# Evolutionary patterns and morphological diversification within the European members of the *Euphorbia illirica* (*E. villosa*) group: one or several species?

**Evoluce a morfologická diferenciace evropských zástupců okruhu *Euphorbia illirica* (*E. villosa*) – jeden nebo více druhů?**

Božo Frajman[1], Maja Graniszewska[2] & Peter Schönswetter[1]

[1]*Institute of Botany, University of Innsbruck, Sternwartestraße 15, A-6020 Innsbruck, Austria, e-mail: bozo.frajman@uibk.ac.at;* [2]*Faculty of Biology, University of Warsaw, Ilji Miecznikowa 1, 02-096 Warsaw, Poland*

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The Alps and the Carpathians are important centres of plant endemism in Europe, but there are fewer phylogenetic studies on the patterns in biodiversity of Carpathian biota than there are for the Alps. Here, we use nuclear ribosomal ITS, the plastid *trn*T–*trn*F region and amplified fragment length polymorphism (AFLP) fingerprinting to determine the phylogenetic position of and relationships within the *Euphorbia illirica* group and determine the biogeographic links between the Alps and the Carpathians. In addition, we use morphometric data to re-evaluate the controversial taxonomic status of several endemic taxa belonging to this group. ITS and AFLP data indicate that *E. austriaca*, *E.* "*beskidensis*", *E. carpatica*, *E. semivillosa*, *E. sojakii* and *E. illirica* (*E. villosa*) are members of the *E. illirica* group and *E. palustris* is their sister, whereas in the plastid dataset *E. palustris* is nested within the *E. illirica* group. Additionally, AFLP data indicate a genetic split into two geographical groups, one including Carpathian populations and the other comprising all other populations. The split thus supports the role of the Carpathians as an important Pleistocene refugium, but does not offer support for traditionally recognized taxa within the group. Moreover, the previously suggested biogeographic link between the Alpine *E. austriaca* and the Carpathian *E. sojakii* is not supported by molecular data. Instead, it appears likely that the similar morphology of subalpine populations in the *E. illirica* group developed in parallel in both genetic groups, in *E. austriaca* in the north-eastern Alps and independently in the Carpathian high altitude taxa. Morphometric analyses show strong overlap both among the taxa and between the two genetic groups, which, in connection with the morphological plasticity of the group, prevents recognition of morphologically identifiable evolutionary units. It thus seems reasonable to treat the members of this group as a single polymorphic species, *E. illirica,* following the concept proposed in Flora Europaea.

K e y w o r d s: AFLP, Carpathians, Eastern Alps, endemic species, *Euphorbia* sect. *Helioscopia*, Europe, ITS, multivariate morphometrics, taxonomy

## Introduction

The Alps and the Carpathians are important centres of plant endemism in Europe (Pawłowski 1970, Davis et al. 1994, Finnie et al. 2007). Whereas the Alps are among the best studied mountain systems in the world (Tribsch & Schönswetter 2003) and several studies in the past decade have addressed different aspects of plant diversification in this part of Europe (e.g. Schönswetter et al. 2005, Alvarez et al. 2009, Taberlet et al. 2012),

much less is known about the processes that have contributed to the high levels of plant diversity and endemism in the Carpathians (reviewed by Ronikier 2011). A common phylogeographic pattern observed in several plant groups is a close genetic link between populations in the Eastern Alps and the Carpathians (Kropf et al. 2003, Fér et al. 2007, Paun et al. 2008, Slovák et al. 2012), which earlier was acknowledged on the basis of patterns in the distribution of species (Pawłowski 1928, Meusel et al. 1978, Meusel & Jäger 1992). In other plant groups, however, Carpathian and Alpine populations are only distantly related (Frajman & Oxelman 2007, Puşcaş et al. 2008) and Carpathian populations have closer phylogeographic links with the mountains of the Balkan Peninsula (Ronikier 2011).

High levels of endemism and species diversity are attributes commonly used in conservation biology (Médail & Quézel 1999) for defining hotspot areas at a global scale (Myers et al. 2000), as well as for designating protected areas at national and regional scales (Rabitsch & Essl 2009). At the intraspecific level, centres of plant endemism correspond well with areas harbouring genetically divergent populations, but not necessarily with areas of high intrapopulation genetic diversity, in the Alps (Tribsch & Schönswetter 2003, Taberlet et al. 2012). This underlines the importance of endemics as criteria for decision-making in nature conservation. Unfortunately, the delimitation of endemic taxa often relies exclusively on traditional, morphology-based taxonomic concepts, and has often not been assessed using molecular phylogenetic approaches. Even more important, minor regional morphological differences, which were highlighted without taking the variability of populations from the entire distribution area into account, have sometimes served as a basis for a description of endemic taxa that were later shown to be neither phylogenetically nor morphologically supported (Schönswetter et al. 2004, Frajman & Oxelman 2007, Schönswetter et al. 2009, Bardy et al. 2011, Caković et al. 2015).

The *Euphorbia illirica* group includes sturdy, about one meter high rhizomatous perennials growing in western Eurasian lowland wetlands, forest clearings and montane to subalpine tall herbaceous plant communities (Meusel et al. 1978). The species belonging to this group are diploids with 2n = 20 (Frajman & Schönswetter 2011); the count 2n = 36 published for *E. illirica* (as *E. villosa*; Benedi & Blanché 1992) might rather relate to another species, for instance *E. pilosa*, for which 2n = 32 is reported (Graniszewska 2007, Frajman & Schönswetter 2011). The *E. illirica* group is an ideal system for studying the biogeographic links between the Alps and the Carpathians and for re-evaluating the controversial taxonomic status of several endemic taxa. Whereas in Flora Europaea *E. austriaca*, *E. carpatica*, *E. semivillosa* and *E. tauricola* are listed in synonymy to *E. illirica* (Smith & Tutin 1968), most other authors recognize them as independent species belonging to the *E. illirica* group. Govaerts et al. (2000), Geltman (2009), Barres et al. (2011) and Riina et al. (2013) acknowledge that the name *E. illirica* has priority over the better known name *E. villosa*. Therefore, Frajman (2014) proposes the rejection of the former to permit the continued use of *E. villosa*, but the Nomenclature Committee for vascular plants rejected this proposal (Applequist 2016), therefore we use the name *E. illirica* for this species. Polatschek (1971), who revised the central-European members of the *E. illirica* group, defines this group broadly and includes *E. palustris* along with *E. austriaca*, *E. carpatica* and *E. illirica*. This was followed by Greuter et al. (1986), who include *E. illirica* (with *E. semivillosa* in synonymy), *E. palustris* and *E. tauricola* in the same group. Meusel et al. (1978) and Geltman (2009) do not include *E. palustris* in their

circumscriptions of the *E. illirica* group. Meusel et al. (1978), however, include, alongside *E. illirica* distributed from the south-western Pontic area to the Atlantic coasts of Spain and France, other low altitude taxa such as *E. semivillosa* (south-western Pontic area to western Kazakhstan) and *E. valdevillosocarpa* (north-western coast of the Black Sea). In addition, they include high altitude taxa such as the geographically restricted *E. austriaca* (north-easternmost Alps and western Carpathians), *E. carpatica* (north-eastern Carpathians) and *E. tauricola* (Crimea). *Euphorbia austriaca* has been further subdivided into subsp. *austriaca* (north-eastern Alps) and subsp. *sojakii* (Western Carpathians; Chrtek & Křísa 1970, Polatschek 1971, Meusel et al. 1978); the latter is often considered to be a separate species (e.g. Čeřovský et al. 1999, Geltman 2009). Govaerts et al. (2000) treat *E. carpatica* and *E. sojakii* as independent species, but consider *E. austriaca*, *E. tauricola* and *E. semivillosa* conspecific with *E. illirica*, recognizing *E. semivillosa* as an independent subspecies. Alongside the seven above-mentioned taxa Geltman (2009) includes *E. procera* from the Caucasus in the *E. illirica* group and suggests that *E. semivillosa* and *E. valdevillosocarpa* should be considered to be subspecies of *E. illirica*, whereas all others deserve specific rank. Graniszewska (2007) studied the morphological differentiation of the Carpathian taxa and *E. austriaca*, and concluded that *E. austriaca* is well differentiated from the Carpathian populations, whereas within the Carpathians only *E. carpatica,* with four subspecies, forming a geographic sequence from north-west to south-east, should be recognized: *E. carpatica* "subsp. *beskidensis*", "subsp. *sojakii*", subsp. *carpatica* and subsp. *jasiewiczii*. The first was never validly described and the second proposed combination never validly published.

