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Genetic and morphological differentiation within *Euphorbia japygica* (Euphorbiaceae) suggests divergence of populations from the south-eastern Apennine Peninsula

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ABSTRACT

The Mediterranean Basin is a hotspot of animal and plant diversity. Contrary to the Balkan and Iberian Peninsulas that were subject of many phylogeographic studies in past decades, the Apennine Peninsula and, in particular, the diversification of its flora has been neglected in contemporary phylogenetic studies. The few available studies showed a complex pattern of north–south differentiation of genetic diversity in Italy, both among the Alps, the Apuan Alps, and the Apennines, as well as within the Apennines. Here, we explore phylogeographic patterns within recently described *Euphorbia adriatica*, distributed in the central and northern Apennine Peninsula, the southern margin of the Eastern Alps and the north-western Balkan Peninsula, and its relationship to the southern Italian *E. japygica*. Our integrative approach using nuclear ITS sequences, amplified fragment length polymorphisms, relative genome size estimation combined with chromosome counting, as well as multivariate morphometrics inferred a weak genetic differentiation that only partly corresponds to the morphological differentiation. Whereas all southern populations have hairy capsules characteristic for *E. japygica*, only those in south-eastern Italy (Puglia and Basilicata) are genetically divergent. There are, however, additional morphological characters that differentiate them from other populations. Our data are thus in favour of recognizing a single species, *E. japygica*, which includes *E. adriatica* that should be treated as a subspecies, named *E. japygica* subsp. *prostrata*. We provide a revised taxonomic treatment for *E. japygica*, including the typification. Our study shows the necessity of further in-depth investigations of diversification of Italian biota.

Keywords: Apennine Peninsula; AFLP; Balkan Peninsula; *Euphorbia adriatica*; *Euphorbia nicaeensis*; genome size; ITS; phylogenetic analyses; morphometry

INTRODUCTION

The Mediterranean Basin is one of the richest areas in the world in terms of animal and plant diversity and is considered one of the Earth's 25 biodiversity hotspots (Myers *et al.* 2000), hosting 24 000 plant species, 60% of which are endemic (Nieto Feliner 2014). In particular, the Iberian, Apennine, and Balkan peninsulas are important hotspots of genetic diversity (Petit *et al.* 2003) and areas of high endemism (Bilton *et al.* 1998, Thompson *et al.* 2005), due to their important role as glacial refugia during late 'Tertiary' and Quaternary climate fluctuations (Hewitt 2011).

The flora of the Apennine Peninsula—thus excluding the Alps and the Italian islands, which are rich in endemics (Aeschimann *et al.* 2011, Peruzzi *et al.* 2014)—appears to be less diverse than the one of the Balkan and the Iberian Peninsulas, especially in the number of endemic species (Hernández Bermejo and Herrera Molina 2005, Stevanović *et al.* 2007, Dimopoulos *et al.* 2013, Peruzzi *et al.* 2014, Buira *et al.* 2017, Bartolucci *et al.* 2018). This is partly attributable to the geologically younger age of the Apennine Peninsula; apart from small areas in north-western and south-eastern Italy, which are geologically old. The largest part of the Italian Peninsula owes its origin to the Tertiary alpine folding and the Apennine area was for a long period in the Tertiary either an island or a peninsula (Meulenkamp and Sissingh 2003).

Contrary to the Balkan (e.g. Đurović *et al.* 2021, Španiel and Rešetnik 2022) and Iberian (e.g. Gómez and Lunt 2007, Rodríguez-Sánchez *et al.* 2010) peninsulas that were subjects of many phylogeographic and phylogenetic studies in past decades, the Apennine Peninsula, and in particular the diversification of its flora, has been neglected in contemporary phylogenetic studies. Among the few studies including populations

