and chromosome numbers the studied species can be classified into two groups: Group 1 comprises the polyploid species *R. patientia* ($2n = 60$), *R. confertus* ($2n = 100$) and *R. cristatus* ($2n = 80$), whereas Group 2 contains the diploid species *R. alpinus* ($2n = 20$) and the referent species *R. pulcher* ($2n = 20$). *Rumex pulcher* has proved to be genetically most distant, which corresponds to its morphological distinctions too. In the current study some samples of *R. patientia* (number 700–703) and *R. pulcher* (samples with numbers 200–208) show greater dispersion, which probably indicates a wider variation range, due to infraspecific variation and hybridization typical for the European species from section *Rumex* (Rechinger 1932, 1990).

The selected primers reveal polymorphism between the studied species. Most distinctive is primer 836 (Fig. 3 G). The selectiveness of the primer generates specific bands which clearly distinguish five clades corresponding to the five studied species. Therefore, primer 836 can be considered the most successful in species distinction in the type subgenus *Rumex*.

Taxonomic relations between the remaining members of the subgenus will be clarified in the course of future molecular studies of the Bulgarian species of subgenus *Rumex*.

Conclusion

The ISSR method is appropriate as a source of objective information for taxonomic analysis of wild populations of the genus *Rumex* subg. *Rumex*. The species differentiation in the subgenus (generation of species-specific bands) is successful when using a small number of primers, among which most successful are primers 891, 857, 836, and 817. The results with primer 891 are most consistent in species identification, but it is not equally selective when geographical origin is concerned. Similar ISSR profiles of the studied species were obtained with primers 810 and 811. All results, with minor exceptions, are consistent with the existing morphological classification. Our molecular studies have provided objective and reliable demonstration of the phylogenetic relationships of the Bulgarian species of subgenus *Rumex*.

ISSR data may supply more accurate information with high level of reliability about the relationships between different groups of the *Rumex* species, especially when morphological variability, natural hybridization and speciation are concerned. That is why we consider it appropriate to expand the molecular studies of natural populations of subgenus *Rumex* in Bulgaria, so as to clarify the phylogenetic relationships and the degree of genetic variability.

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