Three Tertiary *Euphorbia* species persisted in the forests of the Balkan Peninsula

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Abstract

The Balkan Peninsula is renowned as a refugium of forest species, which were more widespread in the Tertiary. We here investigated three species from one of the largest genera of flowering plants, *Euphorbia*, which survived the Pleistocene glaciations in the Balkan Peninsula, but responded differently to Holocene warming. Using ITS sequences, multivariate morphometrics and relative genome size (RGS) measurements, we explored relationships among *E. amygdaloides*, *E. heldreichii* and *E. orjeni*, for which different taxonomic treatments have been proposed. The ITS-inferred phylogenies indicate that all three species form independent evolutionary lineages, which have similar RGS, but differ morphologically, and should thus be treated as independent species. The most enigmatic of them, *E. orjeni*, was found for the first time after 100 years at its type locality in Montenegro, and, based on herbarium revision, was discovered also in the surroundings of Belgrade. *Euphorbia heldreichii* is more widespread, distributed in the southern Balkan Peninsula, whereas *E. amygdaloides* occurs in forests throughout Europe, North Africa and Western Asia. Our study emphasises the importance of further botanical exploration of the Balkan Peninsula, a hotspot of European biotic diversity.

Keywords Balkan Peninsula · Endemism · Genome size · ITS · Morphometry · Taxonomy · Tertiary relict

Introduction

The current distribution of biota throughout the Northern Hemisphere has been greatly affected by the climatic fluctuations and changing geography of the last 65 million years, i.e. during the Tertiary and Quaternary periods (Wolfe 1975; Milne and Abbott 2002). Aridification and cooling over the last 15 million years drastically changed the vegetation of Europe, where species of previously widespread Tertiary humid-temperate forests found their refugia in the Balkan Peninsula, Carpathians and adjacent Anatolia (Milne and Abbott 2002; Lendvay et al. 2016). With the decline of evergreen laurel forests, deciduous forest communities as well as evergreen gymnosperm forests expanded, but their ranges shrank again during Pleistocene glaciations (Tiffney 1985) and modern Tertiary relict floras comprise mostly deciduous woody taxa and a small number of herbs (Milne and Abbott 2002).

The Balkan Peninsula is renowned as a refugium of several, previously more widespread, tree and shrub species. *Aesculus hippocastanum* L., *Forsythia europaea* Degen & Bald. (for which it is not clear if previously had larger distribution) and *Picea omorika* (Pančić) Purk. are examples of species that nowadays have very restricted distributions (Xiang et al. 1998; Aleksić and Geburek 2014; Ha et al. 2018), whereas *Pinus heldreichii* Christ, *Syringa vulgaris* L. and *Tilia tomentosa* Moench (Li et al. 2012; Saladin et al. 2017) cover larger areas on the Balkan Peninsula and beyond. In addition, many other tree species that in the Holocene expanded their ranges north of the Alps, had their glacial refugia on the Balkan Peninsula (Petit et al. 2002;
Grivet and Petit 2003; Magri et al. 2006), which enabled persistence and diversification of understory forest species in this part of Europe (Kramp et al. 2009; Willnner et al. 2009; Slovák et al. 2012; Rešetnik et al. 2016).

Euphorbia L., one of the three largest genera of flowering plants, exhibits a large diversity of growth forms (Horn et al. 2012). Its Eurasian radiation, E. subgen. Eusula Pers., including about 480 species, diversified mostly in open, forest-free areas, especially in warm and dry habitats of the Mediterranean and Irano-Turanian regions (Riina et al. 2013). One of the exceptions is E. sect. Patellares (Prokh.) Frajman, which includes several mesophilous forest perennials, characterised by raylet leaves connate at the base (Riina et al. 2013). This lineage originated in the early Miocene, 17.7 Ma, but started to diversify only at the boundary between Miocene and Pliocene 5.6 Ma (Horn et al. 2014), giving rise to 14 species (Riina et al. 2013).

The most widespread of them is the European forest understory species E. amygdaloides L., distributed from Great Britain and Spain in the west over most of Central and Southern Europe to North Africa (Algeria, Tunis) and Western Asia, where it extends its range to the Caspian Sea area in the east (Radcliffe-Smith and Tutin 1968; Meusel et al. 1978; Radcliffe-Smith 1982). In addition, E. heldreichii Orph. was described from the southern Balkan Peninsula, where it grows in deciduous and pine forests in Albania and Greece (Radcliffe-Smith and Tutin 1968; Aldén 1986; Barina 2017). Aldén (1986) argued that it has seeds and capsules identical to E. amygdaloides and differs from it “only in having all the ray leaves and rays in whorls” and should thus be treated as parapatric subspecies E. amygdaloides subsp. heldreichii (Orph.) Aldén, which was followed also by Govaerts et al. (2000) and Dimopoulos et al. (2013).