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from different parts of the Apennine Peninsula, Cozzolino *et al.* (2003) revealed divergent plastid haplotypes in Tuscany, Gargano, and Murge in *Anacamptis palustris* Jacq. Ansell *et al.* (2008) showed two genetic pools corresponding to the Apuan Alps and the Apennines, respectively, using allozyme analyses of *Arabis alpina* L., and indicated divergent plastid haplotypes in the Gargano area. The AFLPs in the study of *Saxifraga callosa* Sm. (Grassi *et al.* 2009) inferred three distinct phylogroups corresponding to the Maritime Alps, the Apuan Alps, and the Apennines, whereas the plastid data showed a clear divergence between the Alps and the northern Apennines on the one, and the central and southern Apennines on the other hand. In *Alyssum diffusum* Ten., di-, tetra-, and hexaploid populations from the central Apennines (Abruzzo and Umbria) were divergent from diploid populations from the Gargano (Puglia) and tetraploid populations from the southern Apennines (Calabria, Basilicata) as indicated by AFLPs (Španiel *et al.* 2011). The plastid DNA analyses confirmed the genetic divergence among the three regions and showed that the central Apennine haplotype was derived from the southern Apennine haplotype. Also in oaks, a divergent plastid haplotype was found in *Quercus ilex* L. from Gargano (Lumaret *et al.* 2002) and the plastid DNA analyses of *Quercus robur* L., *Q. petraea* Matt., *Q. pubescens* Willd. *s.l.*, *Q. frainetto* Ten. (Fineschi *et al.* 2002) showed a clear split between the south-central and the northern part of the peninsula as well as the presence of a divergent haplotype in Gargano. In summary, different studies showed a north–south differentiation of genetic diversity, both among the Alps, the Apuan Alps, and the Apennines, as well as within the Apennines, and in many cases pointed to divergence of the Gargano populations. Accordingly, in a review summarizing 90 phylogeographic studies based on different molecular markers (e.g. mitochondrial, plastid, and nuclear DNA sequences, allozymes) and 66 animal and 12 plant species (Schmitt *et al.* 2021), a pronounced phylogeographic differentiation was revealed within Italy that was partitioned into 17 geographic areas. Specifically, northern Italy (Po Plain, Ligurian, and northern Tyrrhenian area, central Adriatic area) and southern Puglia (Murge, Salento and adjacent areas) were supported as most distinct biogeographical units, whereas the central Tyrrhenian area, Tavoliere delle Puglie, and Gargano showed a transitional character between the southern and the northern regions.

Cresti *et al.* (2019) and Caković *et al.* (2021) suggested isolated Pleistocene survival and thus divergence in three different species of the *Euphorbia verrucosa* L. group in the Iberian, Apennine, and Balkan peninsulas, respectively. In this group, only *E. verrucosa* considerably extended its distribution from the Balkan refugium and spread also across northern Italy, whereas the Apennine endemic *E. gasparrinii* Boiss. remained confined to the central Apennines and Sicily, where it underwent polyploidization (Peruzzi *et al.* 2018, Cresti *et al.* 2019). A similar scenario, however, with different range expansion was recently proposed for the *E. nicaeensis* All. and related species (Stojilkovič *et al.* 2022). Based on allopatric distributions, phylogenetic divergence indicated by RAD sequencing data and accompanied by relative genome size (RGS) and partly morphological diversification, Stojilkovič *et al.* (2022) separated the Apennine and the north-west Balkan populations of *E. nicaeensis* as a new species, *E. adriatica* Stojilkovič, Záveská, and Frajman, closely related to the western Mediterranean (Morocco, Iberia, southern France) *E. nicaeensis* and the central Balkan endemic *E. hercegovina* Beck. Their divergence was dated to the late Pleistocene, 0.4–0.6 Mya (95% Highest Posterior Densities, HPD, 0.1–1.1 Mya). In addition, the southern Italian populations of this group were tentatively treated as a distinct species, *E. japygica* Ten., as the single analysed population had increased RGS and was thus considered of polyploid origin, but Stojilkovič *et al.* (2022) also indicated that additional studies are needed to reveal whether all populations of this taxon are polyploid and to clarify its taxonomic status.

