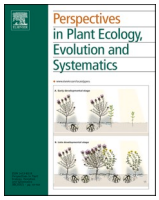
Perspectives in Plant Ecology, Evolution and Systematics 67 (2025) 125861

Contents lists available at ScienceDirect

Perspectives in Plant Ecology, Evolution and Systematics

journal homepage: www.elsevier.com/locate/ppees



Out of Liguria: How polyploidy affected diversification of the sweet spurge (*Euphorbia dulcis*, Euphorbiaceae), European widespread forest species

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ARTICLE INFO

Keywords:
Forest understory species
Morphometry
Phylogeography
Polyploidisation
Taxonomy

ABSTRACT

Polyploidy is an important evolutionary mechanism in flowering plants that can strongly affect their morphological and distributional traits. In this study, we investigated the differences in these traits among di-, tri-, and tetraploid populations of sweet spurge (*Euphorbia dulcis*), a common understory species in deciduous and mixed forests across Europe. We inferred the ploidy of 188 populations of *E. dulcis* by estimating relative genome size and chromosome counting. The data indicate that tri- and tetraploids are more widespread compared to the ancestral diploid populations, which are restricted to Liguria (north-western Italy) and adjacent regions. We suggest that polyploidisation was crucial for range expansion and the colonisation of higher latitudes, but not for the colonisation of higher elevations, where diploids appear to be more successful. Similarly, morphological differentiation after polyploidisation is only partly consistent with the classical hypothesis that polyploids are larger and have larger organs. Using nuclear ITS and plastid *ndhF–trnL* sequences, we inferred the spatio-temporal diversification of *E. dulcis*. It diverged from its diploid sister species *E. duvalii*, an endemic of south-western France and adjacent Spain, in the mid-Pliocene. This divergence was likely due to vicariant speciation accompanied by adaptation to forest and grassland environments in *E. dulcis* and *E. duvallii*, respectively. Whereas the diploid populations of both taxa have restricted ranges today, polyploidisation within *E. dulcis* likely triggered by the Pleistocene climatic oscillations contributed to its significant range expansion and diversification. The species exhibits the highest genetic diversity in the south-western Alps, where all three ploidies co-occur. Based on the ploidy differentiation and the less pronounced genetic and morphological differentiation, we propose treating di- and triploids as well as two geographically and genetically divergent groups of tetraploids (eastern and western) as four subspecies. This challenges various taxonomic treatments previously proposed for this species. Our study highlights the importance of polyploidisation for diversification and range expansion, and indicates the necessity of further research to test hypotheses related to the morphological and distributional characteristics of polyploid organisms.

1. Introduction

Polyploidisation plays a crucial role in the evolution of flowering plants; most angiosperm lineages have undergone at least one whole genome duplication (WGD) event in their evolutionary history (Soltis and Soltis, 1993; Wendel, 2000; Jiao et al., 2011). It can affect genetic, morphological, ecological and thus distributional characteristics of organisms (Balao et al., 2011; Weiss-Schneeweiss et al., 2013; Baduel et al., 2018). The positive correlation between genome size and cell size is well documented (Müntzing, 1936; Otto and Whitton, 2000; Gregory, 2001; Beaulieu et al., 2008), suggesting that polyploidy leads to larger and more robust plants with larger leaves, flowers and seeds (Müntzing, 1936; Garbutt and Bazzaz, 1983; Bretagnolle and Lumaret, 1995; Clo and Kolář, 2021). The genetic variability enhanced by polyploidisation has a positive effect on plant competitiveness, which may ultimately lead to an increased ability to colonise new environments (Moura et al.,

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https://doi.org/10.1016/j.ppees.2025.125861
Received 14 August 2024; Received in revised form 19 December 2024; Accepted 3 March 2025
Available online 13 March 2025

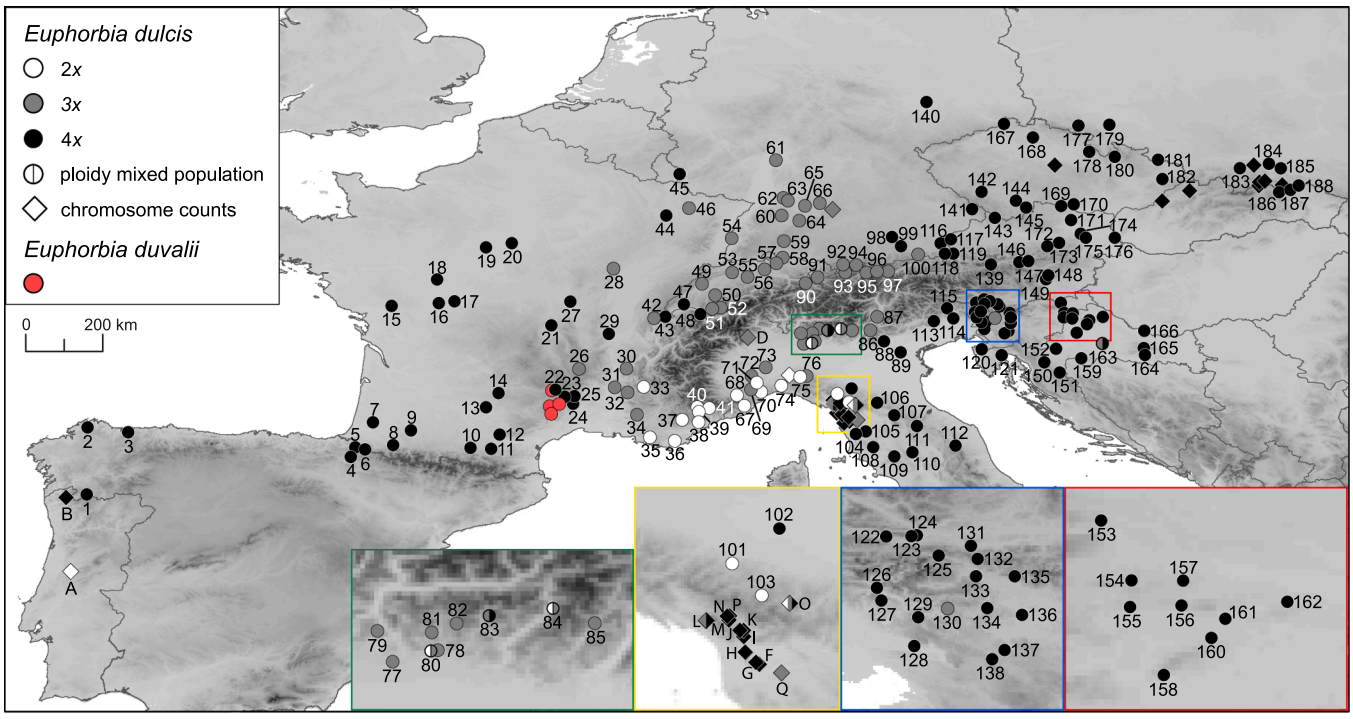

Fig. 1. The geographical distribution of diploid (white), triploid (grey) and tetraploid (black) populations of *Euphorbia dulcis* and those of *E. duvalii* (red) sampled in the study. Ploidy levels based on relative genome size estimates are marked with circles, those based on published chromosome numbers marked with diamonds. Population letters and numbers correspond to Tables S1 and S2.

2021). The successful range expansion and radiation demonstrated in various natural autopolyploids suggest that genome multiplication *per se* may be evolutionarily advantageous (Soltis et al., 2007). Polysomic inheritance and increased genomic flexibility allow them to colonise new environments and persist across heterogeneous landscapes in the long run (Parisod et al., 2010). Polyploids can thus exploit new niches or outcompete their diploid ancestors (Leitch and Leitch, 2008; Shimizu-Inatsugi et al., 2017) and often have larger distributions compared to their parents (Hijmans et al., 2007; Heimer and Frajman, 2023; Pungaršek and Frajman, 2024). In addition, the finding that experimental manipulations of temperature resulted in *de novo* polyploid formation suggests that polyploid species may be more prevalent at higher latitudes and elevations (Manton, 1937; Ramsey and Schemske, 1998). Polyploidy is thought to infer fitness advantages and a higher vigour that enable plants to better adapt to more extreme climatic conditions (Schinkel et al., 2016). It has been shown that the polyploid frequency increases away from the equator and that climate, particularly temperature, appears to be the most influential predictor of polyploid distribution (Rice et al., 2019). Along the same lines, it has been suggested that polyploids are more prevalent at higher elevations (Löve and Löve, 1943; Brochmann et al., 2004), but this has rarely been demonstrated (e.g. Vamosi and McEwen, 2013; Schinkel et al., 2016), and opposite scenarios have been shown in other polyploid systems (Liu et al., 2004; Pungaršek and Frajman, 2024). This indicates that elevational distribution of di-polyploid systems may be more complex than hypothesised.

With over 2000 species, *Euphorbia* L. is one of the largest flowering plant genera (Riina et al., 2013). About 40 % of *Euphorbia* species are polyploid, and diversification in chromosome number is believed to have led to this high diversity and wide geographic distribution (Perry, 1943; Hans, 1973). Within *Euphorbia* subgen. *Esula* Pers., the earliest diverging lineage within the genus consisting of approximately 500 species with highest diversity in Eurasia (Riina et al., 2013), the incidence of polyploidy appears to be unevenly distributed across different lineages (Heimer and Frajman, 2023). Eleven out of 21 sections are diploid, while the remaining ten contain at least some polyploid taxa (Riina et al., 2013). Polyploidy is only extensive in the second largest section of this subgenus, *E.* sect. *Esula* (Pers.) Dumort, where numerous polyploid or ploidy-mixed taxa are found (Heimer and Frajman, 2023). On the contrary, polyploid evolution seems to have been less important for the diversification of *E.* sect. *Helioscopia* Dumort., which, with 135 species, represents the largest section of *E.* subgen. *Esula* (Riina et al., 2013). Most species in this section are diploid with 14 or 16 chromosomes, but some higher chromosome numbers have also been reported, indicating polyploidisations (Riina et al., 2013; Rice et al., 2015).

One of the European forest understorey species that contains multiple ploidies and belongs to *E.* sect. *Helioscopia* is *Euphorbia dulcis* L. It is a widespread Central European species that extends south to the central Apennine and Balkan peninsulas, east to the Carpathians and west to the Iberian Peninsula. It reaches its northern limit in central Germany and northern France (Zimmermann, 1924; Meusel et al., 1978). *Euphorbia dulcis* is a glabrous to pubescent, up to 50 cm tall perennial, with triangular raylet leaves and a fleshy, jointed rhizome that can serve vegetative propagation. It grows in damp or shady places, mostly in deciduous or mixed forests (Radcliffe-Smith and Tutin, 1968). The high morphological variability within the species has led to the recognition of several infraspecific taxa (Govaerts et al., 2000; Geltman, 2008). Phylogenetically, *E. dulcis* belongs to one of the main clades of *E.* sect. *Helioscopia*, which includes most of the species in this section, but with poorly resolved relationships (Riina et al., 2013; Kirschner et al., 2023). It therefore remains unknown which species are most closely related to *E. dulcis*. Simon and Vicens (1999) included it in the *E. flavicoma* DC group, which is, however, highly polyphyletic and all species of this group are only distantly related to *E. dulcis* (Riina et al., 2013; Caković et al., 2021). Similarly, Geltman (2008) considered *E. dulcis* to be closely related to *E. altaica* Ledeb., *E. angulata* Jacq., *E. carniolica* Jacq. and *E. duvalii* Lecoq and Lamotte, but the first three species are phylogenetically distant (Riina et al., 2013). Only *E. duvalii*, a species endemic to France and morphologically slightly similar to *E. dulcis* (Simon and Vicens, 1999), has never been studied phylogenetically and might be the

closest relative of *E. dulcis*.

Different chromosome numbers ($2n = 12$, 18, 24) corresponding to di-, tri- and tetraploids have been reported for *E. dulcis*, all from close vicinity in Tuscany, Italy (Cesca, 1961; Cesca and Muzzi, 1972), but little is known about the occurrence of different ploidies in other parts of the species' distribution. In addition to populations from Italy (Cesca and Muzzi, 1972), chromosome numbers have been reported for populations from Portugal (Queirós, 1975), the Netherlands (Perry, 1943), Germany (Cesca and Muzzi, 1972), Czech Republic (Javourková-Jarolímová and Mesíček, 1992), Slovakia (Májovský et al., 1987; Mičieta, 1981; Marhold et al., 2007) and Poland (Bauer, 1971; Supplementary Table S1), suggesting that tetraploids may be most widespread. In addition to polyploidy, facultative polyembrionic apomixis has been reported for *E. dulcis* (Hegelmaier, 1901; Carano, 1926; Gustafsson, 1946), where several nuclear embryos can develop from different parts of a single nucellus without fertilisation (Kapil, 1961).