Another Balkan endemic species similar to E. amygdaloides was described by Beck Mannagetta (1920) from Mt. Orjen in Montenegro (Veselý 1890) and to our knowledge remained known only from the type specimen until now. The species was ignored by Radcliffe-Smith and Tutin (1968), but also by Rohlena (1942) and Pulević (2005), who provided the most recent comprehensive treatments of the flora of Montenegro. On the other hand, Govaerts et al. (2000) included it in synonymy with E. amygdaloides and Riina et al. (2013) recognised it as a valid species, but it remained unclear whether this taxon is just a richly branched form of E. amygdaloides, as could be hypothesised based on the type material (B. Frajman, personal observation), or it deserves independent taxonomic status. Moreover, it is unknown where this species is distributed.

Taking into account uncertainties regarding the status of and relationships among widespread E. amygdaloides and the Balkan endemics E. heldreichii and E. orjeni, our aim is to explore phylogenetic relationships among these taxa and other co-occurring members of E. sect. Patellares and to investigate the molecular and morphological differentiation as well as possible relative genome size differences among the three before-mentioned taxa. To achieve this, (1) we searched for the existence of E. orjeni at its type locality and explored whether it is present elsewhere in the Balkan Peninsula by revising different herbarium collections. (2) We further inferred phylogenetic relationships among the taxa using nuclear ribosomal ITS sequences and (3) performed a morphometric analysis of all three taxa to elucidate which morphological characters potentially differentiate among them. (4) Using flow cytometry, we investigated variation in relative genome size in the three taxa, as 2n = 20 has been established for E. amygdaloides and E. heldreichii, but for E. amygdaloides occasionally also 18 chromosomes have been reported (Rice et al. 2015 and references therein). Finally, (5) we provide a revised taxonomic treatment for all three taxa and provide an identification key.

Materials and methods

Plant material

Plant material of E. amygdaloides, E. heldreichii and E. orjeni for RGS estimation, molecular and morphometric analyses was collected in the field between 2005 and 2019. Euphorbia orjeni was searched for at its classical locality by taking the path described by Veselý (1890), who collected the only known herbarium specimen of this taxon. Molecular and RGS analyses were based on silica gel-dried leaf material, whereas morphometric measurements were performed on herbarium vouchers. In addition, we used four ITS sequences (one accession of E. amygdaloides and three of outgroup taxa) from previous studies (Frajman and Schönswetter 2011; Riina et al. 2013) deposited in GenBank and sequenced eight additional ingroup herbarium vouchers (deposited at BEOU and W) as well as six outgroup accessions of E. characias L. and E. macroceras Fisch. & C.A.Mey. (Online Resource 1). Moreover, 20 additional herbarium specimens from the herbaria W and WU were used for morphometric analyses. In total, 56 populations of E. amygdaloides (41), E. heldreichii (12) and E. orjeni (3) were studied, the numbers reflecting the differences in species’ distributions. In most cases, the populations were represented by a single specimen in ITS and morphometric analyses, as we were not interested in intra-population variability, but in differences among the three taxa. In RGS analyses, one to four individuals per population were analysed. For 25 populations, ITS was sequenced and one sequence was included from GenBank, for 23 RGS was measured and 48 specimens from 39 populations were included in morphometric analyses (for details see Online Resource 1 and Fig. 1 as well as the text given as follows). In four cases, three specimens and in one case two specimens from the same collection were studied morphologically, which was
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important especially in the case of *E. orjeni*, for which only two collections were available.

**DNA extraction, ITS sequencing and analyses of sequence data**

Extracts of total genomic DNA and ITS sequencing were performed as described by Frajman and Schönswetter (2011), with the exception that sequencing was carried out at Eurofins Genomics (Ebersberg, Germany). In all cases, only a single individual per population was sequenced, exception being population 42 from Gramos, from which four individuals were sequenced, one morphologically corresponding to *E. heldreichii* and three morphological intermediates between *E. amygdaloides* and *E. heldreichii*. Contigs were assembled, edited and sequences aligned using Geneious Pro 5.5.9 (Kearse et al. 2012). Base polymorphisms were coded using NC-IUPAC ambiguity codes. GenBank numbers are given in Online Resource 1, and the alignment is available in Online Resource 2. Outgroup species were selected from all major clades resolved in *E. sect. Patellares* (Riina et al. 2013), including *E. characias* s.l., which occurs in the area of our focal taxa, *E. heldreichii* and *E. orjeni*, as well as the more distant *E. erubescens* and *E. macroceras*; the latter was used for rooting.