Euphorbia japygica was described by Tenore (1830) from hills around Lecce and Gravina in Puglia; 'Japygia' in the 19th century referred to southern part of Puglia (Lecce, Taranto, and Brindisi; Wagensommer *et al.* 2014). According to Tenore (1830), the species was similar to *E. nicaeensis* and *E. myrsinites* L., but it differed from them in having pubescent capsules, smooth seeds, dichotomous rays, and crenate nectarial glands ('petals') with appendices. Meanwhile, *E. myrsinites*, which is morphologically clearly divergent, was shown to belong to another section (Riina *et al.* 2013). Later, Boissier (1862) listed *E. japygica* as a form with pilose capsules in the synonymy of *E. nicaeensis*, whereas Nyman (1881) considered it a variety and Arcangeli (1882) a subspecies, *E. nicaeensis* subsp. *japygica* (Ten.) Arcang. This was followed by Palanza (1900), who listed several localities for this taxon in Puglia: Murge of Cassano, Toritto, Ruvo, Andria, Minervino, Gravina, and Altamura, where it thrives on dry gravelly soils. Fiori and Béguinot (1901), who had peculiar views on the taxonomy of *Euphorbia* (cf., Cresti *et al.* 2019, Stojilkovič *et al.* 2022), treated *E. japygica* as a subspecies of *E. seguieriana* Neck., along with *E. gerardiana* Jacq. and *E. nicaeensis*, and later (Fiori 1929) along with *E. prostrata* Ait. and *E. nicaeensis*, and listed it for Murge and Lecce in Puglia. Pignatti (1982, 2017) again treated *E. japygica* as subspecies of *E. nicaeensis* and listed it for Puglia. Such a treatment was applied in all recent Italian accounts (Conti *et al.* 2005, Del Guacchio 2010, Medagli *et al.* 2014, Fenu *et al.* 2016, Pignatti 2017, Bartolucci *et al.* 2018, Licht and Wagensommer 2020, Licht 2021), and hairy capsules constituted the main morphological character that differentiates it from typical *E. nicaeensis* (Fenu *et al.* 2016, Pignatti 2017), i.e. from the Italian populations now treated as *E. adriatica* (Stojilkovič *et al.* 2022). Based on this character, this taxon has also been reported—apart from Puglia—in the adjacent Gravina di Matera in Basilicata (Medagli and Gambetta 2003, Medagli *et al.* 2014), but also in the more distant Montenero at the Gargano Peninsula in Puglia (Licht 2008, 2021, Licht and Wagensommer 2020), as well as for Monte Polveracchio in Campania (Del Guacchio 2010). However, Fenu *et al.* (2016) neglected this last report and listed *E. japygica* only for Puglia (including Gargano) and Basilicata. On the other hand, geographically close populations from the central Apennines (Gran Sasso) that were considered to belong to *E. japygica* (Tammaro 1995) actually belong to *E. adriatica* according to Conti (2007).

Given the unclear phylogenetic origin and taxonomic status of *E. japygica* in relation to *E. adriatica*, we here use an integrative approach to disentangle the relationships between both taxa. Specifically, based on a sampling covering the entire distribution, we (i) investigate using RGS and chromosome number estimations whether the origin of *E. japygica* involved polyploidization,