Given the morphological and ploidy-related variability within *E. dulcis*, we aimed to infer the distribution of the three ploidy levels across near-complete range of the species. Using flow cytometry, we estimated the relative genome size (RGS) of 188 populations of *E. dulcis* and inferred their ploidy, calibrated with chromosome numbers. Based on these results, we investigate the incidence of genome downsizing (Leitch and Bennett, 2004) in polyploid populations and test the hypotheses that tri- and tetraploids (1) have larger distributions compared to their diploid progenitor, (2) occur at higher elevations, and (3) are bigger and have larger organs. In addition, we infer the evolutionary origin of *E. dulcis*, particularly in relation to *E. duvalii*, using the nuclear ribosomal internal transcribed spacer (ITS) and the plastid *ndhF–trnL* region. We also investigate how the distribution of different ploidies correlates with the phylogeographic patterns of ITS ribotypes and plastid haplotypes, and generate hypotheses regarding the origin of polyploids. Finally, we use multivariate morphometric analyses to explore the morphological differentiation among different ploidies and phylogroups and provide a taxonomic treatment for *E. dulcis*.

2. Materials and methods

2.1. Plant material

Plant material for molecular analyses and RGS measurements (silica gel dried leaf material), chromosome number estimation (seeds) and morphometric analyses (herbarium vouchers) was collected in the field between 2012 and 2022. We sampled 188 populations of *E. dulcis* from most of its distribution and four populations of *E. duvalii* (Fig. 1, Table S2). Leaf material for molecular analyses and RGS estimation was collected from one to seven individuals per population and dried in silica gel. Herbarium vouchers are deposited at the herbarium of the University of Innsbruck (IB; see Table S2 for details).

2.2. Relative genome size estimation and statistical analyses

RGS was measured using flow cytometry as described by Suda and Trávníček (2006), with modifications described by Stojilkovič et al. (2022). Nuclei of silica gel dried leaf material of *E. dulcis* from 188 populations and *E. duvalii* from four populations as well as fresh leaves of reference standards *Bellis perennis* L. (2C = 3.38 pg; Schönswetter et al., 2007) or *Pisum sativum* L. cv. Kleine Rheinländerin (2C = 8.84 pg; Greilhuber and Ebert, 1994) were stained with 4′,6-diamidino-2-phenylindole (DAPI). A CyFlow space flow cytometer (Partec, GmbH, Münster, Germany) was used to record the relative fluorescence of 3000 nuclei and FloMax software (Partec) to evaluate the histograms and to calculate the coefficients of variation (CVs) of the standard and sample peaks. The RGS was calculated as the ratio between the mean relative fluorescence values of the sample and the standard.

For statistical analyses and visualisation of RGS data, we used R 4.2.2 and RStudio 2022.12.0 + 353 (R Core Team, 2021; R Studio Team, 2020) with the visualisation package "ggplot2" (Wickham, 2016). We generated scatterplots of holoploid RGS for individual populations and boxplots of three ploidies within *E. dulcis* and that of *E. duvalii*. In addition, to test for genome downsizing, we generated the boxplots of monoploid RGS of the three ploidies within *E. dulcis*. We tested the holo- and monoploid RGS values within each group for normal distribution using the Shapiro-Wilk test. Subsequently, we compared the groups using ANOVA with the following Tukey's HSD (Tukey's honest significant difference) test or the Kruskal-Wallis H test and the Conover-Iman *post hoc* test.

A distribution map of populations of different ploidy levels was created in ArcGIS 10.3 (ESRI, 2014). We also included ploidy data based on chromosome numbers from the literature (Table S1). The coordinates of these populations are approximate and were estimated based on location descriptions using Google Earth.

2.3. Chromosome numbers estimation

To establish a correlation between the measured RGS values and the chromosome numbers of di-, tri- and tetraploid populations ($2x = 12$, $3x = 18$, $4x = 24$) known from the literature (Cesca, 1961), we counted the chromosomes of one to two individuals from one triploid (61) and three tetraploid populations (6, 7, 125) of *E. dulcis*, respectively, as well as those of presumably closely related *E. duvalii* (population 2), for which two chromosome counts ($2x = 12$, 14) have been reported (Wiebecke, 1989; Simon et al., 1997). The chromosomes of diploid *E. dulcis* were not counted, as none of the few available seeds germinated.

After removing the caruncles, we incubated the seeds in the dark under alternating temperature conditions (12 hours at 20°C, 12 hours at 10°C). Root tips of germinated seeds were pretreated with 0.002 M aqueous solution of 8-hydroxyquinoline for two hours at room temperature and two hours at 4°C, fixed in 96 % ethanol and glacial acetic acid (3:1) and stored at −20°C until use. The root tips were hydrolysed in 5 M HCl for 30 min at room temperature, washed with dH_2O and stained with Schiff's reagent for one hour. Excess dye was rinsed off twice with fresh SO_2-water for 10 minutes (Greilhuber and Ebert, 1994; Weiss-Schneeweiss et al., 2009). Chromosome spreads were prepared by squashing a stained apical root meristem in a drop of acetic acid (45 %) under the coverslip and analysed with an AxioImager M2 microscope (Carl Zeiss, Vienna, Austria). The images were acquired with an AxioCam camera using AxioVision 4.8 software (both Carl Zeiss, Vienna, Austria).

2.4. Elevational distribution of ploidy levels

We compared the elevational distribution of di-, tri-, and tetraploid populations based on their field-recorded elevation. We treated phylogenetically divergent eastern and western tetraploids (see Results) separately. For statistical analysis and data visualization, we used R 4.2.2 (R Core Team, 2021) with the visualization package 'ggplot2' and RStudio 2022.12.0 + 353 (R Studio Team, 2020). The elevational distribution within each group was tested using the Shapiro-Wilk test and the groups were then compared using the Kruskal-Wallis H test and Dunn's *post hoc* test with the Bonferroni adjustment.

2.5. DNA extraction and sequencing

Total genomic DNA extractions as well as internal transcribed spacer (ITS) amplification and sequencing were performed for 56 individuals from 53 populations of *E. dulcis* and two individuals from two populations of *E. duvalii* (Table S2) as described by Frajman and Schönswetter (2011). In addition, we sequenced one accession of *E. alatavica* Boiss., three of *E. mazandaranica* Pahlevani and three of *E. squamosa* Willd. and supplemented the dataset with 90 accessions of

89 species from GenBank (Table S3). We amplified and sequenced the plastid *ndhF–trnL* region for 56 individuals from 53 populations of *E. dulcis* as described by Pahlevani and Frajman (2023). In ploidy-mixed populations, we sequenced two individuals of different ploidies from the same population for both ITS and *ndhF–trnL*. Sequencing was performed at Eurofins Genomics (Ebersberg, Germany). The contigs were assembled and edited and sequences aligned using Geneious Pro 5.5.9 (Kearse et al., 2012).

2.6. Phylogenetic analyses and divergence time estimation

Maximum parsimony (MP) and MP bootstrap analyses of ITS were performed using PAUP 4.0b10 (Swofford, 2002). *Euphorbia coniosperma* Boiss. & Buhse was used for rooting, based on previous analyses (Riina et al., 2013). The most parsimonious trees were searched for heuristically with 100 replicates of random sequence addition, TBR swapping and MulTrees on. The swapping was performed on a maximum of 1000 trees (nchuck = 1000). 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+ Γ 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. In addition, a NeighbourNet was generated with ITS sequences of *E. dulcis* using SplitsTree4 12.3 (Huson and Bryant, 2006).

Based on the results of the ITS analyses described above, only two accessions of *E. dulcis* and one of *E. duvalii* were retained and added to the ITS alignment used for the dating analyses by Kirschner et al. (2023). Divergence times were estimated using BEAST 1.8.2 (Drummond et al., 2012). The birth-death speciation prior (Gernhard, 2008) and the GTRI+ Γ substitution model with estimated base frequencies were used for phylogeny inference. A lognormal relaxed clock with a weakly informative prior on the clock rate (exponential with mean 0.001) was applied. The prior age of the root was set to 23.4 million years with a normally distributed standard deviation of 3.5, which corresponds to the median age and 95 % highest posterior densities (HPD) interval of the corresponding node (split between *E.* sect. *Holophyllum* (Prokh.) Prokh. and *E.* sect. *Helioscopia*) obtained from the dating analysis by Horn et al. (2014), i.e. 23.4 Ma (HPD 16.1–31.3). Two independent MCMC chains were run for 10,000,000 generations, saving trees and parameters every 1000 generations. The performance of the analysis was checked in Tracer 1.6.0 (Rambaut et al., 2014); both the effective sample sizes (ESS>200) and mixing were appropriate. Log and tree files from both runs were combined using Log Combiner (part of the BEAST package) after discarding 10 % of each run as burn-in. The maximum clade credibility tree (MCC) was then produced and annotated with Tree Annotator (part of the BEAST package) and visualised with FigTree 1.4.2 (Rambaut, 2014).

Finally, we constructed a statistical parsimony network of the *ndhF–trnL* alignment using TCS (Clement et al., 2000) with the connection limit set to 95. Gaps were treated as a fifth character state, and an indel longer than 1 bp (alignment positions 287–292) was reduced to a single base pair column allowing the structural mutations to be counted as single base pair mutations only. In addition, parts of the poly-A region of different lengths (alignment positions 204–205) were removed prior to the analyses.

Table 1
Morphological characters studied in *Euphorbia dulcis*.

	Stem
1	Plant height, cm
2	Stem length, cm
3	Stem width, mm
4	Number of trichomes along 1 cm × 1 mm of the stem (middle part of the lower half of the stem)
	Axillary rays
5	Number of axillary rays
6	Length of the longest axillary ray, cm
7	Length of the stem from the basis to the lowest axillary ray, cm
8	Ratio Length of the stem from the basis to the lowest axillary ray / Stem length
	Pleiochasium
9	Number of terminal rays
10	Length of the longest terminal ray, cm
11	Number of branchings of (the longest) terminal ray
12	Ratio Length of (the longest) terminal ray / Length of a Ray leaf
13	Ratio Length of (the longest) terminal ray / Plant height
	Middle stem leaves
14	Length of the largest middle stem leaf, mm
15	Width of the largest middle stem leaf, mm
16	Size of the largest middle stem leaf (leaf length × leaf width / 2), mm^2
17	Distance from the base to the widest part of the largest middle stem leaf, mm
18	Ratio Length / Width of the largest middle stem leaf
19	Ratio Distance from the base to the widest part of (the largest) middle stem leaf / Length of (the largest) middle stem leaf
20	Width of the largest middle stem leaf two millimetres below the apex, mm
21	Ratio Width of the largest middle stem leaf two milometers below the apex / Width of the largest middle stem leaf
22	Number of trichomes along one mm of the leaf margin (middle part of the leaf)
23	Number of trichomes in one mm^2 upper leaf surface
24	Length of three trichomes on one mm^2 upper leaf surface
25	Number of trichomes in one mm^2 lower leaf surface
26	Length of three trichomes on one mm^2 lower leaf surface
27	Leaf petiole length, mm
	Ray leaves
28	Length of a ray leaf, mm
29	Width of a ray leaf, mm
30	Ratio Length / Width of a ray leaf
31	Distance from the base to the widest part of a ray leaf, mm
32	Ratio Distance from the base to the widest part of a ray leaf / Length of a ray leaf
33	Width of a ray leaf two millimetres below the apex, mm
34	Ratio Width of a ray leaf two millimetres below the apex / Width of a ray leaf
	Raylet leaves
35	Length of a raylet leaf, mm
36	Width of a raylet leaf, mm
37	Ratio Length / Width of a raylet leaf
38	Distance from the base to the widest part of a raylet leaf, mm
39	Ratio Distance from the base to the widest part of a raylet leaf / Length of a raylet leaf
40	Width of a raylet leaf two millimetres below the apex, mm
41	Ratio Width of a raylet leaf two millimetres below the apex / Width of a raylet leaf
	Cyathium
42	Length of a cyathophyll, mm
43	Width of a cyathophyll, mm
44	Width of a cyathophyll two millimetres below the apex, mm
45	Ratio Width of a cyathophyll two mm below the apex / Width of a cyathophyll
46	Distance from the base to the widest part of a cyathophyll, mm
47	Ratio Length / Width of a cyathophyll
48	Ratio Distance from the base to the widest part of a cyathophyll / Length of a cyathophyll
49	Length of cyathial involucre, mm
50	Width of cyathial involucre, mm
51	Ratio Length / Width of cyathial involucre
52	Length of cyathial gland, mm
53	Width of cyathial gland, mm
54	Ratio Length / Width of cyathial gland
	Fruit
55	Fruit length, mm
56	Fruit width, mm
57	Ratio Fruit length / Fruit width
58	Distance from the base to the widest part of the fruit, mm

(*continued on next page*)

Table 1 (*continued*)

59	Ratio Distance from the base to the widest part of the fruit / Fruit length
60	Length of a wart on the fruit, mm
61	Width of a wart on the fruit, mm
62	Ratio Length / Width of a wart on the fruit
63	Distance from the base to the widest part of a wart, mm
64	Ratio Distance from the base to the widest part of a wart / Length of a wart on the fruit
65	Number of warts on a carpel surface
66	Style length, mm
67	Number of trichomes in one mm² capsule surface
68	Length of three trichomes on one mm² capsule surface
	Seed
69	Seed length, mm
70	Seed width, mm
71	Ratio Seed length / Seed width
72	Distance from the base to the widest part of a seed, mm
73	Ratio Distance from the base to the widest part of a seed / Seed length
74	Caruncle length, mm
75	Caruncle width, mm
76	Ratio Caruncle length / Caruncle width
77	Distance from the base to the widest part of the caruncle, mm
78	Ratio Distance from the base to the widest part of the caruncle / Caruncle length

2.7. Morphometric analyses

A total of 126 individuals from 122 populations were analysed morphometrically; we examined 78 morphological traits, including 25 ratios calculated from the measured characters (Table 1). Besides classical *Euphorbia* terminology following Riina et al. (2013), we here introduce the term "cyatophyll" for upper raylet leaves, whereas for the lower, bigger raylet leaves we use "raylet leaves". Plant height and stem, ray and leaf characters were measured or scored manually. Cyathium, fruit and seed characters were measured on microscopic images taken with an Olympus SZX9 and Carl Zeiss Stemi SV11 stereomicroscopes using Olympus analySIS pro and AxioVision image analysis software, respectively. Not all characters were developed in all specimens; fruits and seeds were present in only 98 and 71 individuals, respectively. As not all characters could be scored in all individuals, we replaced the missing values in the final data matrix with respective groups mean values.