Maximum parsimony (MP) and MP bootstrap (MPB) analyses were performed using PAUP 4.0b10 (Swofford 2002). The most parsimonious trees were searched for heuristically with 100 replicates of random sequence addition, TBR swapping, and MulTrees on. All characters were equally weighted and unordered. The data set was bootstrapped using full heuristics, 1000 replicates, TBR branch swapping, MulTrees option off, and random addition sequence with five replicates. Bayesian analyses were performed using MrBayes 3.2.1 (Ronquist et al. 2012) applying the GTR substitution model proposed by the Akaike information criterion implemented in MrAIC.pl 1.4 (Nylander 2004). 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 each, sampling trees every 1000th generation using default priors. The posterior probabilities (PP) of the phylogeny and its branches were determined from the combined set of trees, discarding the first 1001 trees of each run as burn-in.

**Relative genome size measurements**

RGS was measured using flow cytometry (FCM) as described by Suda and Trávníček (2006). Nuclei of silica gel-dried material of the reference standard (*Bellis perennis* L., 2*C* = 3.38 pg; Schönswetter et al. 2007) and of the target *Euphorbia* sample were stained using 4′,6-diamidino-2-phenylindole (DAPI). The RGS was estimated for one to
four individuals per population for 23 populations, 18 of
E. amygdaloides, three of E. heldreichii and two of E. orjeni
(see Online Resource 1). A CyFlow space flow cytometer
(Partec, GmbH, Münster, Germany) was used to record the
relative fluorescence of 3000 nuclei, and FloMax software
(Partec) was used to evaluate histograms and to calculate
coefficients of variation (CVs) of the standard and sample
peaks. The RGS was calculated as the ratio between the val-
ues of the mean relative fluorescence of the sample and the
standard. Mean value and standard deviation were calculated
for each population.

**Morphometric analyses**

Twenty-eight individuals from 25 populations of E. amygd-
aloïdes, 14 individuals from 12 populations of E. heldre-
ichii and six individuals from two populations of E. orjeni
were analysed morphometrically (see Online Resource 1 for
details).

Thirty-five metric characters were measured or scored;
based on them, 12 ratio characters were calculated (Table 1).
Leaf characters were measured on mid-stem leaves and ray-
let leaves from the basal bifurcation of rays. Plant height,
stem length and width, stem leaf characters, number and
length of axillary and terminal rays as well as number of
branchings of terminal rays were measured manually. All
the other characters (ray and raylet leaf, cyathium, fruit, seed
and trichome characters) were measured on microscopic
images taken with a stereomicroscope Olympus SZX9 using
the Olympus image analysis software analySIS pro. Since
fruits and seeds were developed only in a limited number of
specimens (see Online Resource 1), we performed statistical
analyses separately for this set of characters. Since fruits and
seeds were not available in two and four specimens (two of
E. amygdaloides, E. heldreichii and four of E. orjeni), respec-
tively, we replaced their character values in the data matrix with mean
values of the respective taxon.

Statistical analyses were performed using Statistica vers.
5.1. (StatSoft 1996). Correlation among metric characters
was tested employing Pearson and Spearman correlation
coefficients but as correlation coefficients did not exceed
0.95 for any character pair, all measured characters were
retained for further analyses. Box plot diagrams were pro-
duced for all characters, in order to visualise and show the
variation among the three species. After standardisation
to zero mean and one unit variance, principal component
analysis (PCA) was performed. As a Tukey’s HSD post-hoc
test showed no discriminatory importance of fruit and seed
characters, as well as of 17 other characters (LYSL, BLASL,
LW, TrN, RyN, RyLL, RyLMW, RLL, CL, IL, IW, CGAL,
including five ratios LMW/LL, RLL/RLW, RLWM/RLL,
IL/IW, GL/GW), we excluded them from canonical discri-
minant analysis (DA). This analysis was applied to explore
morphological differentiation between E. amygdaloides,
E. heldreichii and E. orjeni and to clarify the relative impor-
tance of characters as discriminators between them. Values
presented in the species descriptions and in the identification
key correspond to the 25 and 75 percentiles, supplemented
by extreme values in parentheses.

**Results**

**ITS phylogeny**

The ITS alignment was 693 characters long, which was
also the length of all ingroup sequences. Sixteen characters
(2.3%) were parsimony informative. Homoplasy index was
0.05 (0.06 after exclusion of uninformative characters), and
the retention index was 0.99. Totally, 5301 most parsimoni-
ous trees were found and their score was 20. Bayesian and
maximum parsimony reconstructions resulted in congru-
ent topologies (Fig. 2). One main clade excluding E. mac-
roceras, which was used for rooting, was resolved (posterior
probabilities, pp 1, MPB 98%), including in a polytomy the
outgroups E. erubescens and E. characias, as well as two
clades of ingroup taxa. One (pp 1, MPB 82%) included all
accessions of E. amygdaloides, whereas the other (pp 0.75,
MPB 65%) included one clade with E. heldreichii (pp 1,
MPB 95%) and one with E. orjeni (pp 0.99, MPB 66%).