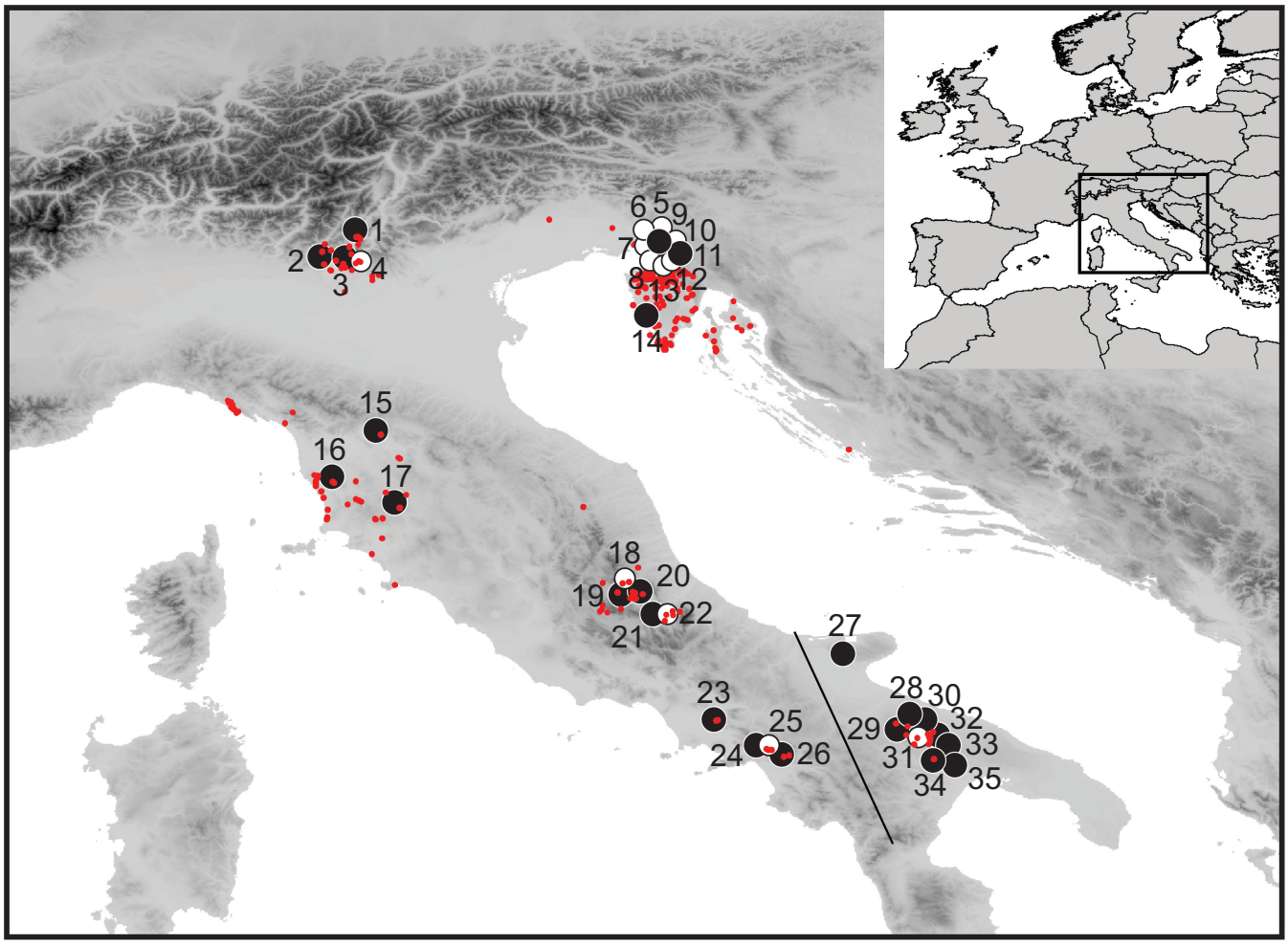

Figure 1. Distribution of populations of *Euphorbia japygica* subsp. *prostrata* (*E. adriatica;* populations 1–26) and *E. japygica* subsp. *japygica* (27–35) used in this study; the division between both subspecies is indicated with a line. Black circles indicate populations used in AFLP and partly also ITS, relative genome size (RGS), and morphometric analyses, and white circles those used in ITS, RGS, and/or morphometric analyses. Population numbers correspond to Supporting Information, Table S1. Red dots indicate distribution of additional populations based on different sources (see Materials and methods).