Statistical analyses were performed in SPSS 24.0. Correlations among metric characters were tested using Pearson and Spearman correlation coefficients, which exceeded 0.9 in four character pairs: Plant height / Stem length, Length of the largest middle stem leaf / Distance from the base to the widest part of the largest middle stem leaf, Length of a ray leaf / Distance from the base to the widest part of a ray leaf, Number of trichomes in 1 mm² capsule surface / Length of three trichomes on 1 mm² capsule surface. The second character of each pair was therefore excluded from further analyses. After standardization to zero mean and one-unit variance, principal component analysis (PCA) and discriminant analysis (DA) were performed, separately for (1) vegetative parts of the plants including cyathium characters, for (2) fruit, and (3) seed characters (Tables S4 and S5), both for the complete dataset and for the dataset without diploids, which were found to be morphologically most divergent (see Results). The "vegetative and cyathium" matrix included 54 characters measured/scored on 126 individuals; the missing values that were replaced by groups' mean values represented 1.86 % of the data. The "fruit" matrix included 14 characters measured/scored on 98 individuals; the missing values that were replaced by groups' mean values represented 13.35 % of the data. The "seed" matrix included ten characters measured on 71 seeds and contained no missing values. Whereas in the PCA all objects (individuals) belong to one group and the components are computed to encompass the total variation among all samples, in the DA the samples are allocated to the groups (in our case ploidies) and the axes (discriminant functions) are computed to maximize the separation of the groups and therefore provide information about which characters contribute most to this separation (Marhold, 2011).

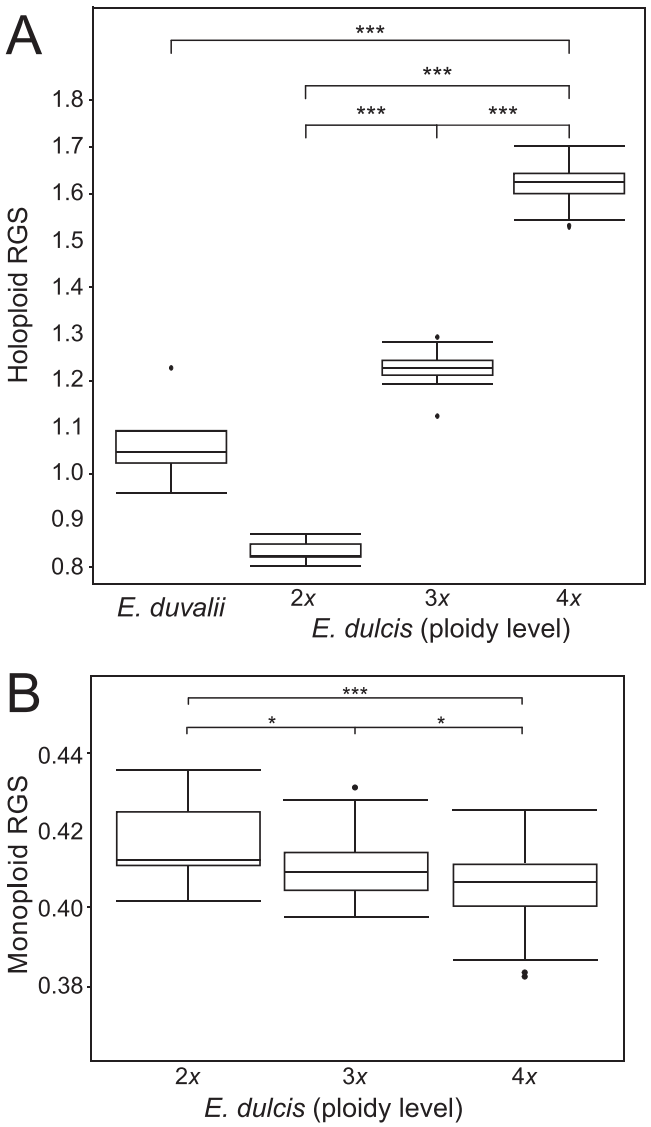

Fig. 2. (A) Holoploid relative genome size (RGS) variation within diploid (number of samples, n = 18), triploid (n = 52) and tetraploid (n = 122) populations of *Euphorbia dulcis* and those of *E. duvalii* (n = 4). (B) Monoploid RGS variation within populations of *E. dulcis* of different ploidies. Outliers putatively belonging to the same ploidy level as most of the samples are presented as dots. Statistically significant differences are marked with asterisks (*: $p < 0.05$, ***: $p < 0.001$).

To test for differences in the size of plants and their organs (plant height, leaf size, seed size) across ploidy levels, we used either a one-way ANOVA with Tukey's HSD *post hoc* test or a Kruskal-Wallis H test followed by a Dunn's test with Bonferroni adjustment method, depending on the normality of the distributions within the compared groups (Fig. S1). The latter was tested with the Shapiro-Wilk test. We did the same for the characters that contributed most to the separation along the first component in the discriminant analysis (DA), i.e. those with the highest absolute values of the discriminant function coefficients.

3. Results

3.1. Relative genome size, chromosome number and ploidy variation

The RGS of *E. dulcis* varied between 0.803 and 1.702 (Fig. 2 A, Fig. S2A, Table S2). Three discrete groups of RGS values corresponded to

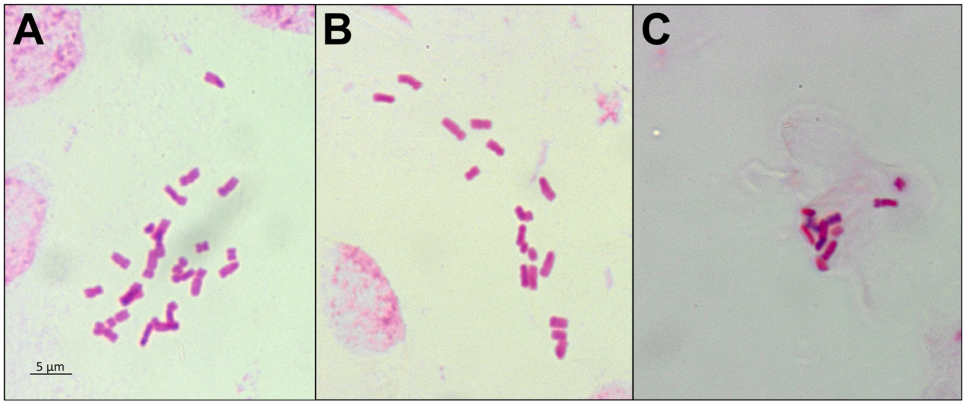

Fig. 3. Chromosomes of a (A) tetraploid ($2n = 24$, population 6) and (B) triploid ($2n = 18$, population 61) individual of Euphorbia dulcis and (C) of E. duvalii ($2n = 12$).

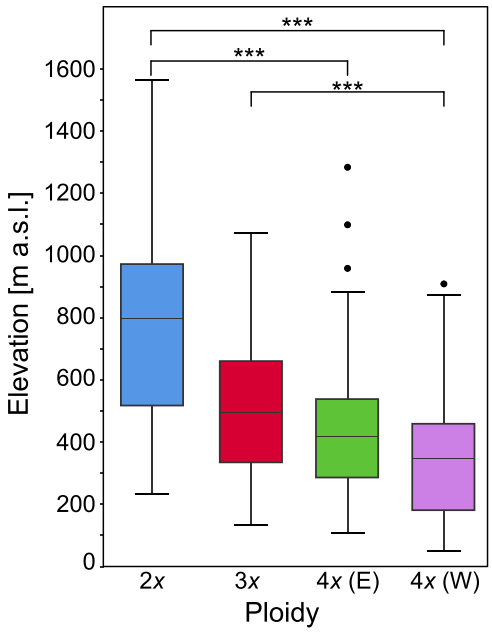

Fig. 4. Elevational distribution of the diploid (blue), triploid (red), Eastern tetraploid (green) and Western tetraploid (violet) populations of Euphorbia dulcis. Statistically significant differences are marked with asterisks (***: $p < 0.001$).

di-, tri- and tetraploid populations; tri- and tetraploids were calibrated with chromosome numbers of one triploid population (61) with $2n = 18$ and three tetraploid populations (6, 7, 125) with $2n = 24$ (Fig. 3A, B). In addition, *E. duvalii* had 12 chromosomes (Fig. 3C) and its RGS ranged from 0.959 to 1.227 (Fig. 2 A; Table S2).

There were 16 exclusively diploid, 48 triploid and 120 tetraploid populations of *E. dulcis*. Two populations (80, 84) from Lombardy in Italy consisted of both diploid and triploid individuals. One population (163) from Croatia and one from Lombardy (83) contained tri- and tetraploid plants (Fig. 1). The RGS of diploids ranged from 0.803 to 0.871, that of triploids from 1.193 to 1.293 and that of tetraploids from 1.528 to 1.702. In addition, one putatively triploid population from Emilia-Romagna (76) had a divergent RGS of 1.124 and one individual from a tetraploid population from Umbria (110) had an RGS of 1.437 (Fig. 2 A, Fig. S2A). Both were excluded from the statistical tests for monoploid RGS and are therefore not shown in Fig. 2B.

Monoploid genome sizes of *E. dulcis* ranged from 0.402 to 0.436 for diploids, from 0.375 to 0.431 for triploids, and from 0.359 to 0.426 for tetraploids (Fig. S2B). The values within all three ploidy levels were normally distributed. ANOVA with *post hoc* Tukey's HSD test showed statistically significant differences in monoploid genome sizes between all compared groups, whereas in the case of holoploid RGS, significant differences were found between *E. duvalii* and tetraploid *E. dulcis*, between di- and tetraploid, and between tri- and tetraploid *E. dulcis* (Fig. 2).

While the diploids were restricted to the western and southern fringes of the Western Alps and the northernmost Apennines (north-western Italy and south-eastern France), the triploids were mainly found in the northern adjacent areas, extending across the Alps to central Germany. The tetraploids were most widespread, distributed west of the Alps to the Iberian Peninsula (Western tetraploids hereafter), as well as east and south of the Alps, in the northern Apennine Peninsula, the north-western Balkan Peninsula and central Europe (Eastern tetraploids hereafter; Fig. 1). The ploidy levels based on published chromosome numbers had a similar distribution (Table S1; Fig. 1), with the exception of the diploid numbers from the westernmost Iberian Peninsula and the Netherlands; since the sample from the Netherlands originated from Delft (Perry, 1943), where *E. dulcis* is not native (Holverda et al., 2009), we assume that the material was from a cultivation of unknown, probably Italian origin (therefore not shown in Fig. 1).

There were significant differences in elevational distribution of some ploidy levels (Fig. 4). The diploids were collected at elevations from 235 to 1565 m a.s.l. (median 800 m), the triploids between 134 and 1075 m (median 495 m), the Western tetraploids between 50 and 910 m (median 340 m), and the Eastern tetraploids between 109 and 1285 m (median 419 m). The comparison among the groups using the Kruskal-Wallis H test and *post hoc* Dunn's test with Bonferroni adjustment showed statistically significant differences between diploids and Eastern ($p = 0.0021$) as well as Western ($p = 0.0001$) tetraploids, and between triploids and Western tetraploids ($p = 0.0219$).

3.2. *Phylogenetic origin of* Euphorbia dulcis *and temporal diversification based on ITS sequences*

The ITS alignment was 728 characters long, 197 characters were parsimony-informative. In the heuristic search, 12,000 trees were saved, each 851 steps long. The consistency index (CI) was 0.476 (without uninformative characters 0.407), and the retention index (RI) was 0.769. Bayesian and parsimony analyses resulted in congruent topologies (Fig. S3). *Euphorbia dulcis* formed a clade (maximum parsimony bootstrap, MPB, 97 %, posterior probabilities, PP, 1) with unresolved relationships among accessions of all three ploidies. This clade was sister (MPB 76 %, PP 1) to *E. duvalii* (MPB 100 %, PP 1) and both taxa were in a clade (PP 0.96) containing several different species of *E.* sect. *Helioscopia* with poorly resolved relationships.