**Relative genome size**

RGS ranged between 1.57 (population 26) and 1.87 (popu-
lation 8) in E. amygdaloides, whereas the RGS values of
E. heldreichii (1.62, population 42, to 1.65, population 48)
and E. orjeni (1.78, population 55, to 1.82, population 56)
were within the range of E. amygdaloides (Online Resource
1, Fig. 3).

**Morphometry**

The morphological character states are presented in Online
Resource 3. Box plot diagrams of the most important dif-
ferential characters are shown in Online Resource 4. The
PCA scatter plot (first three axes explaining 21.5, 11.7 and
10.0% of the total variation; Fig. 4a) based on vegetative and
cyathium characters showed a separation trend with slight
overlap between E. orjeni and E. amygdaloides/E. heldre-
ichii along the first axis and between E. heldreichii and
E. amygdaloides/E. orjeni along the second axis. The char-
acters contributing most to the first separation were habitus
characters (Plant height, Stem width, Number of branch-
ings of terminal rays, Number of branchings of the longest
| No. | Character                                                                 | Abbreviation |
|-----|---------------------------------------------------------------------------|--------------|
| **Total plant**                                                                 |              |
| 1   | Plant height, mm                                                          | PH           |
| **Stem**                                                                 |              |
| 2   | Stem length, mm                                                           | SL           |
| 3   | Stem width, mm                                                            | SW           |
| 4   | Length of the last year’s stem, mm                                        | LYSL         |
| 5   | Length of the stem from the basis to the lowest axillary ray, mm          | BLASL        |
| **Middle stem leaves**                                                       |              |
| 6   | Length of a middle stem leaf, mm                                          | LL           |
| 7   | Width of a middle stem leaf, mm                                           | LW           |
| 8   | Ratio length of a middle stem leaf/width of a middle stem leaf            | LL/LW        |
| 9   | Distance from the base to the widest part of a middle stem leaf, mm       | LMW          |
| 10  | Ratio distance from the base to the widest part of a middle stem leaf/length of a middle stem leaf | LMW/LL |
| 11  | Number of trichomes in 1 mm² on the lower leaf surface                    | TrN          |
| **Terminal rays**                                                            |              |
| 12  | Number of terminal rays                                                   | RyN          |
| 13  | Length of terminal rays, mm                                               | RyL          |
| 14  | Number of branchings of terminal rays                                      | RyBN         |
| **Axillary rays**                                                            |              |
| 15  | Number of axillary rays                                                   | ARyN         |
| 16  | Length of the longest axillary ray, mm                                    | ARyL         |
| 17  | Number of branchings of the longest axillary ray                          | ARyB         |
| 18  | Number of axillary ray whorls                                             | ARyW         |
| **Ray leaves**                                                               |              |
| 19  | Length of a ray leaf, mm                                                 | RyLL         |
| 20  | Width of a ray leaf, mm                                                   | RyW          |
| 21  | Ratio length/width of a ray leaf                                          | RyLL/RyLW   |
| 22  | Distance from the base to the widest part of a ray leaf, mm               | RyLMW        |
| 23  | Ratio distance from the base to the widest part of a ray leaf/length of a ray leaf | RyLMW/RyLL |
| **Raylet leaves**                                                            |              |
| 24  | Length of a raylet leaf, mm                                               | RLL          |
| 25  | Width of a raylet leaf, mm                                                | RLW          |
| 26  | Ratio length/width of a raylet leaf                                       | RLL/RLW     |
| 27  | Distance from the base to the widest part of a raylet leaf, mm            | RLMW         |
| 28  | Ratio distance from the base to the widest part of a raylet leaf/length of a raylet leaf | RLMW/RLL |
| 29  | Length of the connation, mm                                               | CL           |
| **Cyathium**                                                                 |              |
| 30  | Length of cyathial involucre, mm                                         | IL           |
| 31  | Width of cyathial involucre, mm                                          | IW           |
| 32  | Ratio length of cyathial involucre/width of cyathial involucre            | IL/IW        |
| 33  | Length of cyathial gland without appendage (horn), mm                     | GL           |
| 34  | Width of cyathial gland, mm                                              | GW           |
| 35  | Ratio length of cyathial gland without appendage/width of cyathial gland  | GL/GW        |
| 36  | Length of cyathial gland with appendage (horn), mm                        | GAL          |
| **Fruits**                                                                   |              |
| 37  | Fruit length, mm                                                          | FL           |
| 38  | Fruit width, mm                                                           | FW           |
| 39  | Ratio fruit length/fruit width                                            | FL/FW        |
| 40  | Distance from the base to the widest part of the fruit, mm                | FMW          |
| 41  | Ratio distance from the base to the widest part of the fruit/fruit length  | FMW/FL       |
axillary ray, Length of the longest axillary ray) and those to the second separation were Number of axillary ray whorls, Width of a ray leaf, Length of a raylet leaf, Width of a raylet leaf, Length of cyathial involucre. Accordingly, the DA of the same characters (Fig. 4b) showed a clear separation of all three taxa. *Euphorbia heldreichii* was separated from the other taxa along the first axis, and the characters contributing most to this separation were Number of axillary ray whorls, Ratio of Distance from the base to the widest part of a ray leaf/Length of a ray leaf, Number of branchings of terminal rays and Width of a ray leaf. *Euphorbia orjeni* was separated from the other two taxa along the second axis, and the most important characters contributing to this separation were Stem length, Plant height and Number of branchings of the longest axillary ray. For fruit and seed characters, both PCA and DA showed a strong overlap among the three taxa (not shown).