Prokhanov (1949) includes *E. illirica* with 13 additional taxa in *E.* sect. *Helioscopia* subsect. *Lutescentes* Prokh., but recent phylogenetic studies (Barres et al. 2011, Frajman & Schönswetter 2011, Riina et al. 2013) have shown that this subsection is polyphyletic, whereas this section as currently circumscribed (Riina et al. 2013) is monophyletic. According to Meusel et al. (1978) and Polatschek (1971) *E. palustris* and allied taxa (e.g. *E. ceratocarpa*, *E. soongarica*, *E. velenovskyi*) are closely related to the narrowly circumscribed *E. illirica* group, but *E. ceratocarpa* and *E. velenovskyi* do not belong to the *E. illirica – E. palustris* alliance (Frajman & Schönswetter 2011, Riina et al. 2013). Within that alliance, ITS sequences reveal that *E. alpina*, *E. aristata*, *E. lamprocarpa*, *E. palustris*, *E. pilosa*, and *E. procera* are closely related to the *E. illirica* group (comprising *E. austriaca*, *E. carpatica*, *E. illirica*, *E. semivillosa*, *E. sojakii* and *E. valdevillosocarpa*); relationships within the latter are unresolved. On the contrary, *E. palustris* is positioned within the *E. illirica* group in the plastid *trn*T–*trn*F phylogeny (Frajman & Schönswetter 2011), whereas the relationships within this group and with the outgroup taxa are unresolved in a plastid *ndh*F phylogeny (Riina et al. 2013). However, in both studies, with the exception of *E. semivillosa,* only a single accession per taxon was used, rendering the conclusions on the interspecific relationships unreliable. In addition, the exact phylogenetic position of the *E. illirica* group remains unclear.

The main aim of this study is to determine the phylogenetic history of the European members of the *E. illirica* group. To this end, we generated sequences of nuclear ribosomal ITS and the plastid *trn*T–*trn*F region as well as amplified fragment length polymorphisms (AFLP) for a taxonomically and geographically broad population sample. In addition, we aimed to reassess the morphological diversification within this group. In particular, we (i) address the question of monophyly of the group and its position

within *Euphorbia* sect. *Helioscopia* using available and new sequence data. Further, (ii) we explore the relationships between the widely distributed, low altitude *E. illirica* and the regionally endemic, high altitude taxa. Finally, (iii) we intersect the genetic results with patterns of morphological differentiation, and (iv) based on our results suggest a taxonomic treatment of members of the *E. illirica* group.

## Materials and methods

### *Plant material*

Twenty-five populations of European taxa belonging to the *E. illirica* group were sampled for the genetic analyses (Electronic Appendix 1, Fig. 1). Leaf material of one to five individuals per population was collected and immediately stored in silica gel. We considered all previously recognized taxa including the not validly published *E. carpatica* "subsp. *beskidensis*" (Graniszewska 2007) as potentially independent taxonomic entities. For simplicity, we treat all entities at the species level (*E. austriaca*, *E.* "*beskidensis*", *E. carpatica*, *E. semivillosa* and *E. sojakii*). Populations 24 and 25 were collected in the vicinity of the locus classicus of *E. sojakii* and *E. carpatica*, respectively. In the case of *E. austriaca* the locus classicus lies between populations 15 and 16. For *E. illirica* the type locality is not clear (see Frajman 2014), but two populations were included from Hungary and one from adjacent Austria, the region, where the type locality of *E. villosa* should be situated (see Taxonomic treatment). The Carpathian endemic *E. jasiewiczii* was not included in our study. All species were determined based on their morphology and distribution; for *E. austriaca* Fischer et al. (2008) for *E. semivillosa* Prokhanov (1949) and for the Carpathian taxa Graniszewska (2007) were used. Population 12 of *E. illirica* from Bosnia and Herzegovina morphologically resembles *E. austriaca*, but we assigned it to *E. illirica* based on its geographic origin. Nomenclature follows Riina et al. (2013) and Govaerts et al. (2000).

Sequences of *E. procera*, *E. semivillosa* and *E. valdevillosocarpa*, and several outgroup taxa from *Euphorbia* sect. *Helioscopia* were obtained from previous studies (Steinmann & Porter 2002, Kryukov et al. 2010, Frajman & Schönswetter 2011, Riina et al. 2013). The ITS sequence of *E. illirica* HQ900616 from Barres et al. (2011) and *E. semivillosa* GU979430 and *E. valdevillosocarpa* GU979431 from Kryukov et al. (2010) were not included in the analyses as they contained several nucleotide polymorphisms (not coded in the sequences uploaded to GenBank) that are not recorded in other studies of the *E. illirica* group and may have originated from contamination (original chromatograms were kindly provided by L. Barres and R. Riina). These GenBank accessions should therefore be treated as doubtful. Voucher data and GenBank numbers of the accessions used in our study are presented in Electronic Appendices 1 and 2. Among the 28 ingroup populations (*E. illirica* group, *E. palustris*) ITS and plastid sequences were newly produced for 22 populations, whereas the other sequences were from previous studies. One individual per population was sequenced.

For morphological analyses herbarium specimens of 254 individuals from 111 localities (including most of the genetically studied populations) were investigated. The sample comprised 78 individuals from 36 localities (78/36) of *E. austriaca*, 23/9 of *E.* "*beskidensis*", 26/11 of *E. carpatica*, 75/44 of *E. illirica*, 3/3 of *E. semivillosa* and 49/8 of *E. sojakii*;