The ITS chronogram (Fig. 5) was largely congruent with the Bayesian

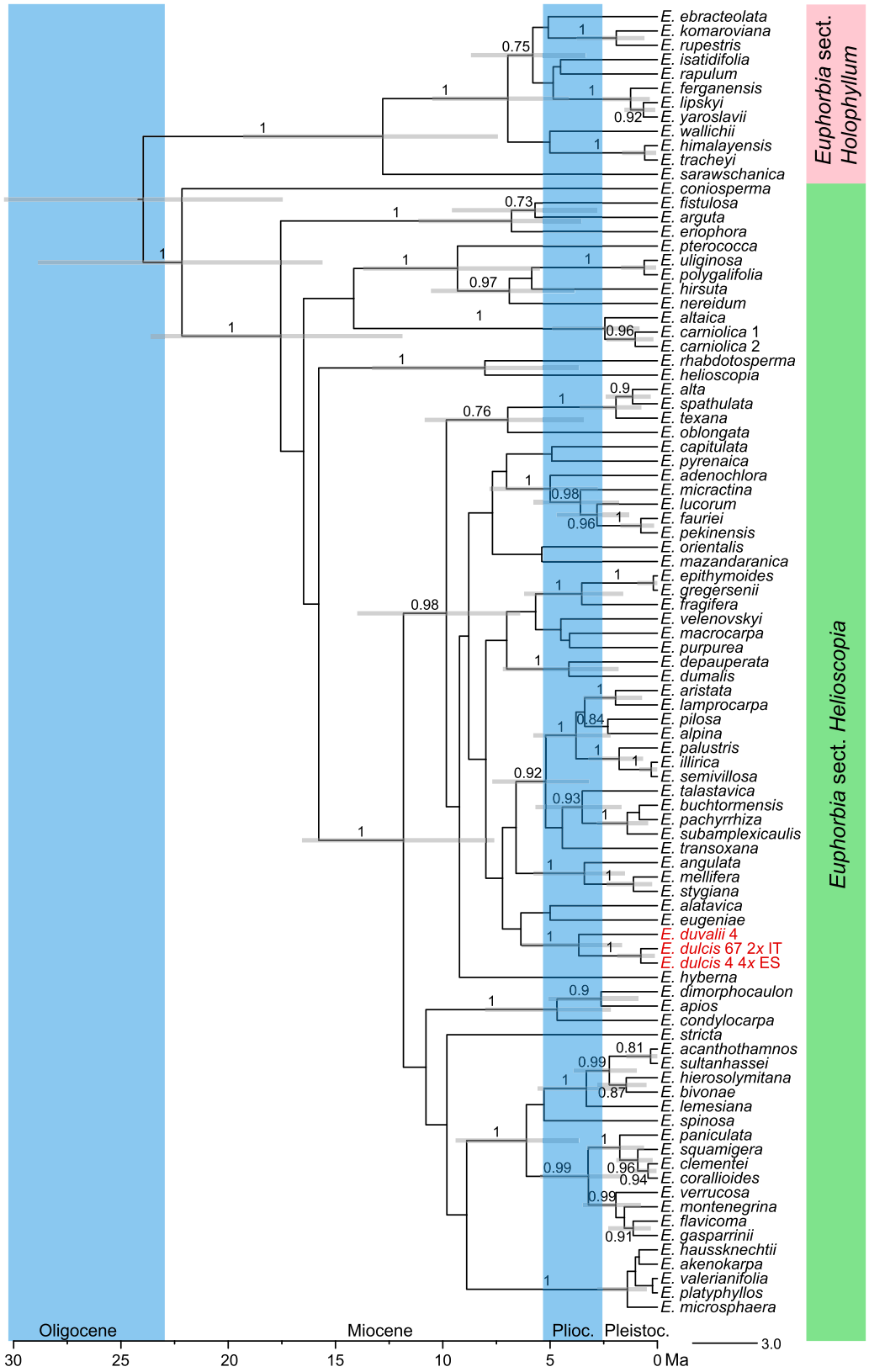

Fig. 5. Bayesian consensus chronogram (Maximum Clade Credibility tree) based on Internal Transcribed Spacer (ITS) sequences showing temporal diversification within *Euphorbia* sect. *Helioscopia*. Numbers above the branches are posterior probabilities > 0.70, and the grey bars represent 95 % highest posterior densities (HPD) of the age estimates. Population numbers correspond to Table S2 and are supplemented with ploidy and country in *E. dulcis*.

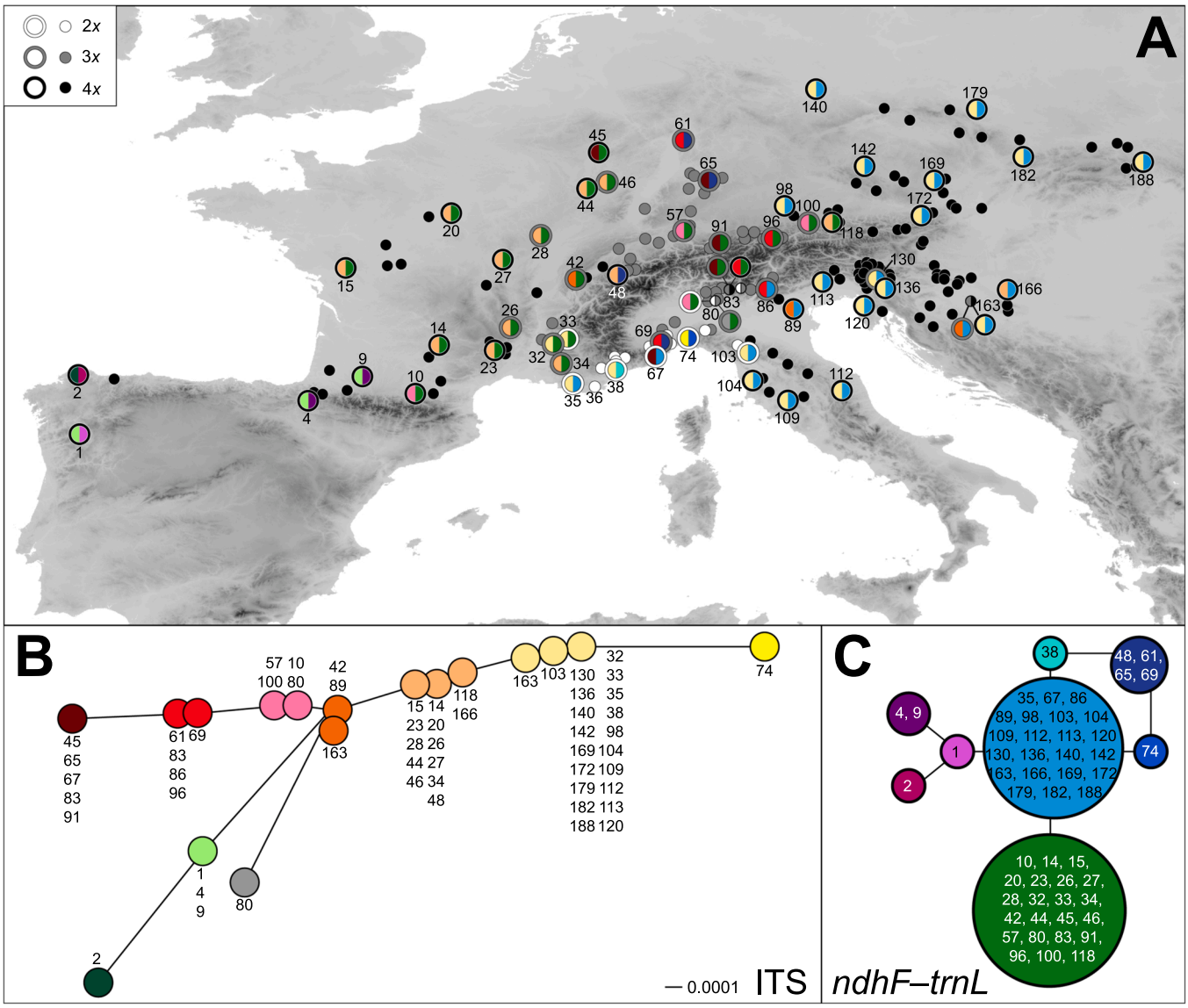

Fig. 6. Phylogeographic relationships within *Euphorbia dulcis*. (A) Geographic distribution of ribotypes (left halves of large circles; colours as in B; a simplified map showing only ribotypes is in Fig. S4) and haplotypes (right halves of large circles; colours as in C; a simplified map showing only haplotypes is in Fig. S5). Margin of the large circles and colours of the small circles representing non-sequenced populations indicate ploidy levels: white, diploid; grey, triploid; black, tetraploid. (B) NeighbourNet of internal transcribed spacer (ITS) sequences showing the relationships among ribotypes. (C) Plastid *ndhF–trnL* haplotype network. The size of the circles is proportional to the number of populations belonging to respective haplotype. Population numbers correspond to Table S2.

phylogram (Fig. S3). The early diverging lineages within *E.* sect. *Helioscopia,* which were in a polytomy in the ITS tree, appeared gradually diverging in the chronogram. *Euphorbia* sect. *Helioscopia* originated in the late Oligocene 24.0 Ma (HPD 17.4–30.4) and started to diversify in the early Miocene 22.2 Ma (HPD 15.6–28.9), when *E. coniosperma* Boiss. and Buhse originated. Other major clades, including the monophyletic lineage containing *E. dulcis* and *E. duvalii* as well as most other species, originated in the mid-Miocene 15.9 Ma (HPD 10.5–21.1). The common ancestor of *E. duvalii* and *E. dulcis* originated in the late Miocene 6.5 Ma (HPD 3.8–9.3), while the two species diverged in the mid-Pliocene 3.8 Ma (HPD 1.6–6.3). The onset of diversification within *E. dulcis* was dated to the mid-Pleistocene, ca. 0.9 Ma (HPD 0.1–1.9).

3.3. *Phylogeographic patterns within* Euphorbia dulcis

The ITS NeighbourNet of *E. dulcis* (Fig. 6B) was almost linear, with two additional branches branching off from the central part and leading to five divergent populations. The four ribotypes to the left of the NeighbourNet (yellow) were present in several diploid and one triploid population distributed along the western and southern margins of the south-western Alps and northern Apennines as well as in most of the Eastern tetraploids (Fig. 6A, Fig. S4). Slightly more divergent light orange ribotypes were present in most of the Western tetraploid and some triploid populations, with the exception of the ribotype found in populations 118 and 166 of the Eastern tetraploids. The central orange and pink ribotypes were found in seven populations scattered across the species distribution, whereas the most divergent red and brown ribotypes on the right of the NeighbourNet were found mainly in triploid populations in the central part of the distribution, together with the grey ribotype from population 80. Finally, all green divergent ribotypes were found in the tetraploids of the Iberian Peninsula and adjacent south-western France.

The *ndhF–trnL* alignment of *E. dulcis* was 558 characters long. There were two main haplotypes differentiated by one substitution (Fig. 6C). The blue haplotype predominated in the eastern part of the distribution and the green haplotype in the western part (Fig. 6A, Fig. S5). More