**Discussion**

Many Tertiary species found a shelter in the Mediterranean Basin that is recognised as one of the most important refugia for species of this ancient flora (Thompson et al. 2005) and as a biodiversity hotspot with an exceptional role in the preservation of unique species and genetic diversity (Myers et al. 2000; Nieto Feliner 2014). *Euphorbia amygdaloides, E. heldreichii* and *E. orjeni* likely originated in Tertiary, since the divergence between *E. amygdaloides* and *E. characias* was dated to the boundary between Miocene and Pliocene 5.6 Ma (Horn et al. 2014) and our phylogenetic analyses show that *E. orjeni* and *E. heldreichii* form a clade.

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**Table 1** (continued)

| No. | Character | Abbreviation |
|-----|-----------|--------------|
| 42  | Style length, mm | StL |
| 43  | Seed length, mm | SeL |
| 44  | Seed width, mm | SeW |
| 45  | Ratio seed length/seed width | SeL/SeW |
| 46  | Distance from the base to the widest part of a seed, mm | SeMW |
| 47  | Ratio distance from the base to the widest part of a seed/seed length | SeMW/SeL |

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![Fig. 2](image-url)  
**Fig. 2** Bayesian consensus phylogram inferred from ITS sequences showing phylogenetic relationships among *Euphorbia amygdaloides, E. heldreichii, E. orjeni* and closely related outgroup species; numbers above branches are posterior probabilities, those below branches maximum parsimony bootstrap values. Population numbers correspond to Online Resource 1 and Fig. 1

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![Fig. 3](image-url)  
**Fig. 3** Mean relative genome size (RGS) in *Euphorbia amygdaloides, E. heldreichii* and *E. orjeni*. Population numbers (ID) correspond to Online Resource 1 and Fig. 1
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in polytomy with *E. characias* and *E. amygdalooides*. The sister relationship between *E. heldreichii* and *E. orjeni* is supported by only one synapomorphic base in ITS sequences (alignment position 625) and their divergence, supported by several differences in ITS sequences, happened later, possibly in the Pliocene. All three species survived the Pleistocene glaciations in the Balkan Peninsula but reacted differently to Holocene warming.

*Euphorbia amygdalooides* is distributed throughout Europe, North Africa and Western Asia (Fig. 5), suggesting that it might have had multiple Pleistocene refugia, from which it extended its range considerably in parallel with the spread of forests in the Holocene, similar to other understory forest species in this part of Europe (Kramp et al. 2009; Willner et al. 2009; Slovák et al. 2012; Rešetnik et al. 2016).

On the other hand, *E. heldreichii*, the most thermophilous of all three species, remained confined to the southern Balkan Peninsula, similar to *Aesculus hippocastanum* and *Forsythia europaea* (Xiang et al. 1998; Ha et al. 2018). In its northern distribution area in Albania and northern Greece, its range overlaps with that of *E. amygdalooides* (Aldén 1986; Barina 2017; Fig. 5). Both species can occur in close vicinity, e.g. in the region of Mt. Gramos in Greece (Fig. 1), and Aldén (1986) reported that intermediate individuals occur in this area. However, forms of *E. heldreichii* with less pronounced whorls of axillary rays from Mt. Gramos (population 42; three individuals sequenced), thus resembling *E. amygdalooides*, had the same ITS sequence as co-occurring typical *E. heldreichii* (one individual sequenced). This suggests that they are probably not hybrids between *E. amygdalooides* and *E. heldreichii*, but just atypical forms of the latter and that the two species do not hybridise.