(ii) infer its phylogenetic position using nuclear ITS sequences, and (iii) explore its divergence from *E. adriatica* using amplified fragment length polymorphisms (AFLP) that also provide clues on the phylogeographic differentiation of both taxa. Finally, we (iv) explore morphological differentiation of both taxa using multivariate morphometrics, and based on all data we (v) provide a revised taxonomic treatment.

MATERIALS AND METHODS

Plant material and distribution data

Plant material for RGS estimation, molecular, and morphometric analyses was collected in the field between 2013 and 2022. Molecular and RGS analyses were based on silica-gel dried leaf material, and morphometric measurements were performed on herbarium vouchers. In total, 35 populations of *E. adriatica* and *E. japygica* were studied: 17 of these were included in ITS analyses (nine taken from Stojilkovič *et al.* 2022), 26 (14) in RGS analyses, 23 in AFLP, and 33 (20) in morphometric analyses, respectively (Fig. 1, Supporting Information, Table S1). In addition, two populations of *E. hercegovina*, and four of *E. nicaeensis* were included as outgroups in AFLP analyses. As the AFLP analyses showed an optimal separation of the populations into two groups (see Results), from here on for simplicity we apply the names *E. adriatica* and *E. japygica* for them.

Distribution data of *E. adriatica* and *E. japygica* presented in Figure 1, in addition to our own field records (Supporting Information, Table S1), include herbarium data from herbarium FI, occurrence data on iNaturalist (https://www.inaturalist.org) revised by B. Frajman, non-revised records published at Wikiplantbase for Italy (Peruzzi *et al.* 2023), as well as distributional data for Slovenia from www.bioportal.si partly published by Jogan *et al.* (2001) as well as those from Croatia from Flora Croatica Database (Nikolić 2023). Coordinates of all these additional occurrences are listed in Supporting Information, Table S2.

DNA extraction, ITS sequencing, and analyses of sequence data

Extraction of total genomic DNA and ITS sequencing were performed as described by Frajman and Schönswetter (2011), with the exception that the sequencing was carried out at Eurofins

Genomics (Ebersberg, Germany). Contigs were assembled, edited, and sequences aligned using Geneious Pro v.5.5.9 (Kearse *et al.* 2012). Base polymorphisms were coded using NC-IUPAC ambiguity codes. Sixty-eight ITS sequences of outgroup taxa (Genbank numbers in Supporting Information, Table S3) and nine of *E. adriatica* and *E. japygica* (Supporting Information, Table S1) were taken from Stojilković *et al.* (2022). Maximum parsimony (MP) and MP bootstrap (MPB) analyses were performed using PAUP v.4.0b10 (Swofford 2002) as described by Frajman *et al.* (2019). Bayesian analyses were performed using MrBayes v.3.2.1 (Ronquist *et al.* 2012) applying the HKY+Γ substitution model and the settings as in Frajman *et al.* (2019). We also produced a NeighbourNet with ITS sequences of *E. japygica* and *E. adriatica* using SplitsTree4 v.12.3 (Huson and Bryant 2006).

AFLP analyses

The AFLP procedure followed Vos *et al.* (1995) with the modifications described by Cresti *et al.* (2019). The three primers for selective PCR (fluorescent dye in parenthesis) were: EcoRI (Fam6)-ATC/MseI-CTG, EcoRI (Vic1)-AAG/MseI-CTT, and EcoRI (Ned2)-ACC/MseI-CAG. Here, 1.3 µL of the elution product was mixed with 10 µL of formamide and 0.13 µL of GeneScan 500 ROX (ThermoFisher Scientific, Waltham, MA, USA) and run on a 3130xl Genetic Analyzer (Applied Biosystems). One blank (DNA replaced by water) was included to test for contamination and 14 samples were used as replicates between the two PCR batches to test the reproducibility of the technique.