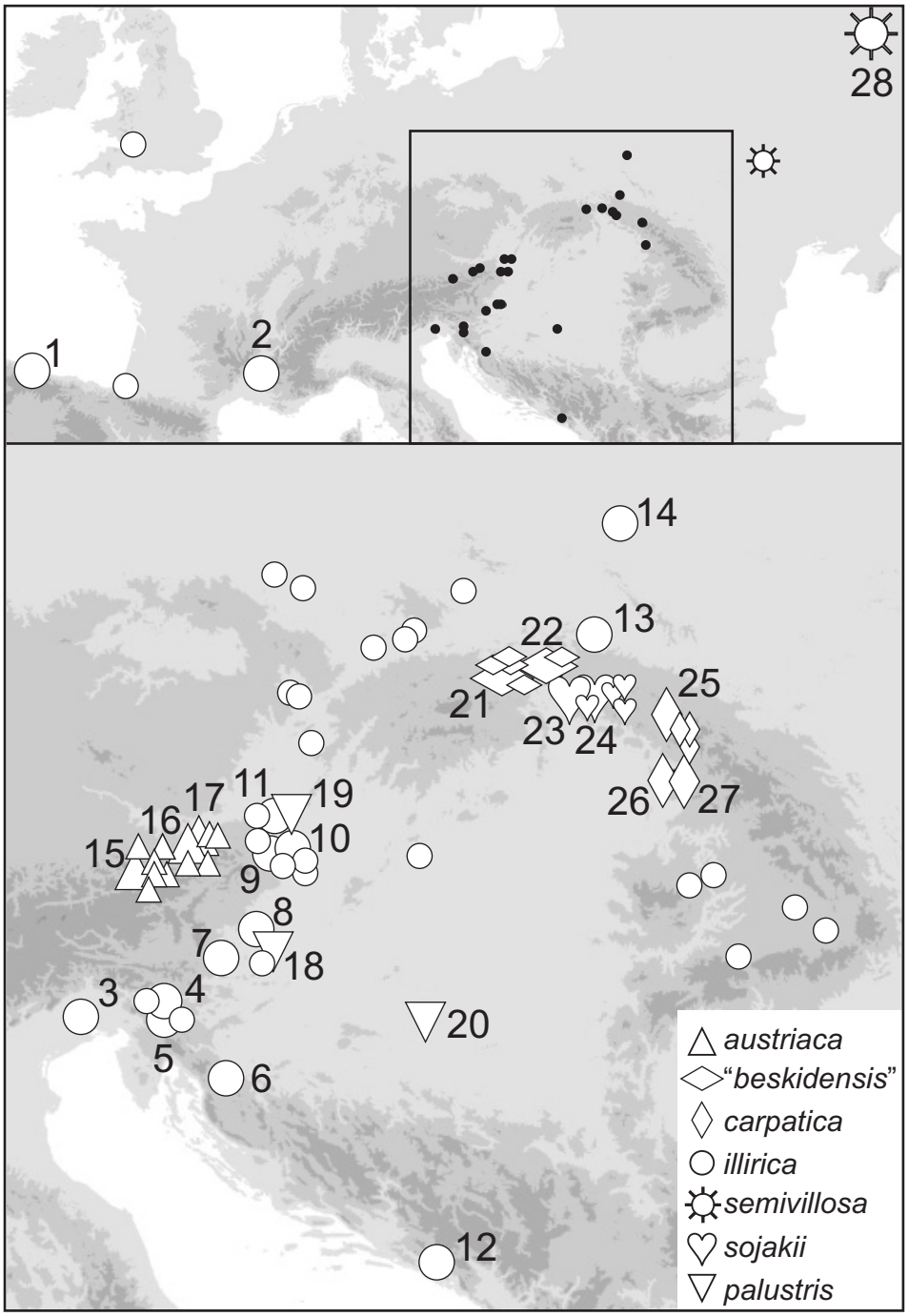



Fig. 1. – Populations of the *Euphorbia illirica* group and three of *E. palustris* that were sampled and used in the genetic analyses (large symbols; their numbers correspond to Electronic Appendix 1), supplemented with additional populations used in the morphometric analysis (small symbols without numbers).

several localities were geographically close, corresponding to a single genetically investigated population (Electronic Appendix 3, Fig. 1).

*Laboratory work*

DNA isolation, PCR and sequencing of ITS and *trn*T–*trn*F were performed as described by Frajman & Schönswetter (2011). The AFLP procedure followed Vos et al. (1995) with the modifications described in Schönswetter et al. (2009). An initial screening of selective primers using 12 primer combinations with three nucleotides was performed. The three final primer combinations for the selective PCR (fluorescent dye in brackets) were EcoRI (6-Fam)-ACA/ MseI-CAC, EcoRI (VIC)-AGG/ MseI-CTG, EcoRI (NED)-ACC/ MseI-CAT. One to five individuals per population from 28 populations totalling 113 individuals were analysed (Electronic Appendix 1). Samples (5 µl) of each selective PCR product were purified as described in Schönswetter et al. (2009); 1.2 µl of the elution product was combined with 10 µl formamide and 0.1 µl GeneScan ROX (Applied Biosystems, Foster City, CA, USA) and separated on an ABI 3130xl Genetic Analyzer automated capillary sequencer (Applied Biosystems).

*Contig assembly, sequence alignment and phylogenetic analyses*

Contigs were assembled and edited using Staden (Staden et al. 1998). Base polymorphisms in the ITS sequences were coded using NC-IUPAC ambiguity codes. Sequences were manually aligned using QuickAlign (Müller & Müller 2003). Ten positions of a polymorphic poly-T region (positions 1593–1602 in the original alignment) were removed from the plastid alignment due to the high degree of homoplasy of single-nucleotide repeats over large geographical scales (Ingvarsson et al. 2003, Vachon & Freeland 2011). Gaps (indels) were coded for the plastid alignment as binary characters using SeqState version 1.25 (Müller 2005) applying simple gap coding (Simmons & Ochoterena 2000), adding 55 binary characters to the alignment.

Maximum parsimony (MP) analyses as well as maximum parsimony bootstrap (MPB) analyses of both data sets were performed using PAUP 4.0b10 (Swofford 2002). The most parsimonious trees were searched for heuristically using 1000 replicates of random sequence addition, TBR swapping and with the MulTrees option on. All characters were equally weighted and unordered. The data set was bootstrapped using full heuristics, 1000 replicates, TBR branch swapping, with the MulTrees option off and a random addition sequence with five replicates. Based on previous studies *Euphorbia helioscopia*, *E. hirsuta* and *E. pterococca* were used as outgroups for rooting the trees (Frajman & Schönswetter 2011, Riina et al. 2013). No analyses of combined datasets were performed due to strong incongruences between both trees, for instance in the position of *E. palustris*.

Bayesian analyses were performed employing MrBayes 3.1 (Ronquist & Huelsenbeck 2003) applying the substitution models proposed by the Akaike information criterion implemented in MrAIC.pl 1.4 (Nylander 2004; Table 1). The plastid data was partitioned into a nucleotide set and an indel set; the latter was treated as morphological data according to the model of Lewis (2001). Values for all parameters, such as the shape of the gamma distribution, were estimated during the analyses. The settings for the Metropolis-coupled Markov chain Monte Carlo process included four runs with four chains each (three heated ones using the default heating scheme), run simultaneously for 10,000,000 generations

Table 1. – Statistics of the parsimony analyses of the two DNA regions analysed and substitution models proposed by MrAIC and used in the Bayesian analyses.

| Region | *trn*T–*trn*F | ITS |
|---|---|---|
| Number of terminals | 104 | 54 |
| Number of included characters | 1816 | 713 |
| Number / percentage of parsimony informative characters | 45 / 2.5% | 98 / 13.7% |
| Length of MP trees | 137 | 321 |
| Consistency index (CI; excluding uninformative characters) | 0.905 (0.783) | 0.701 (0.566) |
| Retention index (RI) | 0.937 | 0.828 |
| Substitution model | GTR+Γ | SYM+Γ |

each, sampling trees every 1000th generation using default priors. The posterior probability (PP) of the phylogeny and its branches was determined from the combined set of trees, discarding the first 1001 trees of each run as burn-in.

Plastid sequence data were analysed using statistical parsimony as implemented in TCS (Clement et al. 2000) with the connection limit set to 95, gaps were treated as a fifth character state. For this analysis, indels longer than 1 bp were reduced to single base pair columns, which allowed those structural mutations to be counted as single base pair mutations. Nested indels were coded as missing data.

*AFLP analyses*

Raw data were collected and aligned with the internal size standard using ABI Prism GENESCAN analysis software 3.7.1 (Applied Biosystems). Subsequently, the GeneScan files were imported into GENOGRAPHER ver. 1.6.0 (version no longer available) for scoring of the fragments. Each AFLP fragment was scored using the 'thumbnail' option, which allows the signal of each fragment over all samples to be compared. The results of the scoring were exported as a presence/absence matrix.

For all populations with at least three samples, Nei's (1987) gene diversity index and frequency down-weighed marker values (DW; Schönswetter & Tribsch 2005) as an estimate of rarity were calculated using the R script AFLPdat (Ehrich 2006). DW was calculated for individuals and population values correspond to the average of the individual values ("rarity 1"). A Neighbor-Joining (NJ) analysis of the complete AFLP dataset (113 individuals) based on Nei-Li genetic distances (Nei & Li 1979) was conducted and bootstrapped (1000 pseudoreplicates) with TREECON v.1.3b (Van de Peer & De Wachter 1997). Additionally, a non-model based approach, non-hierarchical K-means clustering (Hartigan & Wong 1979), was chosen and performed for 109 individuals excluding *E. palustris* (which was clearly in the outgroup in the NJ tree) using a script of Arrigo et al. (2010) in R. We performed 50,000 independent runs (i.e. starting from random points) for each assumed value for K clusters ranging from 2 to 10. We followed the approach of Evanno et al. (2005) to identify the optimal number of clusters. For the same dataset a NeighborNet diagram was produced with SplitsTree 4.12 (Huson & Bryant 2006) from a matrix of uncorrected P distances.