*Euphorbia orjeni* (Fig. 6), the most enigmatic of the three species, is the most restricted. It is only known from its type locality below Mt. Orjen in Montenegro and from one other

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**Fig. 4** Morphological differentiation of the vegetative and cyathium characters among *Euphorbia amygdalooides* (dots), *E. heldreichii* (triangles) and *E. orjeni* (asterisk). a Principal component analysis based on 28 metric characters and eight ratios; b discriminant analysis based on 16 metric characters and three ratios after excluding those characters for which Tukey’s HSD post-hoc test showed no discriminatory importance.

**Fig. 5** Distribution of *Euphorbia amygdalooides* (from Meusel et al. 1978), *E. heldreichii* (from Flora Hellenica Database, prof. A. Strid; Barina 2017, pers. observ.; data in Online Resource 1) and *E. orjeni* (Online Resource 1)
location near Belgrade in Serbia that was discovered during this study (population 54 from Višnjička kosa; see Fig. 5). Leaf material was sampled from this specimen deposited at BEOU for DNA extraction due to its monstrous appearance and several prolonged axillary rays, and its ITS sequence revealed that it belongs to *E. orjeni*. It is thus possible that *E. orjeni* has a larger distribution in this part of the Balkan Peninsula (Montenegro, Serbia, and possibly adjacent Bosnia and Herzegovina). Despite hosting a big collection of specimens from the Balkan Peninsula, none of the herbarium specimens of *E. amygdaloides* from the Balkans deposited at W and WU morphologically corresponded to *E. orjeni*, indicating that the species is rare. However, due to its resemblance to *E. amygdaloides*, which is a common species and thus not attractive for herbarium collectors, and its in general bigger and more branched habit, which is impractical for preparing herbarium specimens, it has likely remained overlooked and undersampled. Future, more focused search for *E. orjeni* will reveal where the species is actually distributed. *Euphorbia orjeni* thus appears to be another Balkan endemic species, which, along with several others, such as *Androsace komovensis* Schönsw. & Schneew. (Schönswetter and Schneeweiss 2009; Frajman et al. 2014), *Campanula comosiformis* (Frajman and Schneeweiss 2009), *Cerastium hekuravense* Jáv. (Caković et al. 2018) and *Heliosperma oliverae* Niketić & Stevan. (Niketić and Stevanović 2007), was only recently (re)discovered, which emphasises the importance of further botanical exploration of the Balkan Peninsula.

Our morphometric survey has shown that the three species are also morphologically distinct and confirmed the discriminatory power of characters that were traditionally deemed to distinguish between *E. amygdaloides* and *E. heldreichii*, i.e. the presence of whorled leaves and axillary rays on the stem. Similarly, our study confirmed some of the characters that were considered diagnostic for *E. orjeni* in relation to *E. amygdaloides* by Beck Mannagetta (1920). However, since Beck Mannagetta (1920) inspected only two flowering shoots lacking the basal parts, some characters stated in the protologue show more variation than suggested. For instance, the nectar glands are not always green but can also be yellowish or light brown, and their appendages/horns are not significantly longer than in the other two species and are only sometimes dilated and emarginate at the tip. In addition, the plants are not always glabrous, but can sometimes be sparsely pubescent. Its leaves are somewhat thicker and coriaceous compared to *E. amygdaloides*, and thus similar in consistence to *E. heldreichii*. Finally, the plants did not have a notably unpleasant odour as stated by Beck Mannagetta (1920: “odore fastidioso”), probably based on the Veselý’s note on the herbarium label of the type specimen (“sehr übel riechend”), and were mostly only up to 0.5 m high, and not “c. 1 m”, as stated by Beck Mannagetta (1920), also based on herbarium label (“1/2 mannhoch”—“half-man high”), as he had not seen the entire plant (“partes inferiores non vidi”).

The RGS data of the three species were not significantly different, as the RGS values of *E. heldreichii* and *E. orjeni* were within the range of those of *E. amygdaloides*, which exhibited considerable variation in RGS among populations and a certain trend of increasing RGS from the east towards the west. The RGS of *E. heldreichii* was lower than that of *E. orjeni* and was in the range
of geographically close populations of *E. amygdaloides* from the southern Balkan Peninsula, whereas the RGS of *E. orjeni* rather corresponded to the values of western populations of *E. amygdaloides* from the Apennine Peninsula and the Pyrenees. Our data suggest that all three taxa are diploids, likely with the same chromosome number 2n = 20 as was several times reported for *E. amygdaloides* and *E. heldreichii* (Aldén 1986; Rice et al. 2015 and references therein).