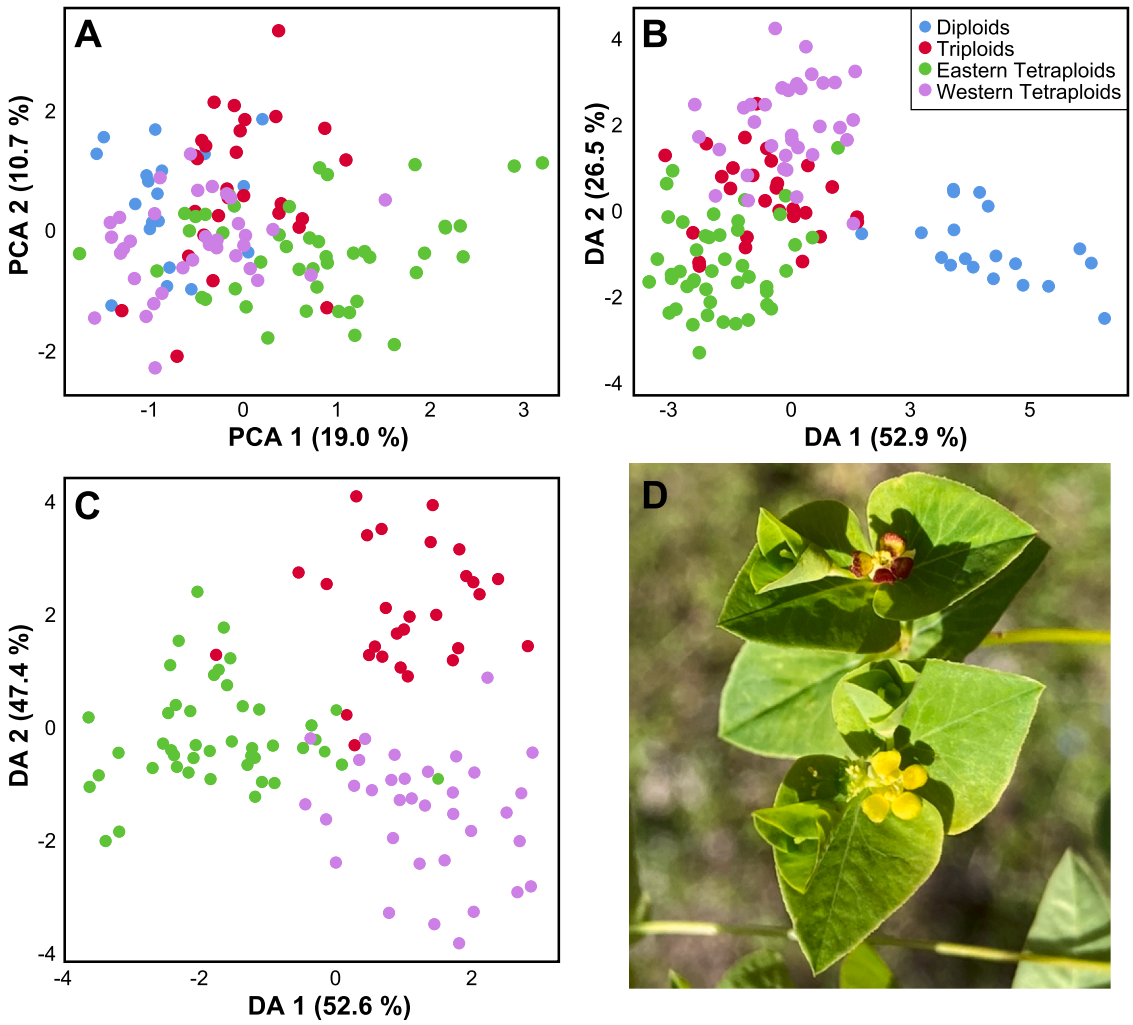

Fig. 7. Morphological differentiation among populations of *Euphorbia dulcis* of different ploidies: diploid (blue), triploid (red), Eastern tetraploid (green) and Western tetraploid (purple). Principal component analysis (PCA; A) and discriminant analyses (DA; B, C) based on 37 metric and 12 ratio vegetative and cyathium characters. Cyathia and raylet leaves of *E. dulcis* (D; photo: B. Frajman).

divergent purple-violet haplotypes were found in the Iberian and south-western French populations (which also shared divergent ribotypes). Finally, more divergent dark blue and turquoise haplotypes were found in di-, tri- and tetraploid populations in the central part of the distribution.

In summary, ribo- and haplotypes showed partly congruent distribution patterns (Fig. 6A). Most Western tetraploids shared a light orange ribotype and a green haplotype, and most Eastern tetraploids shared a light yellow ribotype and a light blue haplotype. In addition, the westernmost tetraploid populations from the Iberian Peninsula and adjacent France shared divergent green ribotypes and purple-violet haplotypes. The greatest diversity of ribotypes and slightly less extensive haplotypes was found in the central part of the species distribution, where di- and triploid populations prevailed.

3.4. Morphological differentiation

The states for all morphological characters, including ratios, are presented in Table S4. The PCA scatterplots showing the overall variation within *E. dulcis* (the first three components explaining 19.0 %, 10.7 % and 9.7 % of the total variation) based on vegetative and cyathium characters showed a high overlap among populations of different ploidies (including Western and Eastern tetraploids) along the three components (Fig. 7A, Fig. S6A).

The DA scatterplot based on vegetative and cyathium characters (Fig. 7B, Fig. S6B) showed separation of diploids along the first factor (Wilks' Lambda = 0.034, χ^2 = 342.617, df = 132, P < 0.001) and a differentiation trend, albeit with high overlap among triploids and Eastern and Western tetraploids along the second (Wilks' Lambda = 0.152, χ^2 = 190.163, df = 86, P < 0.001) and the third (Wilks' Lambda = 0.421, χ^2 = 87.421, df = 42, P < 0.001) factor. Along the second factor, the Eastern and the Western tetraploids were slightly separated, with triploids intermediate and overlapping with them (Fig. 7B), whereas along the third factor, all the ploidies overlapped strongly (Fig. S6B). The characters that contributed most to the separation along the first factor, i.e. those with the highest absolute values of the total canonical structure coefficients (between 0.263 and 0.380; Table S5) were: Ratio length / width of a ray leaf, Width of cyathial gland, Length of a ray leaf, Length and the Ratio length / width of the largest middle stem leaf. Length and Width of a cyathophyll, Length and Ratio length / width of a raylet leaf, as well as the Length of a ray leaf contributed most to the separation along the second factor (absolute values of total canonical structure coefficients between 0.289 and 0.436; Table S5), while the Stem width, Average length of three trichomes on one mm^2 of the upper leaf surface, Length and Number of branchings of the longest terminal ray and the Ratio of the distance from the base to the widest part of a cyathophyll and its length contributed most to the separation along the third factor (absolute values of total canonical structure

Table 2
Statistical differences in morphological characters among populations of different ploidies of *Euphorbia dulcis*. Asterisks indicate statistically significant differences (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). E, Eastern tetraploids; W, Western tetraploids.

Character	Significance level					
	2*x* – 3*x*	**2*x* – 4*x* (E)**	**2*x* – 4*x* (W)**	**3*x* – 4*x* (E)**	**3*x* – 4*x* (W)**	**4*x* (E) – 4*x* (W)**
Plant height, cm				*		
Stem width, mm	**				**	
Length of the longest terminal ray, cm					*	
Ratio length of the longest terminal ray / length of a ray leaf		***		**		
Length of the largest middle stem leaf	**	***	*			*
Width of the largest middle stem leaf					**	**
Size of the largest middle stem leaf (leaf length × leaf width / 2)	**	***				**
Ratio length / width of the largest middle stem leaf		***	***			
Ratio width of the largest middle stem leaf 2 mm under the apex / width of the largest middle stem leaf						*
Length of a ray leaf	**	***				***
Width of a ray leaf					*	**
Ratio width of a ray leaf 2 mm below the apex / width of a ray leaf					*	**
Length of a raylet leaf		***			**	***
Width of a raylet leaf					*	
Ratio length / width of a raylet leaf		***		***		***
Ratio width of a raylet leaf 2 mm below the apex / width of a raylet leaf	***	**			***	***
Length of a cyathophyll, mm		**		***		***
Width of a cyathophyll, mm				*		***
Ratio length / width of a cyathophyll		**		**		***
Distance from the base to the widest part of a cyathophyll, mm	*	***				***
Ratio width of a cyathophyll 2 mm bellow the apex / width of a cyathophyll		**		***		***
Length of cyathial involucre, mm		***		***		*
Length of a cyathial gland, mm		***		**		*
Seed width, mm		*		***		

coefficients 0.332 and 0.235; Table S5). Some of these characters were significantly different among at least some of the four groups compared (see Table 2 and Fig. S1). Other characters that were significantly different between at least two of the four compared groups are also shown in Table 2.

For fruit and seed characters, the PCA (the first three components explaining 22.6 %, 18.9 %, 13.6 % and 26.6 %, 22.1 %, 17.2 % of the total variation, respectively) showed a strong overlap along all three components (not shown). The DA of fruit characters showed only a slight separation of diploids and Eastern tetraploids along the first factor (Wilks' Lambda = 0.379, $\chi 2 = 86.841$, df = 39, $P < 0.001$), with triploids and western tetraploids intermediate and overlapping with both (Fig. S6C). The characters that contributed most to the separation along the first factor were Length of the style, Number of trichomes on one mm^2 of the capsule surface, and Ratio between the distance from the base of a wart to its widest part and its length (absolute values of the total canonical structure coefficients between 0.305 and 0.566; Table S5). Along the second and third factors, there was a high overlap among all four groups, which was also the case for DA of seed characters along all three factors (not shown).

After excluding the diploid populations, the DA scatterplot based on vegetative and cyathium characters revealed a slight separation of Eastern and Western tetraploids along the first factor (Wilks' Lambda = 0. 111, $\chi^2 = 181.532$, df = 88, $P < 0.001$) and a weak separation between triploids and Western tetraploids along the second factor (Wilks' Lambda = 0. 345, $\chi^2 = 87.878$, df = 43, $P < 0.001$, Fig. 7C); with the exceptions of a few individuals, the groups were well separated. The characters that contributed most to the separation along the first factor were Lengths and Ratios length / width of ray and raylet leaves and Length of a cyathophyll (absolute values of the total canonical structure coefficients between 0.387 and 0.488; Table S5). The separation along the second factor was mainly due to differences in the Length of the longest axillary and terminal rays, Number of branchings of (the longest) terminal ray, Number of trichomes along 1 cm × 1 mm of the stem and Length of (three) trichomes on one mm^2 upper leaf surface (absolute values of total canonical structure coefficients for these characters ranged from 0.217 to 0.349).

4. Discussion

4.1. *The origin of* Euphorbia dulcis *and its divergence from* E. duvalii

Our phylogenetic analyses of the ITS sequences of numerous species from *Euphorbia* sect. *Helioscopia* clearly showed, that *E. duvalii* is a sister species of *E. dulcis*. *Euphorbia duvalii* is a species of dry grasslands, gravelly sites and open pine forests on carbonate substrate in southern France and adjacent north-eastern Catalonia in Spain (Wiebecke, 1989; Simon et al., 1997; Bou and Tomàs, 2018). The species is dysploid and the two cytotypes ($2n = 2x = 12, 14$) co-occur in some populations, but the former, which was also detected in our study (Fig. 3C), is more widespread (Simon et al., 1997). Dysploidy might be responsible for high variability of RGS within *E. duvalii* (0.959–1.227). The populations 1–3 had RGS around 1.0 and among them was also the individuum from population 2, for which we counted 12 chromosomes. It is possible that the population 4 with RGS of 1.227 has 14 chromosomes. Alternatively, differential accumulation of retrotransposons and other repetitive elements, which are considered main factors of GS variation in angiosperms along polyploidy (Pellicer et al., 2018), could have been responsible for the observed variation in RGS of *E. duvalii*.

The chromosome number $2n = 12$ is also characteristic for diploid populations of *E. dulcis* (Cesca, 1961; Cesca and Muzzi, 1972) and is therefore considered ancestral to $2n = 14$. Together with the phylogenetic analyses, this supports a common ancestry of *E. duvalii* and *E. dulcis*. The lineage originated in the late Miocene 6.5 Ma (HPD 3.8–9.3), while the two species diverged in the middle Pliocene 3.8 Ma (HPD 1.6–6.3). During the Pliocene (5.3 Ma to 2.6 Ma), the Earth's climate became cooler, drier and more seasonal (Suc, 1984; Lisiecki and Raymo, 2005; Suc and Popescu, 2005; Jiménez-Moreno et al., 2013). The global cooling correlated with the Brunssumian-Reuverian transition 3.5 Ma (Zagwijn, 1960) corresponds to the emergence of thermal seasonality (cool winters), which led to the gradual disappearance of forests and the spread of grasslands and *Artemisia* steppes in the western Mediterranean in the late Pliocene (Suc and Zagwijn, 1983; Suc et al., 1995). These environmental changes may have led to a fragmentation of the previously larger distribution of the common ancestor of *E. duvalii* and *E. dulcis*, followed by vicariant speciation. While grasslands dominated the western Mediterranean, parts of the northern Apennine Peninsula were still forested at this time (Fauquette and Bertini, 2003) and could have supported the persistence of forest understory species such as *E. dulcis*. This differentiation of climate and habitats thus likely triggered the ecomorphological divergence between the thermophilic *E. duvalii*, which thrives in open sub-Mediterranean grasslands and pine

forests, and the mesophilic *E. dulcis*, a typical understory species of broadleaf and mixed forests; the divergence was also accompanied by RGS differentiation (Fig. 2). Today, diploid populations of *E. dulcis* are restricted to north-western Italy and adjacent France (Fig. 1). In this area, the species probably also persisted the Pleistocene glaciations, as this region was partly forested even during the glacial maxima (Ricci et al., 2015; Guido et al., 2020). Our study highlights the importance of north-western Italy (Liguria and adjacent regions) as an important glacial refugium; along *E. dulcis,* several other plant species survived the Pleistocene glaciations in this part of Europe (Taberlet et al., 2012; Casazza et al., 2016).