*Morphometric analyses*

Material for morphometric analyses included 254 individuals from 111 localities, including one to 14 individuals per locality (Electronic Appendix 3, Fig. 1). In a preliminary study (Graniszewska 2007) 57 morphological characters were studied in the *E. illirica* group, of which only 11 proved to be discriminatory for these taxa and were thus used in this study. The only exception was capsule size, which could not be determined as most vouchers of the molecularly investigated populations did not have mature fruits. The 10 scored characters and one ratio character are listed in Table 2. In 17 cases we were not able to score the character state; those states were replaced with the population means (14 cases), or in the case of the single individual per population with the species mean (three cases).

Correlation among metric characters was tested using Pearson or Spearman correlation coefficients dependent on character distribution. Length and width of leaves, ray leaves and raylet leaves were strongly correlated, therefore the characters 1, 2, 5 and 7 (Table 2) were omitted from further analyses and instead the ratio of leaf length and width was included. We produced boxplot diagrams for quantitative characters and histograms for the qualitative and semi-quantitative (ordinal) characters in order to explore the variation among the taxa and between the two AFLP groups. Individuals from populations not included in the genetic analyses were assigned to the two AFLP groups based on geographic proximity, thus including all Carpathian samples in the Carpathian group. Additionally, a principal component analysis (PCA) was performed to explore the relative position of taxa and genetic groups. Moreover, a canonical discriminant analysis (CDA) was performed to test whether the two AFLP groups can be defined morphologically and to clarify the relative importance of characters as discriminators between the two groups. The qualitative multistate character 11 (Table 2) was excluded from both PCA and CDA analyses. All analyses were performed using IBM SPSS Statistics (version 21).

Table 2. – Morphological characters studied in the *Euphorbia illirica* group.

| | Character | Abbreviation |
|---|---|---|
| 1 | Leaf length (mm) | LL |
| 2 | Leaf width (mm) | LW |
| 3 | Ratio of leaf length and leaf width | LL/LW |
| 4 | Ray leaf length (mm) | RyLL |
| 5 | Ray leaf width (mm) | RyLW |
| 6 | Raylet leaf length (mm) | RLL |
| 7 | Raylet leaf width (mm) | RLW |
| 8 | Terminal ray length (mm) | RL |
| 9 | Stem hair density: glabrous (1), few hairs (2), sparse (3), dense (4) | SHD |
| 10 | Capsule hair density: sparse (1), moderate (2), dense (3), very dense (4) | CHD |
| 11 | Capsule appendages: smooth (1), minutely tuberculate (2), hemispherical (3), cylindrical (4), crested (5) | CA |

## Results

### *Phylogenetic relationships*

The number of terminals, included characters, parsimony informative characters, percentage of parsimony informative characters, number and lengths of MP trees, consistency and retention indices for both DNA regions, as well as the model of evolution proposed by MrAIC and used in MrBayes analyses are presented in Table 1.

The ITS sequences of the ingroup taxa (*E. illirica* group including *E. palustris*) contained zero (11 accessions) to three (one accession) polymorphic positions; nine accessions contained one polymorphism and seven contained two polymorphisms. Most of the polymorphisms were autapomorphic. Those shared by more individuals were mostly stochastically distributed, whereas in two cases they were shared by geographically close populations and in one case they were restricted to *E. palustris*. In the ITS tree (Fig. 2) the accessions of *E. austriaca*, *E.* "*beskidensis*", *E. carpatica*, *E. illirica*, *E. semivillosa* and *E. sojakii* formed a monophyletic clade with strong support (96% MPB, PP 1), which from here on is referred to as the *E. illirica* group, but its internal relationships were unresolved. The sister of the *E. illirica* group is *E. palustris* (63% MPB, PP 1); further closely related species are *E. alpina*, *E. pilosa* and *E. procera*, all included in a clade with strong support (91% MPB, PP 1). Consecutive sisters of the before-mentioned clade are *E. aristata* and *E. lamprocarpa* (86% MPB, PP 1), whereas the relationships to other taxa of *Euphorbia* sect. *Helioscopia* remain unresolved.

In the *trn*T–*trn*F tree (Fig. 3, Electronic Appendix 4) *E. palustris* was nested within the *E. illirica* group with strong support (96% MPB, PP 1). In a large polytomy containing haplotypes H1–H7, three clades with intermediate support were resolved; accessions of *E. palustris* were positioned in two of them. The statistical parsimony network (Fig. 3) illustrates the relationships among the haplotypes of the *E. illirica* group and *E. palustris*, their frequencies and the presence of alternative connections. Most of the main haplotype groups have wide distributions; only haplotypes H10–H12 are more restricted, occurring in the SE Alps and northern Dinarides (Fig. 3B).

### *AFLP fingerprinting*

We scored 320 AFLP fragments ranging from 79 to 545 base pairs. The error rate, based on ten replicated samples, amounted to 1.1% based on phenotypic comparisons. After exclusion of 19 unrepeatable fragments, we ended up with 301 AFLP fragments, of which 72 (23.9 %) were monomorphic or lacking in only a single individual. These fragments were excluded from further analyses.

Genetic diversity ranged from 0.05 in population 5 to 0.11 in population 24, and DW ranged from 1.05 in population 17 to 3.66 in population 12 (Electronic Appendix 5). A comparison between populations from the Alps and adjacent areas on the one hand and the Carpathians and adjacent areas on the other (i.e. excluding populations 1 from Spain and population 12 from Bosnia and Herzegovina) revealed no differences in DW (t-test, $T = -1.84$, $df = 18$, $P = 0.082$), but a significantly higher genetic diversity in the Carpathians ($T = -3.13$, $df = 18$, $P = 0.006$).