**Taxonomic treatment**

Based on our phylogenetic and morphological data, we provide the following taxonomic treatment, recognising *E. amygdaloides*, *E. heldreichii* and *E. orjeni* as independent species.

**Identification key**

1a Middle stem leaves 5.4–12.5 × 1.8–2.7 cm. Terminal rays 9–25 cm long, 2–5 times dichotomous, axillary rays 7–24 cm long, 3–5 times dichotomous .......... *E. orjeni*

1b Middle stem leaves 2.7–9.7 × 8.0–3.5 cm. Terminal rays 0.7–10 cm long, 1–2 times dichotomous, axillary rays 1.2–8.5 cm long, 1–2 times dichotomous. .......... 2

2a Axillary rays 8–39, arranged together with upper stem leaves in 2–6 whorls below the umbel ...... *E. heldreichii*

2b Axillary rays 4–19, not in whorls, upper stem leaves alternate .................. *E. amygdaloides*

**Euphorbia amygdaloides** L., Sp. Pl. 1: 463. 1753. ≡ *Tithymalus amygdaloides* (L.) Garsault, Fig. Pl. Méd.: 594. 1764. Described from: “Habitat in Gallia, Germania”.—LECTOTYPE (designated by Geltman (2002)): s.loc, s.d., s.col. (LINN 630.71 [web http://linnean-online.org/4604/]).

For the complete list of synonyms see Govaerts et al. (2000), under “subsp. amygdaloides”.

**Description:** Pubescent to glabrescent perennial, with stem (14.2)28.1–37.4(62.2) cm high and 2.0–4.0(6.0) mm thick. Middle stem leaves oblanceolate to ovate, (27)43.5–54.5(80) × (9)13.5–18.5(26) mm, (1.9)2.6–3.7(5.7) times longer than wide, widest at (0.4)0.0–0.7(0.8) of the length. Leaves pubescent, rarely glabrescent, with (0)1–5(9) hairs in 1 mm² on the lower surface. Terminal rays 5–6, (7)33–69(98) mm long, 1–2 times dichotomous. Axillary rays (4)9–11(19), (12)35–65(80) mm long, 1–2 times dichotomous. Ray leaves broadly ovate, (7.0)10.5–14.6(18.3) × (4.3)8.2–11.9(20.4) mm, (0.8)0.1–1.5(1.9) times longer than wide, widest at (0.06)0.4–0.6(0.7) of the length. Lowermost ray-leaves semioblong or semiobovate, (5.5)7.3–10.0(14.1) × (5.3)14.2–18.3(26.2), (0.4)0.5–0.6(4.3) times longer than wide, widest at (0.1)0.2–0.4(0.9) of the length, connate along (3.2)8.4–12.2(17.4). Cyathial involucre narrowly campanulate, (1.2)2.1–2.7(3.3) × (1.5)1.9–2.4(2.6) mm, (0.7)0.9–1.3(1.5) times longer than wide. Nectar glands dark brown, elliptic (0.3)0.5–0.7(1.0) × (0.9)1.0–1.7(2.0) mm, 0.3–0.5(0.9) times longer than wide, with two appendages/horns 1.3–1.6(2.2) mm long. Capsule subglobose, trilobate, smooth, (2.1)2.3–3.3(3.8) × (2.1)2.8–4.2(5.7) mm, (0.6)0.7–0.8(1.0) times longer than wide, widest at (0.4)0.5–0.6 of the length. Styles (0.7)0.8–1.4(1.5) mm long. Seeds ovoid, smooth, greyish to black, (1.9)2.1–2.4(2.6) × (1.2)1.7–1.8(2.0) mm, (1.1)1.2–1.3(1.7) times longer than wide, widest at 0.4–0.5(0.7) of the length. 2n = 20 (count of 18 chromosomes needs to be verified!)

**Distribution:** Widespread throughout Europe with the exception of the northern and easternmost regions; the Krym and Pontic region east of the Black Sea, West Asia (regions south of the Caspian Lake), and North Africa (Algeria, Tunisia); Fig. 5.

**Habitat:** Forests, rarely scrubland above timberline.

**Euphorbia heldreichii** Orph. ex Boiss., Diagn. Pl. Orient. ser. 2, 4: 90. 1859. ≡ *Euphorbia amygdaloides* subsp. heldreichii (Orph. ex Boiss.) Aldén in A.Strid (ed.), Mount Fl. Greece 1: 575. 1986. ≡ *Tithymalus heldreichii* (Orph. ex Boiss.) Soják, Čas. Nár. Mus., Odd. Přír. 140: 173. 1972.—TYPE: “Habit. in monte Malevo [Laconiae] prope Kaston- itza (rara), Fl. Jul-aug. alt. 4000”, 10/22 Aug 1850, T.G. Orphanides (lectotype designated here: WU 0078174 [web https://herbarium.univie.ac.at/]); isolecotypes: HAL barcode 0136163, WAG barcode 0004321, 0004322, WU 0078175, YU barcode 244735). Other original material: “in regione sylvatica Olympi Thessali”, T. Heldreich (WAG 1794797).