4.2. *Consequences of polyploidisation in* Euphorbia dulcis

Our results indicate that polyploidisation was crucial for the expansion of *E. dulcis*. While the diploids remained confined to the presumed refugial area in north-western Italy and south-eastern France, the triploids colonised the areas northwards across the Alps to central Europe (Germany), and the tetraploids successfully colonised all other areas of the species' current range (Fig. 1). In contrast to *E. dulcis*, the sister species *E. duvalii*, which did not undergo polyploidization, remained restricted to a small area in southern France, mirroring restricted distribution of the diploid populations of *E. dulcis*. The successful colonisation of new areas by tri- and tetraploids resulted in the current wide distribution of *E. dulcis*, one of the most widespread *Euphorbia* species in Europe. This highlights the importance of polyploidisation for range expansion. The duplicated genomes of polyploids may result in a higher adaptive capacity, which, combined with greater genetic variability, may enhance their ability to colonise new areas (Soltis and Soltis, 2000; Comai, 2005; Doyle et al., 2008; Parisod et al., 2010; Te Beest et al., 2012; Moura et al., 2021). This is consistent with studies indicating greater competitive success of polyploids compared to their diploid ancestors (Leitch and Leitch, 2008; Shimizu-Inatsugi et al., 2017), leading to their greater distributions (Hijmans et al., 2007; Rejlová et al., 2019; Heimer and Frajman, 2023; Pungaršek and Frajman, 2024). The distribution of the three ploidies in *E. dulcis* is also consistent with evidence that polyploids are more abundant at higher latitudes (Rice et al., 2019). On the other hand, the hypothesis suggesting higher frequency of polyploids at higher elevations (Löve and Löve, 1943; Brochmann et al., 2004) has not been rigorously tested yet. Only seldomly have higher-level cytotypes been shown to colonise higher elevations compared to their diploid progenitors (e.g., Vamosi and McEwen, 2013; Schinkel et al., 2016; Rejlová et al., 2019), but also opposite scenario or no elevational differentiation among ploidies have been documented (Liu et al., 2004; Kiedrzyński et al., 2021; Pungaršek and Frajman, 2024). The elevational distribution of the different ploidies in *E. dulcis* (Fig. 4) also shows an opposite pattern. The diploids, although having the smallest distribution, span the largest elevational range and occupy the highest elevations. They are followed by triploids, whereas tetraploids, albeit highly overlapping their elevational distribution with both di- and triploids, occur at relatively lower elevations.

Polyploidisation and further diversification of *E. dulcis* was accompanied by a divergence of morphological traits among ploidies (Fig. 7B–C). Whereas we observed a clear shift in morphology from the diploids to the tri-and tetraploids, morphological divergence among the triploids, Western and Eastern tetraploids was less pronounced, with considerable overlap among them in multivariate morphological space (Fig. 7C). Similar observations were made by Chansler et al. (2016) in *Phlox amabilis* Brand (Polemoniaceae), in which diploid individuals differ from polyploids to a greater extent than tetraploid and hexaploid plants from each other. Further studies are needed to clarify whether this is a general trend or an exception in autopolyploid systems.

The hypothesis that polyploids tend to be larger, more robust, and have larger leaves, flowers and seeds compared to their diploid progenitors (Müntzing, 1936; Garbutt and Bazzaz, 1983; Bretagnolle et al., 1995; Stevens et al., 2020; Chan et al., 2022) could also not be entirely confirmed in *E. dulcis*. Instead, we revealed complex patterns in the variability of morphological traits related to size. In most cases, the Eastern tetraploids show significantly larger trait values (except for involucre and gland lengths) compared to di- and triploids, whereas the Western tetraploids overlap considerably with both di- and triploids (see Fig. S1 and Table S4). In most cases, only the Eastern tetraploids showed a significant increase in certain traits compared to the lower ploidies. These traits include Seed width, Stem leaf size, and the Lengths of ray and raylet leaves and cyathophylls. In terms of organ size, there were no significant differences between most (Plant height, Length of the longest terminal ray, Width of a raylet leaf; Table 2) or all (Width of a cyathophyll 2 mm below the apex, Seed length, Fruit length and width) of the groups compared.

For several characters, there were greater differences between the Eastern and the Western tetraploids than compared to lower ploidies (Table 2, Fig. S1). Our results thus suggest that although polyploids may have larger organs, this is not always the case, and the pattern may be opposite. It is likely that other factors besides polyploidisation, such as environmental conditions, contribute to organ development and size, leading to more complex patterns than assumed.

Finally, our comparison of monoploid genome sizes among the three ploidy levels shows a significant decreasing trend of the monoploid RGS with increasing ploidy (Fig. 2B). Genome downsizing (Verma and Rees, 1974; Leitch and Bennett, 2004; Renny-Byfield et al., 2013) can occur promptly after polyploidization (Raina et al., 1994), and various selection pressures act to reduce polyploid genomes (Wang et al., 2021). These factors may have played a role in the colonisation of new environments and range expansion of the tri- and tetraploid populations of *E. dulcis*, which today cover most of the species' total distribution.

4.3. *Diversification and phylogeographic relationships within* Euphorbia dulcis

Euphorbia dulcis diverged from *E. duvalii* in the Pliocene, whereas the onset of diversification within *E. dulcis* has been dated to the mid-Pleistocene, ca. 0.9 Ma (HPD 0.1–1.9; Fig. 5). Pleistocene climatic oscillations had a significant impact on the cyclic expansions and contractions of species' ranges (Merxmüller, 1952; Hewitt, 2000; Kadereit et al., 2004; Schönswetter et al., 2005), leading to secondary contacts (Stebbins, 1984) that promoted hybridisation and polyploid speciation (Parisod and Besnard, 2007; Guggisberg et al., 2009; Schmickl et al., 2010; Casazza et al., 2012; Skubic et al., 2023). This was probably also the case in *E. dulcis*. The southern margins of the Alps, where the diploids have their restricted distribution, exhibit the greatest intraspecific diversity, particularly where all three ploidies co-occur and sometimes form ploidy-mixed populations (Fig. 1). In this area, there is a high diversity of ribo- and haplotypes, but only minimal congruence in their distribution, which may be a result of Pleistocene population dynamics that led to isolation and bottlenecks as well as hybridization during secondary contacts.

The triploid populations in particular show a high genetic diversity, suggesting their multiple origins. The majority of ribo- and haplotypes were not found exclusively in triploids, indicating gene flow between different ploidies. Triploids are usually sterile in most plant species (Pearson, 2001), and several triploid species, e.g. in hawthorns (*Crataegus* spp.), can reproduce exclusively apomictically (Kolarčik et al., 2022). On the other hand, in pears (*Pyrus* spp.), a small proportion of triploids can reproduce sexually, which results in higher diversity (Phillips et al., 2016). In addition, sexual reproduction can re-establish in triploids within a few generations, leading to increased genetic variability (Hojsgaard, 2018), which may also be the case in *E. dulcis*. Apomictic reproduction has been demonstrated in *E. dulcis* (Hegelmaier, 1901; Carano, 1926; Kapil, 1961), and different genotypes in triploid *E. dulcis* could therefore represent either distinct apomictic lineages that have arisen independently through multiple hybridisations between di- and tetraploids, or triploid populations that have re-established sexual

reproduction, contributing to genetic diversity. Further studies exploring reproductive modes of different populations are needed to disentangle the causes for the high genetic diversity within triploids.

In contrast to the genetically heterogeneous triploids, both groups of tetraploids are genetically more uniform, with the exception of the westernmost tetraploids. The distribution of ribo- and haplotypes suggests independent origins of the Eastern and Western tetraploids, which are separated by a gap inhabited by diploids and triploids (Figs. 1, 6A). The Western tetraploids share the same or similar haplo- and ribotypes with a diploid population 33 from the western edge of the diploids' range in France, suggesting that they originated in this area. Similarly, most of the Eastern tetraploids share the same haplo- and ribotype with diploid populations from the northern Apennine Peninsula, indicating their separate origin and subsequent eastward expansion, which likely followed the expansion of broad-leaf forests from their Pleistocene refugia in the Holocene (Magri et al., 2006; Giesecke et al., 2017).

Within the Western tetraploids, a subgroup of four populations from the northern Iberian Peninsula and adjacent France (1, 2, 4, 9) exhibit haplo- and ribotypes that differ from those of the other Western tetraploids (Fig. 6). This may indicate their long-term isolation from other tetraploids, possibly due to divergent Pleistocene refugia. Alternatively, the Iberian tetraploids may have originated independently from other Western tetraploids, as a single diploid chromosome number of *E. dulcis* has been reported from central Portugal by Queirós (1975; population A in Fig. 1 and Table S1). Since our sampling across the Iberian Peninsula was relatively sparse, it is possible that diploids indeed exist in this area and gave rise to tetraploids divergent from other tetraploid populations.

4.4. *Morphological variability within* Euphorbia dulcis *and taxonomic considerations*

The taxonomic treatment of natural polyploids can be challenging. Allopolyploids are often recognised as separate species because they combine characteristics of both parents (e.g., Clausen et al., 1945; Stebbins, 1947; Grant, 1981, Skubic et al., 2023), whereas autopolyploids typically share their morphology with their diploid parents (Soltis et al., 2007; Ramsey and Ramsey, 2014; Clo and Kolář, 2021; Pungaršek and Frajman, 2024). *Euphorbia dulcis* is a species that exhibits significant morphological variation, with considerable overlap in the traits of different ploidies (Fig. 7). Nevertheless, we identified certain trends in morphological differentiation for several characters, even though none of them differed exclusively among the groups compared. For instance, a combination of characteristics such as the Length of the longest terminal ray, Stem width, Stem and ray leaf width, and Raylet leaf length could be used to distinguish between the Western tetraploids and parapatric triploids (see Table S4 and Fig. S1 for ranges of values). Along the same line, a combination of measurements of Stem width, Length and size of stem leaves, and Length of a ray leaf can discriminate between di- and triploids. Triploids and the Eastern tetraploids differ from each other by Plant height, Ratio length of the longest terminal ray / length of a ray leaf, Ratio length / width of a raylet leaf and cyathophyll, Cyathophyll length and shape (ratio width 2 mm below the apex/ width at their widest part), Cyathial involucre and Cyathial gland length, and Seed width. Diploids can be distinguished from the Eastern tetraploids based on significant differences in Length and Size of the largest stem leaf, Ratio length of the longest terminal ray/length of a ray leaf, Lengths of ray and raylet leaves, Length / width ratios of stem and raylet leaves, Cyathophyll length and shape (ratio width 2 mm below the apex/ width at their widest part), Length / width ratio of a cyathophyll, Cyathial involucre and Gland lengths, and Seed width. Although we did not sample populations of diploids and the Western tetraploids from close proximity in this study, they may occur in close proximity in other areas; they differ significantly in the Length and Length / width ratio of the largest stem leaves. Interestingly, some traits such as Stem leaf width, Ray leaf length and width, Length of a raylet leaf and its length / width ratio, Cyathophyll length, width and their ratio, and Ratio cyathophyll width 2 mm below the apex / cyathophyll width were found to differ more between the Eastern and the Western tetraploids than compared to the lower ploidies. In agreement with our phylogenetic results, this is further evidence that supports separate evolutionary history of the two groups of tetraploids.

The morphological variability within *E. dulcis* has led to the recognition of two or three subspecies that were originally described as distinct species (Thuillier, 1799; Cesati, 1838; Nyman, 1881; Rothmaler, 1963): *E. dulcis* subsp. *dulcis, E. d.* subsp. *purpurata* (Thuill.) Rothm., and *E. d.* subsp. *incompta* (Ces.) Nyman. The last two are believed to differ in the shape of the cyathophylls, fruit warts, and the time of flowering and fruiting (Thuillier, 1799; Cesati, 1838). However, most authors (e.g., Zimmermann, 1924; Schönfelder, 1970, 1971; Fischer et al., 2008) consider them as synonyms, differing from the nominal subspecies in plant height, size and roundness of stem leaves, ray leaves and cyathophylls, colour of cyathium glands, fruit indumentum and seed size. On the other hand, Govaerts et al. (2000) considered *E. dulcis* subsp. *incompta* a synonym of the widespread subsp. *dulcis*, while *E. dulcis* subsp. *purpurata* should be restricted to southern France, Corsica and Italy. Finally, Geltman (2008) considered *E. purpurata* Thuill. as a synonym of *E. dulcis,* because the morphological traits that are used to distinguish them from each other, vary and are inconsistent across the entire distribution.

According to our results *E. dulcis* subsp. *purpurata* and *E. d.* subsp. *incompta* are certainly not synonyms if these subspecies are to be recognised, since the former was described from close vicinity of Paris (Thuillier, 1799) and therefore most likely pertains to the Western tetraploids, whereas the latter was described from northern Italy (Lago di Pusiano; Cesati, 1838) and could pertain either to diploids, triploids or Eastern tetraploids. Finally, since *E. dulcis* was first described by Linne (1753) from France, Italy and Germany, and the type is presumably from Meissen, eastern Germany (collected by J. Burser, from whom Linne received the herbarium specimen), the nominal subspecies likely pertains to the Eastern tetraploids.

Based on clear ploidy differentiation accompanied by a less clear genetic, distributional and morphological differentiation, we propose here to treat di-, tri-, and Eastern and Western tetraploids as subspecies of *E. dulcis*. We are aware that not all specimens will be possible to identify to subspecies based solely on morphology, but in combination with the locality data this should be possible in most cases. Further studies are needed to clarify whether the westernmost Iberian-Pyrenean populations should be treated as another subspecies. For the western tetraploids we apply the name *E. dulcis* subsp. *purpurata* (synonym *E. deseglisei* Boreau ex Boiss.), for the triploids *E. dulcis* subsp. *incompta*, for the Eastern tetraploids *E. dulcis* subsp. *dulcis* and for the diploids we propose a new name *E. dulcis* subsp. *diploidea*.