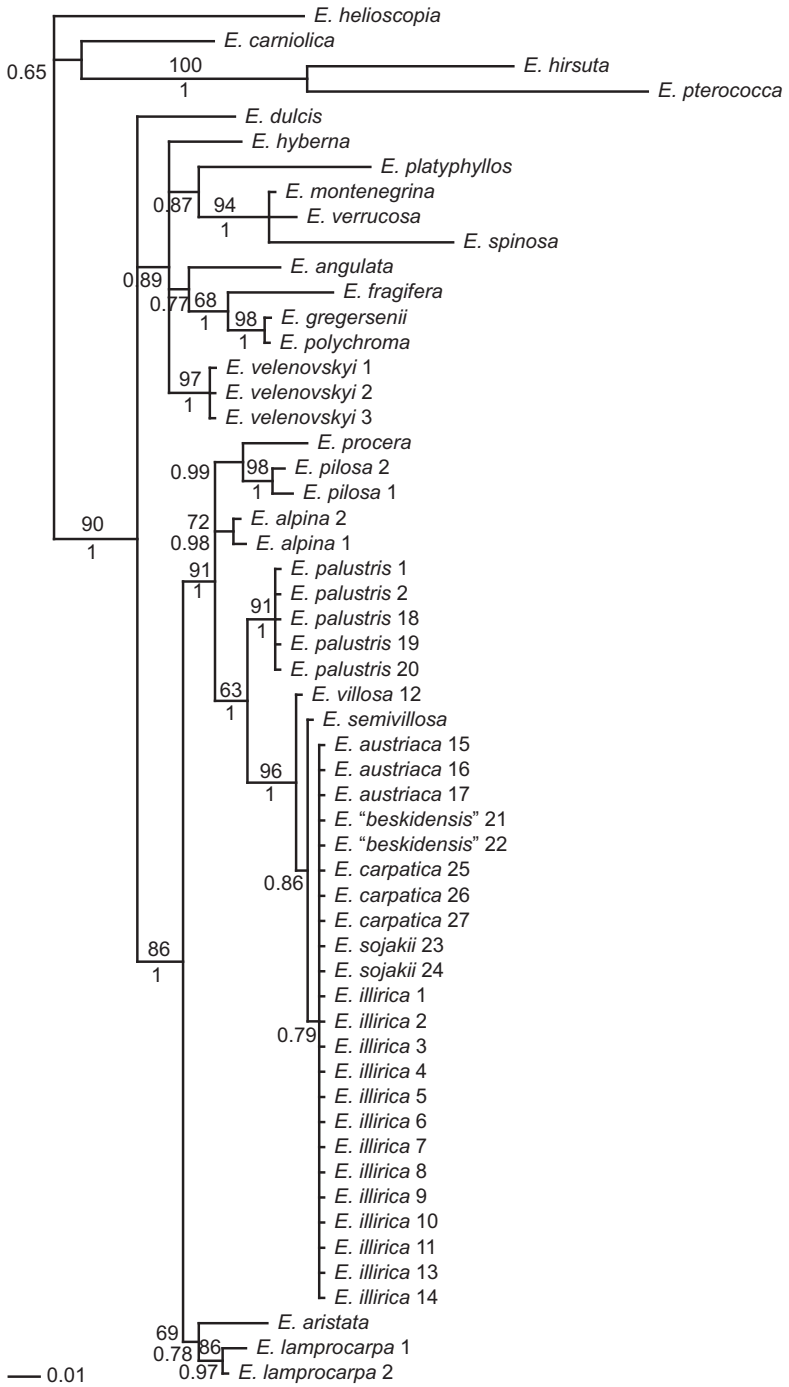



Fig. 2. – Bayesian consensus phylogram of ITS sequences retrieved from accessions of the *Euphorbia illirica* group and outgroup taxa from *Euphorbia* sect. *Helioscopia*. Numbering of multiple accessions per taxon corresponds to Electronic Appendices 1 and 2. Numbers above branches are maximum parsimony bootstrap values > 50%, those below branches posterior probabilities > 0.50.

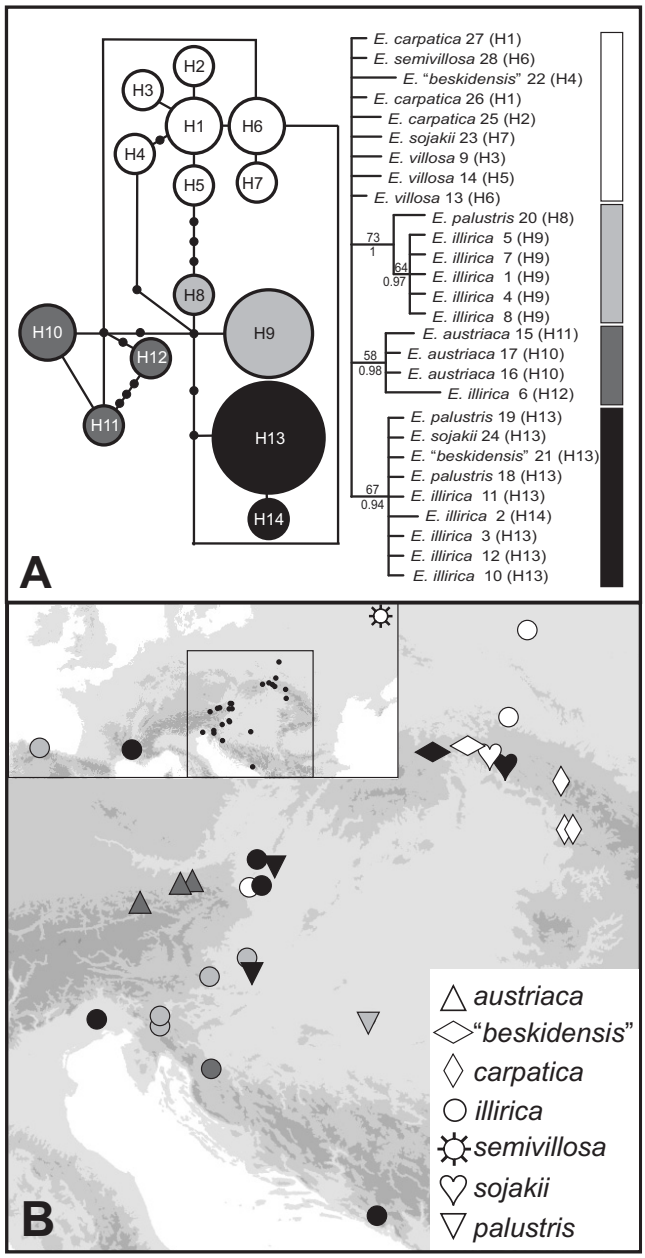



Fig. 3. – Phylogenetic reconstructions of relationships among plastid *trn*T–*trn*F sequences retrieved from accessions of the *Euphorbia illirica* group and *E. palustris*. A, left, statistical parsimony network of plastid DNA haplotypes. The size of the circles is relative to the square root of a haplotype's frequency. Haplotypes that were not sampled are shown as small black dots. Right, Bayesian consensus phylogram of *trn*T–*trn*F sequences pruned to the *E. illirica* group (the complete phylogram is shown in Electronic Appendix 1). Numbering of multiple accessions per taxon corresponds to Electronic Appendices 1 and 2. Numbers above branches are maximum parsimony bootstrap values > 50%, those below branches posterior probabilities > 0.50. B, distribution of the three plastid lineages (indicated by black, dark grey and light grey filled symbols) and the paraphyletic remainder (white) identified in A.

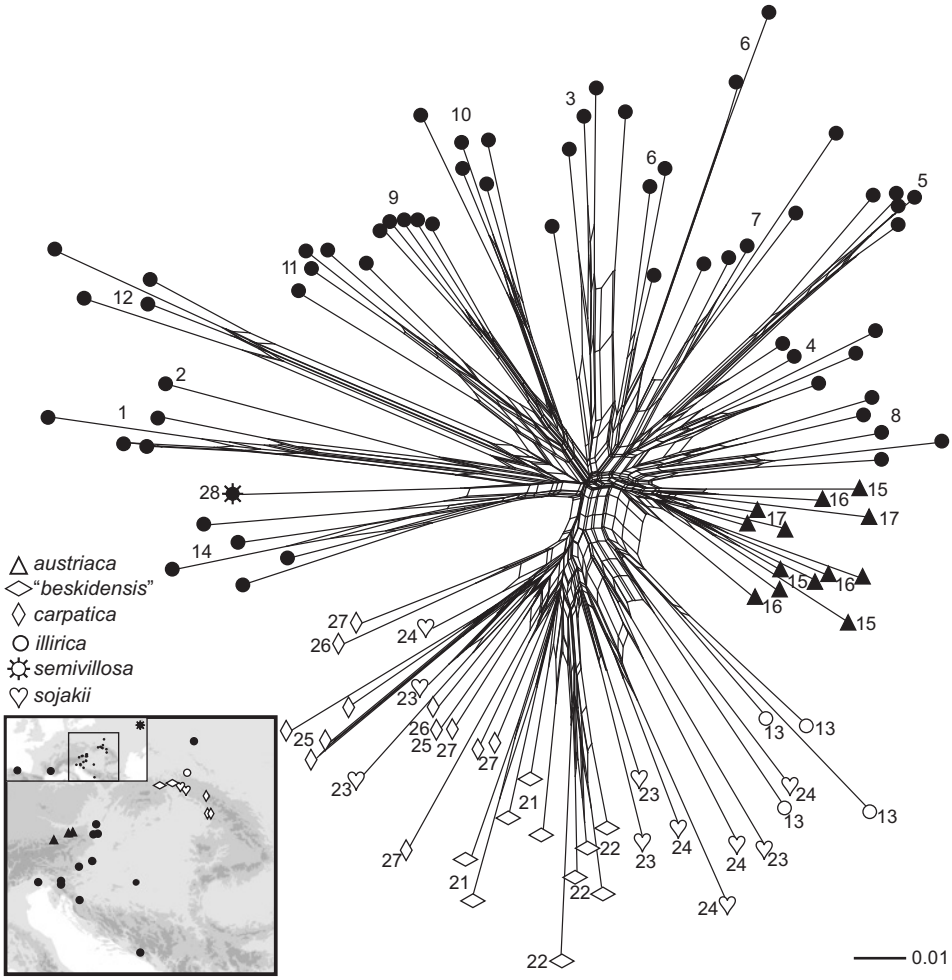



Fig. 4. – NeighborNet derived from AFLP data constructed for 25 populations of the *Euphorbia illirica* group. Population identifiers correspond to Electronic Appendix 1. The insert shows the distribution of the two main groups with black (Extra-Carpathian group) and white (Carpathian group) symbols. The same two groups were also suggested by non-hierarchical K-means clustering.