= *Euphorbia roeseri* Orph. ex Boiss., Diagn. Pl. Orient. ser. 2, 4: 90. 1859. ≡ *Euphorbia heldreichii* var. roeseri Orph. ex Boiss., Diagn. Pl. Orient. ser. 2, 4: 90. 1859.—TYPE: “Hab. in regione superiori Parnassi ad Carcaria”, 3 Jul 1854, T.G. Orphanides (lectotype designated here: WU 0078176 [https://herbarium.univie.ac.at/]); isolecotypes: JE barcode 00002432.

= *Euphorbia semiverticillata* Halácsy, Conspl. Fl. Graec. 3: 103. 1904. ≡ *Tithymalus heldreichii* subsp. semiverticillati (Halácsy) Soják, Čas. Nár. Mus., Odd. Přír. 140: 173. 1972.—TYPE: “Griechenland. Am Fuße des Mte. Taygetus”, Jun 1876, T. Pichler (holotype: WU-Halácsy-Graecum 0078177 [web https://herbarium.univie.ac.at/]).

Note: The type specimens of *Euphorbia roeseri* and *E. semi- verticillata* were not included in our analyses, but based on their morphological resemblance with *E. heldreichii* we consider them synonyms, following also Govaerts et al. (2000),
**Description:** Glabrescent to pubescent perennial, with stem (34.6)35.7–57.0(77.8) cm high and (3.0)4.0–5.0(7.0) mm thick. Middle stem leaves oblong-elliptic, (31)45–70(97) × (8)12–18(35) mm, (2.1)3.2–4.5(8.4) times longer than wide, widest at (0.5)0.6–0.7(0.8) of the length. Leaves glabrous or pubescent on the lower surface, with 0–6(9) hairs in 1 mm². Axillary rays (8)10–23(39), (20)34–63(85) mm long, 1–2 times dichotomous. Lowermost leaflet leaves broadly semiorbicular (5.2)7.0–8.4(13.9) × (9.0)12.1–16.6(20.0), (0.4)0.5–0.6(0.8) times longer than wide, widest at (0.09)0.2–0.3 of the length, conuate along (6.2)7.8–11.0(14.6). Cyathial involucre narrowly campanulate, (1.6)1.9–2.3(3.9) × (1.3)1.7–2.3(2.9) mm, (0.7)1.0–1.3(1.4) times longer than wide. Nectar glands dark brown, elliptic (0.3)0.4–0.5(0.7) × (0.7)0.8–1.3(1.7) mm, (0.3)0.4–0.5(0.7) times longer than wide, with two appendages/horns 1.3–1.6(2.2) mm long. Capsule subglobose, trilobate, smooth, (2.1)2.7–3.3(4.2) × (3.3)3.6–4.0(4.3) mm, (0.6)0.7–0.9(2.1) times longer than wide, widest at (0.4)–0.5(0.6) of the length. Styles (0.9)1.2–1.3(1.6) mm long. Seeds ovoid-ellipsoid to ellipsoid, smooth, greyish to black, (2.0)2.2–2.8(3.0) × (1.5)1.7–1.8(2.0) mm, 1.3–1.5(1.6) times longer than wide, widest at (0.3)0.4–0.5 of the length.

**Distribution:** Southeast Europe: central Balkan Peninsula (Montenegro, Serbia); Fig. 5.

**Habitat:** Deciduous forests.

**Conservation status:** Following the criterion D of the IUCN (2012) for vulnerable species, i.e. population size estimated to number fewer than 1000 mature individuals with a very restricted area of occupancy, we deem *E. orjeni* vulnerable (VU) based on the current knowledge of its distribution.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest and that accepted principles of ethical and professional conduct have been followed.

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Information on Electronic Supplementary Material

Online Resource 1. Studied populations of Euphorbia amygdaloides, E. heldreichii, E. orjeni and four outgroup taxa, including voucher information, GenBank numbers of ITS sequences, relative genome size (RGS) data, and number of individuals included in the morphometric analyses.

Online Resource 2. Alignment of ITS sequences used in this study.

Online Resource 3. Character states of Euphorbia amygdaloides, E. heldreichii and E. orjeni from morphometric analyses.

Online Resource 4. Boxplot diagrams of morphological characters discriminating between Euphorbia amygdaloides, E. heldreichii and E. orjeni.

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