5. Taxonomic treatment

***Euphorbia dulcis* L.**, Sp. Pl.: 457 (1753) ≡ *Tithymalus dulcis* (L.) Scop., Fl. Carniol. ed. 2, 1: 334 (1771) ≡ *Galarhoeus dulcis* (L.) Haw., Syn. Pl. Succ.: 147 (1812). — Lectotype (designated here): "Tithymalus montanus non acris Bauh. / Esula dulcis Lob / In Misnia, Lusatia & c" (UPS-BURSER 16(2):58 = V-174932!). Additional original material: "Euphorbium / Tithym[alus] salicis folio lato et glabro B[auhini] P[inace] montispessulani 17" (LINN 630.39!). *Note:* Geltman (2008) selected the specimen LINN 630.40 from the Linnean Herbarium as a lectotype. However, as this collection came from Arduino and was not received by Linnaeus before 1761, it is not original material for the name and is ineligible as lectotype (Natural History Museum, 2022).

Note: Euphorbia patens Kit. in Linnaea 32: 558 (1864) is listed in synonymy of *E. dulcis* at POWO Plants of the World Online (2024).

However, "Radix annua… petalis 4 bicornibus" in the protologue (https://www.biodiversitylibrary.org/item/335179#page/570/mode/1up) speaks clearly against including this name in synonymy with *E. dulcis*. In addition, *E. lanuginosa* Lam., Encycl. 2: 436 (1788) is listed in synonymy of *E. dulcis* at POWO Plants of the World Online (2024). However, the type associated to this name ("Euph. dulcis Wild. / euphorbia lanuginosa Lam. dict.", Herbier de Lamarck, P00381927!, lectotype designated here) clearly shows that this name is a synonym of *Euphorbia corallioides* L.

Description: Glabrous to pubescent, (15)27–52(65) cm high perennial. Stem (14)20.3–44(55) cm high and (0.9)1.3–2.6(3.4) mm thick, scaly at base, terete, bearing 0–6(9) axillary and (3–)5(–7) terminal rays. A 10 × 1 mm section of the middle part of the lower half of the stem with 0–110(380) hairs. The lowest axillary ray at a height of (8.2)13.5–31.5(44) cm from the base of the stem, i.e. at (0.3)0.5–0.8(0.9) of the stem length; the longest axillary ray (1.4)3.3–10.5(15.5) cm long. The longest terminal rays (1.5)4–12.5(18.5) cm long, 0.1–0.3(0.5) of the plant height, (0)1–2(3) times dichotomously branched. Terminal rays (0.7)1.4–4.2(8.8) times as long as the ray leaves. Mid-stem leaves elliptical to oblong with mostly denticulate margin, (16)29–57.5(72) mm × (7)9.4–16.8(20.5) mm, (1.3)2.5–3.9(5) times longer than wide, widest at (0.1)0.5–0.7(0.9) of their length, with (0)0.3–2.3(4) mm long petiole and acute to rounded apex, which is (3)3.7–6.4(8.9) mm wide 2 mm below its apex, i.e. (0.2)0.3–0.5(0.8) as wide as their widest parts. Upper surface of the middle part of a stem leaf with 0–4(9) trichomes covering a mm^2; trichomes (0.1)0.6–1(2.3) mm long. 0–4(8) trichomes along 1 mm of the margin of the stem leaf. The lower leaf surface is mostly more densely pubescent, with (0)2–14(25) trichomes per mm^2, trichomes (0.1)0.6–0.9(1.1) mm long. Ray leaves broadly ovate to deltoid, sometimes cordate, (12)20–45.5(66) × (5.5)8.5–15.8(21.5) mm, (1.3)2–3.5(4.2) times longer than wide, widest at (0)0.4–0.6(0.7) of their length, with acute or rounded apex, which is (2.6)3.1–5.3(7) mm wide 2 mm below its apex, i.e. (0.2)0.3–0.5(0.6) as wide as their widest parts. Raylet leaves cordate to deltoid, (8.7)10–22.2(36) × (8)11–19(27.5) mm, 0.6(0.8)–1.5(4.4) times longer than wide, widest at 0.1–0.3(0.4) of their length, with mostly denticulate margin and acute or rounded apex, which is (1.6)3.3–6(7.5) mm wide 2 mm below its apex, i.e. 0.2–0.4(0.6) as wide as their widest parts. Cyathophylls broadly ovate to cordate or deltoid, (3.8)5.7–14.1(18.8) × (3.5)5.3–13.1(16) mm, (0.7)0.8–1.3(2.3) times longer than wide, widest at (0.1)0.2–0.4(0.5) of their length, (1.2)3.3–6(8) mm wide 2 mm below its apex, i.e. (0.3)0.4–0.7(1) as wide as their widest parts. Cyathial involucre campanulate, (0.8)1.6–2.7(3.9) × (0.8)1.2–1.9(3) mm, (0.6)1.1–1.7(2.3) times longer than wide. Cyathial glands green to dark purple (often of different colours in the same individuum), (0.4)0.6–1.1(1.4) × (0.4)0.7–1.3(1.7) mm, (0.6)1–1.4(1.8) times wider than long. Capsules (1.8)2.3–3.2(3.4) × (2)2.8–4(4.2) mm, 0.7–0.9(1.1) times longer than wide, widest at (0.3)0.4–0.6(0.7) of their length, deeply sulcate, either glabrous or pubescent, with 0–15(130) trichomes/mm^2 capsule surface, trichomes (0.2)0.3–0.6(0.7) mm long. Style (0.8)1–2(2.3) mm long. Capsules irregularly and sometimes sparsely covered with (6)11–27(34) cylindrical to hemispherical tubercules per carpel surface; tubercules green or purple, often of different colours within single populations, (0.2)0.4–0.9(1.4) × (0.1)0.3–0.6(0.8) mm, (0.8)1.2–2.1(2.6) times longer than wide, widest at (0)0.3–0.7(0.8) of their length. Seeds ovoid to ellipsoid, usually smooth and dark brown (pale yellow to brownish before ripening), (1.9)2.1–2.5(3.2) × (1.4)1.6–2(2.2) mm, (1)1.2–1.4(1.9) times longer than wide, with (0.3)0.5–0.8(1) mm × (0.3)0.6–1.1(1.3) mm caruncle.

Distribution: Across most of Europe, except far north and south. From the Iberian Peninsula (Portugal) in the west to the Carpathians and little beyond (Ukraine) in the east; from central Germany and France in the north, to the central Apennine Peninsula (Italy) and the central Balkan Peninsula (Bosnia and Herzegovina) in the south.

Habitat: Broad-leaved (often beech) and mixed forests.

5.1. Key to subspecies

Although the measured values overlap for most of the examined morphological traits, a combination of characters allows discrimination among the four subspecies of *E. dulcis* proposed here, especially in combination with geographic data. The most discriminating characters are in bold. For most reliable identification the ploidy-level estimation is advisable.

1 Mid-stem leaves **(16)24–41.8(47)** × (8)8.9–16.5(20) mm, **(1.3)2.2–3.1(3.6) times longer than wide**. Ray leaves **(16)17.6–30.2(37)** × (7.5)8.9–15.1(17) mm. Raylet leaves (8.7)10.7–16.7(17.5) × (10.1)12–16.2(17) mm, cordate to triangular. Cyathophylls (3.8)6.5–10.7(14.1) × (4.8)6.8–13(13.5) mm, widest at 0.2–0.3(0.9) of their length, i.e. **(1.2)1.5–3.1(3.6) mm from their base**. Seeds (2)2.1–2.2(2.3) × 1.6–1.7(1.8) mm. $2n = 12$. ***North-west Italy, south-east France*** ……*E. dulcis* subsp. *diploidea*

1* Mid-stem leaves **(19)32–58.4(72)** × (7)9.5–17.2(20.5) mm, **(1.6)2.7–4.1(5) times longer than wide**, ray leaves **(12)21–46(66)** × (5.5)8.5–16.1(21.5) mm, raylet leaves (8.9)10.1–23.3(36) × (8)11–19.3(27.5) mm, triangular to oblong-deltoid. Cyathophylls (4.4)5.6–14.8(18.8) × (3.5)5.1–13(16) mm, widest at (0.1)0.2–0.4(0.5) of their length, i.e. **(1)1.7–4.3(5.5) mm from their base**. Seeds (1.9)2.1–2.5(3.2) × (1.4)1.6–2(2.2) mm…… ……2

2 Mid-stem leaves (19)32–62.6(72) × (7)9.7–17.6(20.5) mm, **ray leaves (18)27.1–58.2(66)** × (7)9–16.4(21.5) mm. **Raylet leaves (11)11.9–26(36)** × (8)11.5–18.9(27.5) mm, **(0.6)1–1.7(2) times longer than wide**. Cyathophylls triangular to deltoid, **(4.5)7.6–16.5(18.8)** × **(3.5)6.7–13.5(16) mm**, **(0.8)0.9–1.3(1.6) times longer than wide**, 0.3–0.6(0.7) as wide 2 mm below their apex as at their widest part. Cyathial involucre **(0.9)1.5–2.3(2.9)** × (0.8)1.1–1.8(2.2) mm, cyathial glands **(0.4)0.5–0.9(1.3)** × (0.4)0.6–1.1(1.2) mm. Seeds (2.1)2.2–2.6 × (1.4)1.7–2(2.2) mm. $2n = 24$. ***Eastern parts of species' distribution*** ……*E. dulcis* subsp. *dulcis*

2* Mid-stem leaves (25)32–51.9(70) × (7.5)9.5–16.4(19) mm, **ray leaves (12)20.6–39(51)** × (5.5)8–15(18) mm. **Raylet leaves (8.9)9.9–19(21)** × (9)10.5–19.5(22) mm, **(0.7)0.8–1.2(1.7) times longer than wide**. Cyathophylls broadly ovate to triangular, **(4.4)5.1–10.9(15.5)** × **(4.2)4.8–11.3(15.5) mm, (0.7)0.8–1.2(2.3) times longer than wide**, (0.3)0.4–0.8(1) as wide 2 mm below their apex as at their widest part. Cyathial involucre **(0.8)1.8–2.8(3.9)** × (1.2)1.3–1.9(3) mm, cyathial glands **(0.5)0.6–1.1(1.4)** × (0.5)0.7–1.3(1.7) mm. Seeds (1.9)2–2.4(3.2) × (1.5)1.6–1.9(2.1) mm … ……3

3 Plant (26)30–57(60) cm high, **stem (1.3)1.5–2.8(3.4) mm wide**. **Terminal rays** 4–7, **(2.4)4.2–10.8(15.5) cm long**. Mid-stem leaves (27)32.7–53.9(60) × **(7.5)10.1–18.2(19)** mm, (1.6)2.4–3.8(4.7) times longer than wide. Ray leaves (18)23.7–41.7(46) × **(6.5)8.7–16.6(18) mm**. Raylet leaves (9.5)12.7–20.2(21) × (9)11.9–20.7(22) mm, 0.2–0.4 as wide 2 mm below their apex as at their widest part. Seeds (2)2.1–2.4(3.2) × (1.5)1.6–1.8(2) mm. $2n = 18$. ***Central parts of species' distribution***……*E. dulcis* subsp. *incompta*

3* Plant (19)26–47(62) cm high, **stem (0.9)1.3–2.3(2.8) mm wide**. **Terminal rays** 4–5(6), **(1.5)3.9–9.6(11.5) cm long**. Mid-stem leaves (25)32–49.4(70) × **(8.4)9.6–14.4(18) mm**, (2.1)2.9–4.2(4.5) times longer than wide. Ray leaves (12)19.3–35.7(51) × **(5.5)8–13.9(16.5) mm**. Raylet leaves (8.9)9.3–17(19.2) × (9.1)10–17.4(19.5) mm, (0.2)0.3–0.5(0.6) as wide 2 mm below their apex as at their widest part. Seeds (1.9)2–2.5(2.8) × (1.5)1.6–2(2.1) mm. $2n = 24$. ***Western parts of species' distribution***……*E. dulcis* subsp. *purpurata*

***Euphorbia dulcis* subsp. *diploidea* Kravanja and Frajman, subspec. nova**

Type: "Italy. Liguria, Imperia: south-east of Pieve di Teco, 290 m, deciduous forest (*Carpinetum*). 7°56'14'' E, 44°2'10'' N". *B. Frajman 16777*, 25.5.2021. Holotype: W0276162 (https://w.jacq.org/W0276162); Isotypes: IB114731, FI086082.