The Neighbor-Joining (NJ) tree (not shown) revealed that *E. palustris* is sister to the *E. illirica* group with 100% bootstrap support. Relationships within the latter were poorly resolved and only the terminal groups (mostly corresponding to populations or a few individuals from the same population) received significant bootstrap support. Similarly, the NeighborNet analysis (Fig. 4) revealed that several of the populations investigated were divergent, but failed to identify a hierarchical structure with the exception of two geographically correlated groups. The first group was composed of the Carpathian populations 21–27 of *E.* "*beskidensis*", *E. carpatica*, *E. sojakii* and population 13 of *E. illirica* from the northern part of the Carpathians, and the second group comprised the remaining

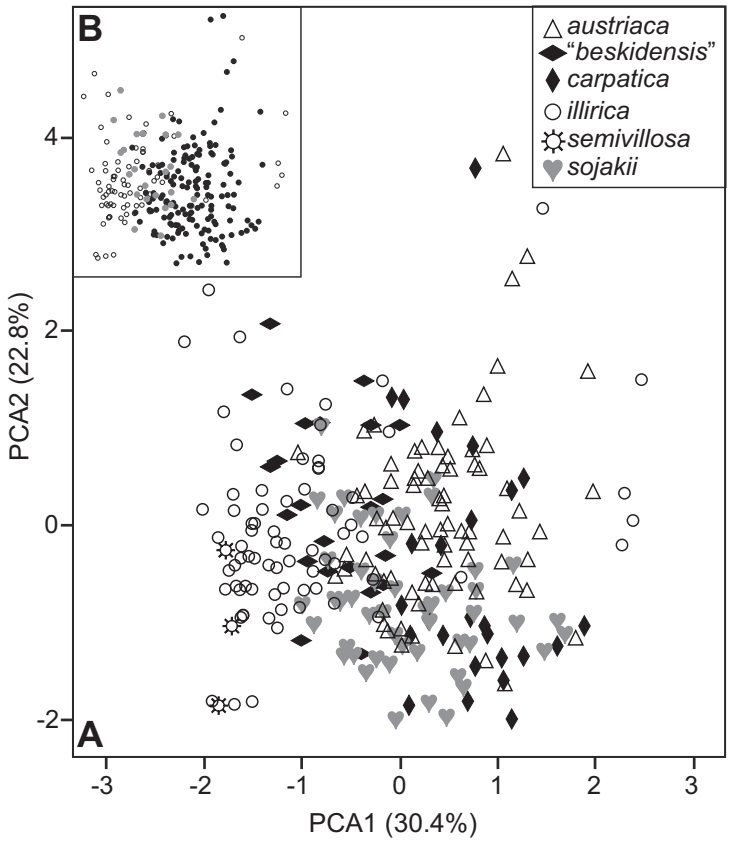



Fig. 5. – Principal component analysis (PCA) based on six morphological characters. (A), symbols indicate assignment to taxa. (B), filling of symbols indicates the altitudinal groups: white, low altitude *E. semivillosa* and *E. illirica*; black, high altitude *E. austriaca*, *E. carpatica* and *E. sojakii*; grey, high altitude *E.* "*beskidensis*", which is morphologically transitional between low and high altitude taxa.

populations of *E. illirica* as well as *E. austriaca* and *E. semivillosa*. From here on we refer to them as the Carpathian and Extra-Carpathian groups. Non-hierarchical K-means clustering revealed an optimal separation of the dataset into two vicariant groups, which were congruent with the two groups identified in the NeighborNet diagram.

*Morphological differentiation*

The measured and observed values for the character states are presented in Electronic Appendix 3. Boxplot diagrams and histograms (Electronic Appendix 6) showed a strong overlap in quantitative morphological characters among the taxa. *Euphorbia semivillosa* was divergent in characters LW and LL/LW, but only three individuals of this species were included. With respect to semi-quantitative characters *E. austriaca*, *E.* "*beskidensis*", *E. carpatica* and *E. sojakii* were similar in SHD, having glabrous to sparsely hairy stems, whereas *E. semivillosa* had glabrous stems and *E. illirica* mostly glabrous, but in a few

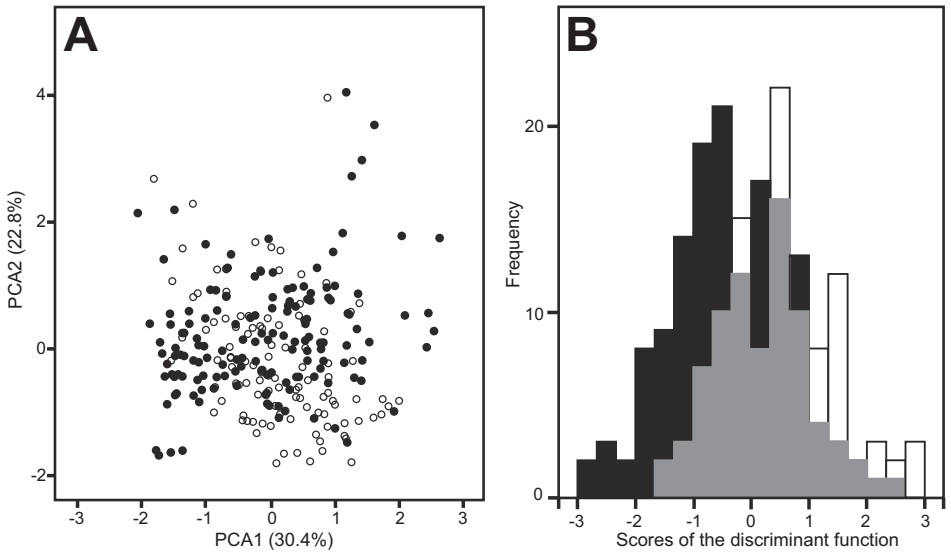



Fig. 6. – (A) principal component analysis (PCA) showing assignment of samples to one of two genetic groups. (B) Histogram of canonical discriminant analysis (CDA). Filling corresponds to the genetic groups retrieved from AFLP data: black, Extra-Carpathian group; white, Carpathian group; overlap of both groups is in grey.

cases also hairy, stems. Scores for CHD were mostly similar for *E. austriaca*, *E. carpatica* and *E. sojakii* with mostly moderately to densely hairy capsules, as well as for *E.* "*beskidensis*", *E. illirica* and *E. semivillosa* with mostly sparsely hairy capsules, but some capsules of *E. illirica* had a moderate to very dense indumentum. *Euphorbia* "*beskidensis*", *E. illirica* and *E. semivillosa* had mostly smooth capsules, whereas the capsules in *E. austriaca* and *E. sojakii* were mostly minutely tuberculate or with hemispherical appendages in *E. sojakii*. *Euphorbia carpatica* mostly had cylindrical, rarely crested appendages.

When both genetic groups were compared the overlap in all characters was strong. The best discriminating character was CA, as some individuals from the Carpathian group had hemispherical, cylindrical and crested appendages, which were absent in the Extra-Carpathian group.