Description: Plant (16)29–47(60) cm high, with (1)1.3–2.3(2.4) mm

thick stem. Terminal rays 5–6, (2.4)4.5–14.3(17) cm long, (1.4)1.8–6.6 (8.8) times longer than the ray leaves. Mid-stem leaves (16)24–41.8(47) × (8)8.9–16.5(20) mm, (1.3)2.2–3.1(3.6) times longer than wide, widest at (0.4)0.5–0.6(0.9) of their length, i.e. at (9)12.6–24.2(27) mm from their base; 2 mm below their apex (0.2)0.3–0.5(0.6) as wide as at their widest parts. Ray leaves (16)17.6–30.2(37) × (7.5)8.9–15.1(17) mm, 2 mm below their apex (0.2)0.3–0.5(0.6) as wide as their widest parts. Raylet leaves (8.7)10.7–16.7(17.5) × (10.1)12–16.2(17) mm, cordate to triangular, (0.7)0.8–1.1(1.2) times longer than wide, 2 mm below their apex 0.3–0.5(0.6) as wide as at their widest parts. Cyathophylls (3.8) 6.5–10.7(14.1) × (4.8)6.8–13(13.5) mm, (0.4)0.8–1.2 times longer than wide, widest at 0.2–0.3(0.9) of their length, i.e. (1.2)1.5–3.1(3.6) mm from their base, 2 mm below their apex (0.4)0.5–0.7(0.9) as wide as their widest parts. Cyathial involucre (1.8)1.9–2.9(3.2) × (1.2)1.4–2.1 (2.4) mm. Cyathial glands 0.7–1.1(1.3) × (0.9)1–1.5(1.6) mm. Seeds [values based on four seeds!] (2)2.1–2.2(2.3) × 1.6–1.7(1.8) mm, (1.2) 1.3 times longer than wide. $2n = 12$.

Distribution: North-western Italy, south-eastern France.

E. dulcis* subsp. *dulcis

= *E. cordata* Schrank in Baier. Fl. 1: 747 (1789) — Type not designated (not in M nor BR!).

= *E. viridiflora* Waldst. & Kit., Descr. Icon. Pl. Hung. 3: 309 (1812) — Lectotype (here designated): "Table 280 / *Euphorbia viridiflora*" in Waldst. & Kit., Descr. Icon. Pl. Hung. 3 (1812): https://library.hungaricana.hu/en/view/MEGY_SOMO_Muzealis_Kit_3/?pg= 252&layout=s. Type locality indicated in the protologue (https://library.hungaricana.hu/en/view/MEGY_SOMO_Muzealis_Kit_3/?pg=91&layout=s): "Habitat in sylvis montanae Croatiae circum arcem vetustam Merszin [Mrsinj grad], rarissima". *Note:* since we were not able to trace any herbarium specimens associated with this name (not at B, BP nor M!), we designate the iconography as a lectotype.

Description: Plant (23)26–51(64) cm high, with (1.1)1.5–2.6(3.1) mm thick stem. Terminal rays (3)4–5, (3)4.2—12.2(14) cm long, (0.7) 1–3.3(5.8) times longer than the ray leaves. Mid-stem leaves (19) 32–62.6(72) × (7)9.7–17.6(20.5) mm, (2.7)2.9–4.1(5) times longer than wide, widest at (0.1)0.5–0.7(0.8) of their length, i.e. at (4.5) 18.4–39(51) mm from their base; 2 mm below their apex (0.2)0.3–0.5 (0.8) as wide as at their widest parts. Ray leaves (18)27.1–58.2(66) × (7)9–16.4(21.5) mm, 2 mm below their apex (0.2)0.3–0.5(0.6) as wide as at their widest parts. Raylet leaves broadly ovate to deltoid, (11) 11.9–26(36) × (8)11.5–18.9(27.5) mm, (0.6)1–1.7(2) times longer than wide, 2 mm below their apex 0.2–0.4 as wide as at their widest parts. Cyathophylls (4.5)7.6–16.5(18.8) × (3.5)6.7–13.5(16) mm, (0.8) 0.9–1.3(1.6) times longer than wide, widest at (0.1)0.2–0.3(0.4) of their length, i.e. (1)1.9–5(5.5) mm from their base; 2 mm below their apex (0.3)–0.6(0.7) as wide as at their widest parts. Cyathial involucre (0.9) 1.5–2.3(2.9) × (0.8)1.1–1.8(2.2) mm. Cyathial glands (0.4)0.5–0.9(1.3) × (0.4)0.6–1.1(1.2) mm. Seeds (2.1)2.2–2.6 × (1.4)1.7–2(2.2) mm, (1) 1.2–1.4(1.5) times longer than wide. $2n = 24$.

Distribution: Eastern part of species' distribution: central and north-eastern Italy, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro?, Hungary, eastern Austria, eastern Germany, Czech Republic, Poland, Slovakia, Ukraine, Romania?.

Euphorbia dulcis* subsp. *incompta (Ces.) Nyman, Consp. Fl. Eur., Suppl. 2: 275 (1890) ≡ *E. incompta* Ces., Bibl. Ital. (Milan) 91: 348 (1838) ≡ *E. dulcis* var. *incompta* (Ces.) Nyman, Consp. Fl. Eur.: 649 (1881). — Lectotype (designated here): "[Italy] In Montibus di Cesana et Suello", 17.6.1834, *Cesati* s.n. (RO – Herbarium Cesatianum s.n.!). Protologue: https://www.digitale-sammlungen.de/de/view/bsb10539031?page= 354,355

= *E. alpigena* A.Kern. in Oesterr. Bot. Z. 16: 337 (1866). — Lectotype (designated here): "Euphorbia alpigena Kern. / [Austria, Tyrol] Im Plätschenthal bei Innsbruck." *Kerner* s.n. (WU 0075691!; https://wu.jacq.org/WU0075691). *Note:* there are several specimens collected by Kerner deposited at HBG, JE, WU and other herbaria that are part of the original material. We selected this specimen as a lectotype as it is accompanied with the species description by Kerner.

= *E. fallax* Hagenb., Tent. Fl. Basil. 1: 435 (1821). — Lectotype (designated here): "*Euphorbia fallax* / [Switzerland, Basel] Grenzach." (BAS-00001972!). *Note:* The specimen was collected by unknown collector and is a part of Hagenbach's herbarium. Hagenbach added a label with the species description corresponding to the protologue, therefore this specimen can serve as a lectotype. Protologue: https://www.biodiversitylibrary.org/item/29509#page/461/mode/1up

Description: Plant (26)30–57(60) cm high, with (1.3)1.5–2.8(3.4) mm thick stem. Terminal rays 4–7, (2.4)4.2–10.8(15.5) cm long, (0.9) 1.7–4.2(5.4) times longer than the ray leaves. Mid-stem leaves (27) 32.7–53.9(60) × (7.5)10.1–18.2(19) mm, (1.6)2.4–3.8(4.7) times longer than wide, widest at 0.5–0.7(0.9) of their length, i.e. at (16) 18.8–35.2(38) mm from their base; 2 mm below their apex 0.2–0.5 as wide as at their widest parts. Ray leaves (18)23.7–41.7(46) × (6.5) 8.7–16.6(18) mm, 2 mm below their apex 0.2–0.5 as wide as at their widest parts. Raylet leaves cordate to triangular, (9.5)12.7–20.2(21) × (9)11.9–20.7(22) mm, (0.7)0.8–1.4(4.4) times longer than wide; 2 mm below their apex 0.2–0.4 as wide as at their widest parts. Cyathophylls (4.7)6.5–10.6(13) × (4.2)5.4–11.3(12.4) mm, 0.8–1.3(2.3) times longer than wide, widest at 1.4(2) –4.1(4.7) mm, i.e. at (0.2) 0.3–0.4(0.5) of their length; 2 mm below their apex 0.4–0.7(0.8) as wide as at their widest parts. Cyathial involucre (1.7)2–2.8(3.4) × (1.2) 1.3–1.9 mm. Cyathial glands (0.5)0.6–1.1(1.3) × (0.5)0.7–1.3(1.6) mm. Seeds (2)2.1–2.4(3.2) × (1.5)1.6–1.8(2) mm, 1.2–1.4(1.9) times longer than wide. $2n = 18$.

Distribution: Central part of species' distribution: northern Italy, eastern France, Switzerland, Liechtenstein, western Austria, Germany.

Euphorbia dulcis* subsp. *purpurata (Thuill.) Rothm. in Repert. Spec. Nov. Regni Veg. 67: 7 (1963) ≡ *E. purpurata* Thuill., Fl. Env. Paris, ed. 2: 235 (1799). — Lectotype (designated here): "Euhorbia (purpurata) perennis: fol. lanceolatis…" (P0551077! - http://mediaphoto.mnhn.fr/media/1441439469333JyAukNhNiE33htUu; excluding the 2nd plant from the left, which is *E. verrucosa*!). *Note:* even if there is no locality provided on the label (in the protologue "à Palaiseau" is written), the full description of the species by Thuillier is given that corresponds fully to the description in the protologue (https://books.google.pt/books?printsec=frontcover&pg=PA454&id=CDEZAAAAYAAJ&hl=de#v=onepage&q&f=false).

= *E. deseglisei* Boreau ex Boiss. in A.P.de Candolle, Prodr. 15(2): 128 (1862). ≡ *E. dulcis* var. *deseglisei* (Boreau ex Boiss.) P.Fourn. in Quatre Fl. France: 269 (1936) ≡ *E. angulata* subsp. *deseglisei* (Boreau ex Boiss.) Nyman in Consp. Fl. Eur.: 649 (1881). — Lectotype (designated here): "Cher, forét du Rhin du Bois" (G00441483!). Additional original material: "Cher, forét du Rhin du Bois" 20. Mai 1862 (P00600774: http://coldb.mnhn.fr/catalognumber/mnhn/p/p00600774)

Description: Plant (19)26.2–46.6(62) cm high, with (0.9)1.3–2.3(2.8) mm thick stem. Terminal rays 4–5(6), (1.5)3.9–9.6(11.5) cm long, (0.7) 1.4–4.2(5.5) times longer than the ray leaves. Mid-stem leaves (25) 32–49.4(70) × (8.4)9.6–14.4(18) mm, (2.1)2.9–4.2(4.5) times longer than wide, widest at (0.4)0.5–0.7 of their length, i.e. at (13)17.8–31(41) mm from their base; 2 mm below their apex (0.2)0.3–0.5 as wide as at their widest parts. Ray leaves (12)19.3–35.7(51) × (5.5)8–13.9(16.5) mm, 2 mm below their apex 0.3–0.5(0.6) as wide as at their widest parts. Raylet leaves cordate to deltoid, (8.9)9.3–17(19.2) × (9.1)10–17.4 (19.5) mm, (0.7)0.8–1.1(1.2) times longer than wide, 2 mm below their apex (0.2)0.3–0.5(0.6) as wide as at their widest parts. Cyathophylls (4.4)4.7–11.2(15.5) × (4.2)4.6–11.2(15.5) mm, 2 mm below their apex (0.3)0.4–0.9(1) as wide as at their widest part. Cyathial involucre (0.8) 1.8–3.2(3.9) × (1.2)1.3–1.9(3) mm. Cyathial glands 0.6–1.1(1.4) × (0.6)0.7–1.3(1.7) mm. Seeds (1.9)2–2.5(2.8) × (1.5)1.6–2(2.1) mm, (1.1)1.2–1.3(1.4) times longer than wide. $2n = 24$.

Distribution: Western part of species' distribution: France, western Switzerland, Luxembourg, western Germany (?), Spain, Portugal.

Funding

The work was supported by a bilateral cooperation between the Slovenian Research Agency (ARRS; BI-AT/23–24–023 to JDK) and the Austrian Agency for Education and Internationalisation (ÖAD; SI 06/2023 to Peter Schönswetter & BF), by the France-focus and the Italy-centre of the University of Innsbruck to BF, by the Young Researcher grant (ARIS No. 58170) and Program research group P4–0107 (Forest Biology, Ecology and Technology; both Slovenian Research Agency ARIS), Erasmus Internship Scholarship as well as Public Scholarship of the Development, Disability and Maintenance Fund of the Republic of Slovenia (all to MK).

CRediT authorship contribution statement

Frajman Božo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dolenc Koce Jasna:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition. **Kravanja Marija:** Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank all the colleagues listed in Supplementary Table S1 for the help with collecting samples, in particular to F. Andrieu, L. Carlón, M. Finner, C. Gilli, P. Koutecký, C. Oberprieler, A. Pahlevani, P. Schönswetter and L. Silbernagl for more than one collection. Inaturalist community contributed valuable locality data and L. Schratt Ehrendorfer, G. Amann, A. Beiser and M. Thalinger provided the distribution data for Austria. J. Paule (B), A. Grall and J. de Vos (BAS), C. Jarvis & M. Carine (BM), N. Bauer (BP), F. Leliaert (BR), L. Loze & F. Stauffer (G), M. Thalinger (IBF), H. Esser (M), C. Aupic and G. Rouhan (P), T. Agnese (RO), M. Hjertson (UPS) helped with the search of the original material. S. Španiel and M. De Groot helped with the literature. M. Magauer and D. Pirkebner helped with the lab work and V. Als, J. Amann, F. Rexhepi, G. Span, and C. Zudrell measured RGS of some samples. We are grateful to M. Bodner and his colleagues from the Botanical Gardens of the University of Innsbruck for successfully cultivating our living collection of *Euphorbia*.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ppees.2025.125861.

Data availability

The data are included as Supplementary Materials

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