The PCA (first three axes explaining 30.4, 22.8 and 17.5% of the total variation; Fig. 5A) showed strong overlap among the taxa, but with a visible taxonomy-correlated gradient in morphological space from strongly overlapping *E.* "*beskidensis*", *E. illirica* and *E. semivillosa* over *E. austriaca* and *E. sojakii* to *E. carpatica*. Population 12 of *E. illirica* from SE Bosnia and Herzegovina was positioned among populations of *E. austriaca* and *E. carpatica*. There is thus a visible gradient in the morphological variability of the six characters studied between the low altitude *E. illirica* and *E. semivillosa* and the high altitude *E. austriaca*, *E. sojakii* and *E. carpatica*; high altitude *E.* "*beskidensis*" is morphologically transitional between the two groups (Fig. 5B). The PCA also showed a strong overlap of the two AFLP groups (Fig. 6). The characters with highest loading were CHD, LW and SHD (1st axis), RL and RLL (2nd axis) and LL/LW (3rd axis). In addition, the CDA (Fig. 6) also revealed a strong overlap between the two genetic groups. The character that contributed most to the weak discrimination was RLL.

## Discussion

### *Phylogenetic relationships among the Euphorbia illirica group and its close relatives*

Analyses of nuclear ITS sequences revealed that the *E. illirica* group is monophyletic and sister to *E. palustris* (Fig. 2); also in the AFLP dataset *E. palustris* was positioned outside of the *E. illirica* group in the NJ tree (not shown). In the plastid data *E. palustris* was included in the same clade, but incongruence between plastid and nuclear markers is common in *Euphorbia*, where relationships inferred from plastid phylogenies often do not follow species boundaries (Frajman & Schönswetter 2011, Riina et al. 2013, Hand et al. 2015). A close relationship of the *E. illirica* group and *E. palustris* was suggested previously, as among the European species *E. palustris* is morphologically and ecologically most similar to the *E. illirica* group (Polatschek 1971, Meusel et al. 1978, Geltman 2009). Closely related (91% MPB, PP 1) to the *E. illirica* group and *E. palustris* are *E. alpina*, *E. pilosa* and *E. procera*, mostly Asian species of mountain grasslands (Prokhanov 1949), but some additional, not-sampled taxa, such as *E. tauricola* or *E. valdevillosocarpa* might also be closely related to this group.

Whereas relationships within the *E. illirica* group were unresolved in the ITS trees (Fig. 2), four plastid haplotype groups were inferred; three of them were weakly to strongly supported in the plastid tree, whereas one of them formed a basal polytomy (Fig. 3). *Euphorbia palustris* was positioned in two plastid lineages. The plastid groups were neither taxonomically correlated nor geographically separated, mostly spanning large, partly overlapping areas. Secondary contacts and occasional hybridization in evolutionary history might have been responsible for the observed incongruence in the position of *E. palustris* in the ITS and plastid trees. *Euphorbia palustris* and *E. illirica* have similar ecology and occasionally grow in close vicinity, e.g. in wet meadows along the river Danube east of Vienna (B. Frajman & P. Schönswetter, field observations). Population 19 of *E. palustris* from this area thus shares the same haplotype with populations 10 and 11 of *E. illirica* from the same region. Even if no interspecific hybrids are currently known, interspecific gene flow might have occurred in the evolutionary history of both taxa; species of *Euphorbia* do not have specialized pollinators (Frajman & Fišer 2001) and flower synchronously (Polatschek 1971). Due to backcrossing of the hybrid with the paternal lineage, in our example *E. palustris*, the maternal nuclear DNA from *E. illirica* may have been almost completely replaced by the paternal DNA, which is not uncommon (Rieseberg et al. 1996). Alternatively, such a pattern could have been caused by differential sorting of ancient polymorphisms in different populations of *E. palustris* and the *E. illirica* group.

### *Phylogenetic relationships within the Euphorbia illirica group*

AFLP analyses of the *E. illirica* group revealed little genetic differentiation (Fig. 4), which was not taxonomically, but rather geographically correlated. One genetic cluster in the NeighborNet and K-means analyses, the Carpathian group, corresponds to the Carpathian populations of *E. sojakii*, *E. carpatica* and the not validly described *E.* "*beskidensis*" (Graniszewska 2007), as well as the geographically close population 13 of *E. illirica*. The other cluster, the Extra-Carpathian group, comprises all other populations of *E. illirica* as well as *E. austriaca* and *E. semivillosa*. Our results clearly show that *E. austriaca* from

the north-eastern Alps and *E. sojakii* from the Western Carpathians, which were often considered conspecific, are not closely related, but have developed similar morphological traits (hairy capsules, axillary rays shorter than the stem, mostly minutely tuberculate capsules; Electronic Appendix 6) independently. It should be noted that hairy capsules occasionally occur also in *E. illirica* and in *E. austriaca* axillary rays may exceed the stem height (B. Frajman & P. Schönswetter, field observations).

High morphological plasticity is likely to be the reason for the strong overlap in character variability among taxa (Electronic Appendix 6) and absence of discrete morphological groups in the multivariate analyses (Figs 5–6). High morphological and ecological variability within the *E. illirica* group has resulted in the description of several taxa (Polatschek 1971, Meusel et al. 1978). This is most pronounced in the Carpathians, where various taxa are distinguished based on the variability of the indumentum on fruit and presence or absence of warty protuberances (Chrtek & Křísa 1970, Graniszewska 2007). However, different ranks (mostly species or subspecies) and different combinations (e.g. within *E. austriaca* or *E. illirica*) are proposed for some of them (Chrtek & Křísa 1970, Polatschek 1971, Meusel et al. 1978, Geltman 2009), suggesting that morphological differentiation is not discrete and that some morphological traits developed in parallel in different parts of the group's distribution area.

Regional morphological diversification is likely to be the result of adaptation to different environmental and ecological selection pressures (Jablonka & Lamb 2005, Pfennig et al. 2010, Flatscher et al. 2012), as members of the *E. illirica* group inhabit a wide altitudinal range from the lowlands to subalpine areas and grow in meadows, open forests and subalpine tall herbaceous plant communities. Differences in morphological and functional traits associated with increased fitness in a specific habitat can result from environmental conditions experienced during ontogeny (phenotypic plasticity) but can also be mediated by heritable (epi)genetic differences (local adaptation via phenotypic differentiation; Pfennig et al. 2010, Flatscher et al. 2012). Recently Trucchi et al. (unpublished), using RAD-sequence data, report parallel evolution in different populations of low altitude *Heliosperma veselskyi* and high altitude *H. pusillum* in the Eastern Alps. Also in other plant groups multiple independent origins of morphologically similar ecotypes adapted to similar environments are reported (e.g. Berglund et al. 2004, Foster et al. 2007, Roda et al. 2013). It thus seems likely that the similar morphology of subalpine populations in the *E. illirica* group developed in parallel in both genetic groups, in *E. austriaca* in the north-eastern Alps and independently in the Carpathian high altitude taxa. Parallel evolution can result from independent origins of the underlying molecular modification leading to a similar phenotype via a recurrent mutation at the same genomic location, through different alterations in the same gene producing a similar product, or through changes in different molecular components involved in the same phenotypic trait (see Stern 2013 for a review). Alternatively, similar selective pressures acting on different populations increase the frequency of adaptive alleles that are available as shared standing genetic variation or as a consequence of admixture among different populations or the hybridization of species (Loh et al. 2013, Pearse et al. 2014).

Most available phylogenetic and phylogeographic studies that include the Alps and the Carpathians have focussed on high-mountain biota, whereas less attention has been given to the patterns of diversification of plants growing at and below the timberline in the montane and subalpine vegetation belts (Kramp et al. 2009). Exceptions are studies of

plants with wide distributions, which among others also include Alpine and Carpathian populations (Despres et al. 2002, Fér et al. 2007, Kramp et al. 2009, Slovák et al. 2012, Stachurska-Swakoń et al. 2012, 2013, Kučera et al. 2013). The available evidence is insufficient to reveal any common, geography-linked patterns in diversification, but support the hypothesis that the Carpathians have played a major role as a Pleistocene refugium for boreo-montane subalpine taxa. Even if our study did not corroborate traditional taxonomic concepts and, consequently, did not support the Carpathians and Alps as centres of endemism (Pawłowski 1970, Davis et al. 1994) within the *E. illirica* group, the main genetic split in the AFLP dataset separating Carpathian from extra-Carpathian populations (Fig. 4) as well as the higher genetic diversity recorded in the Carpathian populations (Electronic Appendix 5) support the role of the Carpathians as an important Pleistocene refugium significant in the diversification of European biota.

In conclusion, even if locally and regionally distributed taxa within the *E. illirica* group are morphologically weakly distinct, their differentiation disappears when contrasted with the morphological variability of the entire group. Based on the results of both genetic and morphological analyses it seems reasonable to treat the members of this group as a single polymorphic species, *E. illirica*, and thus follow the concept proposed by Smith & Tutin (1968). Even if two genetic groups have been resolved across the populations of the *E. illirica* group investigated, which potentially indicates two geographically separated intraspecific taxa, the multivariate analyses of the morphological data (Fig. 6) revealed a strong overlap in morphology. This renders such a treatment impractical, as the two entities would be morphologically inseparable.

## Taxonomic treatment

Here, only the most important synonyms are listed. For further synonyms see Govaerts et al. (2000). Additional studies are needed to prove whether *E. tauricola* and *E. valde-villosocarpa* are also conspecific with *E. illirica*.

*Euphorbia illirica* Lam., Encycl. [J. Lamarck et al.] 2(2): 435, 1788, *nom. utique rej. prop.* (Frajman, 2014). – Type: "euph. illirica hort. Reg. et enc." (P-LA barcode P00381929), holotype.

= *Euphorbia villosa* Waldst. et Kit. ex Willd., Sp. Pl., ed. 4 [Willdenow] 2(2): 909, 1799. ≡ *Tithymalus villosus* (Willd.) Pacher, Fl. Kärnt. 233, 1887. ≡ *Galarhoeus villosus* (Willd.) Prokh. Trudy Kuibyshevsk. Bot. Sada 1: 33, 1941. – Type: "Pl. rar. Hung, in pratis humidis Hung., Kitaibel", Hb. Putterlick (W!), lectotype designated by Polatschek (1971).

= *Euphorbia austriaca* A. Kern., Oesterr. Bot. Z. 25: 397, 1875. ≡ *Tithymalus austriacus* (A. Kern.) A. Löve et D. Löve, Bot. Not. 114: 40, 1961. ≡ *E. villosa* subsp. *austriaca* (A. Kern.) Soó, Acta Bot. Acad. Sci. Hung. 23: 381, 1977 publ. 1978. – Type: "in regione montana et subalpina montis Bodenwies ad confines Stiriae superioris in valle Unterlaussa; solo calc.; 800–1000 m, A. Zimmeter, 1884", Schedae ad Fl. Exs. Austro-Hung. nr 867 (W 5592), lectotype designated by Polatschek (1971); isotype: KRAM, LE.

= *Euphorbia carpatica* Woł., Spraw. Komis. Fizjogr. 27: 153, 1892. ≡ *Tithymalus carpaticus* (Woł.) A. et D. Löve, Bot. Notiser 114: 40, 1961. – Type: "Między Podlutym i Osmołodą przy rz. Łomnicy, 12. lipca 1889, Dr. Wołoszczak" (W), lectotype designated by Polatschek (1971).

= *Tithymalus semivillosus* Prokh., Consp. Syst. Tithymalus As. Med.: 112, 1933. ≡ *Euphorbia semivillosa* (Prokh.) Krylov, F. Zap. Sibiri 8: 1868, 1935 publ. 1934. ≡ *Galarhoeus semivillosus* (Prokh.) Prokh., Trudy Kuibyshevsk. Bot. Sada 1: 31, 1941. ≡ *Euphorbia villosa* subsp. *semivillosa* (Prokh.) Oudejans Collect. Bot. (Barcelona) 21("1992"): 188, 1993. ≡ *E. illirica* subsp. *semivillosa* (Prokh.) Govaerts, World Checkl. & Bibliogr. Euphorbiaceae 2: 755, 2000. – Type: Kazakhstan, "Turgajskaja obl., Kustanajskij uezd, po tečeniju r. Toguzaka, okr. stanici Verinskoj, na beregu reki, 11. 6. 1913, no. 462, M. Korotkij, Z. Lebedeva" (LE), holotype.

= *Euphorbia austriaca* subsp. *sojakii* Chrtek et Křísa, Preslia 42: 262–263, 1970. ≡ *E. sojakii* (Chrtek et Křísa) Dubovik, Ukr. Bot. Zhurn. 29 (6): 80, 1972. ≡ *Tithymalus sojakii* (Chrtek et Křísa) Chrtek et Křísa, Novit. Bot. Inst. Bot. Univ. Carol. Pragensis: 9, 1972 [publ. 1973?]. – Type: "Slovakia boreo-orient., distr. Snina: ad cacumen montis Hrúbky (1186 m) supra vicum Nová Sedlica, 17. 6. 1960, leg. J. Soják" (PR 230644).

= *Tithymalus jasiewiczii* Chrtek et Křísa, Novit. Bot. Inst. Bot. Univ. Carol. Pragensis: 7, 1972. ≡ *Euphorbia jasiewiczii* (Chrtek et Křísa) A. Radcl.-Sm., Kew Bulletin 36 (2): 216, 1981. – Type: "Karpaty Wsch., Góry Czywczyńskie, w potoku Albin, alt. 1130 m s.m., 25. 7. 1935, A. Środoń." (KRAM 020343), holotype. Note: *Euphorbia jasiewiczii* was not included in our study, but based on its morphology and geographical distribution (Graniszewska 2007) we believe that it is conspecific with *E. illirica*.

See www.preslia.cz for Electronic Appendices 1–6

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## Souhrn

Alpy a Karpaty jsou významnými evropskými centry rostlinného endemismu. Výzkum biodiverzity s využitím fylogenetických studií se však dosud mnohem větší měrou zaměřil na alpské rostliny než na karpatské. V této práci jsme použili sekvence jaderné a chloroplastové DNA a metodu AFLP k odhalení fylogenetických vztahů v okruhu *Euphorbia illirica* a k analýze biogeografických vztahů mezi Alpami a Karpatami. S použitím morfometrických dat jsme prověřili oprávněnost rozlišení několika endemických taxonů. Sekvence jaderné DNA a výsledky AFLP podporují příslušnost druhů *E. austriaca*, *E.* "*beskidensis*", *E. carpatica*, *E semivillosa*, *E. sojakii* a *E. illirica* (*E. villosa*) do okruhu *E. illirica*, zatímco druh *E. palustris* byl rozpoznán jako sesterský k celé skupině. Naproti tomu chloroplastová data podporují *E. palustris* jako součást okruhu *E. illirica*. Data z AFLP odhalují genetickou diferenciaci na dvě geograficky korelované skupiny. Jedna zahrnuje karpatské populace, druhá všechny ostatní. Tato diferenciace podporuje roli Karpat jako významného pleistocénního refugia, ale není v souladu s tradičně rozeznávanými taxony ve studované skupině. Molekulární data nepotvrdila dřívější názory o biogeografické spojitosti mezi alpskou *E. austriaca* a karpatskou *E. sojakii*. Naopak se zdá, že obdobné morfologické znaky subalpínských rostlin se v obou skupinách vyvinuly nezávisle paralelní evolucí. Morfometrická analýza prokázala velké překryvy ve variabilitě jak jednotlivých taxonů, tak mezi oběma genetickými skupinami. Vymezení jakýchkoliv morfologicky určitelných evolučních jednotek navíc brání fenotypová plasticita. Jako nejvhodnější řešení se proto jeví považovat všechny příslušníky této skupiny za jeden polymorfní druh, *E. illirica*, jak už byl vymezen v díle Flora Europaea.

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