Electropherograms were analysed with Peak Scanner v.1.0 (Applied Biosystems) using default peak detection parameters except for using light peak smoothing. The minimum fluorescent threshold was set to 50 relative fluorescence units (RFUs). Automated binning and scoring of the AFLP fragments were performed using RawGeno 2.0-1 (Arrigo *et al.* 2009) for RStudio v.2022.12.0 + 353 (RStudio Team 2022) with the following settings: scoring range 75–500 bp, minimum intensity 100 RFUs, minimum bin width 1 bp, and maximum bin width 1.5 bp. Fragments with a reproducibility < 80% based on sample-replicate comparisons were eliminated. The error rate (Bonin *et al.* 2004) was calculated as the ratio of mismatches (scoring 1 vs. 0) to phenotypic comparisons in AFLP profiles of replicated individuals. A matrix of 87 individuals was finally produced and analysed as described next.

A neighbour-joining (NJ) analysis based on Nei-Li genetic distances (Nei and Li 1979) was conducted and bootstrapped (2000 pseudo-replicates) with TREECON v.1.3b (Van de Peer and de Wachter 1997), using *E. hercegovina* and *E. nicaeensis* for rooting. SplitsTree4 v.12.3 (Huson and Bryant 2006) was used to produce a NeighbourNet based on uncorrected P distances for *E. adriatica* and *E. japygica*, and non-hierarchical *K*-means clustering (Hartigan and Wong 1979) was performed using a script of Arrigo *et al.* (2010) in RStudio v.2022.12.0 + 353 (RStudio Team 2022). A total of 50 000 independent runs were performed (i.e. starting from random points) for each assumed value for *K* clusters ranging from 2 to 10. To select the best number of groups, the strategy proposed by Evanno *et al.* (2005) was used and the proportions of individuals assigned to *K*-means groups (within populations) were displayed on a map in ArcMAP v.10.8.2 (ESRI 2021).

Relative genome size measurements

RGS was measured using flow cytometry as described by Suda and Trávníček (2006). Nuclei of the sample and the reference standard *Bellis perennis* L. (2C = 3.38 pg; Schönswetter *et al.* 2007) were stained using 4′,6-diamidino-2-phenylindole (DAPI). The RGS was estimated for one to six (mostly three) individuals per population (see Supporting Information, Table S1). 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 of the standard and sample peaks. The RGS was calculated as the ratio between the values of the mean relative fluorescence of the sample and the standard.

For statistical analyses of RGS data, RStudio v.2022.12.0 + 353 (RStudio Team 2022) with the visualization package 'ggplot2' was used. Scatter plots were produced for all samples and box plots for both taxa. RGS values of *E. japygica* and *E. adriatica* were tested for normality and homogeneity of variance, after excluding the outlier population 30 of *E. japygica* with much higher RGS; significance of differences was tested using a *t*-test. The population 30 was the only one of *E. japygica* included in the study of Stojilković *et al.* (2022) and based on its divergent RGS considered polyploid. To show its divergent RGS experimentally, we simultaneously isolated, stained, and measured the RGS of individuals from population 30 with higher RGS and population 28 with RGS in the range of all other samples, using *Pisum sativum* L. (2C = 8.84 pg; Greilhuber and Ebert 1994) as standard.

Chromosome number estimation

Seeds of population 26 of *E. adriatica* collected in the field were germinated. After the removal of the caruncle, seeds were incubated on filter paper in Petri dishes. Seeds were sterilized with a mix of bleach and water 1:3 for 10 min and then incubated on sterile paper to inhibit the growth of fungi. Root tips of germinated seeds were pre-treated with 0.002 M colchicine for 2 h at room temperature and then for 2 h at 4°C, then fixed in Carnoy solution (3:1 ethanol:acetic acid) for 24 h at 4°C and then stored in ethanol at −21°C.

Hydrolysis was performed in 5 N HCl at room temperature for 60 minutes. The tips were stained with Feulgen's reagent, kept in darkness at room temperature for 2 hours and then rinsed with water. Spreads were prepared by squashing the stained meristem in a drop of 45% acetic acid under the coverslip. Microscopic slides were then dry-ice frozen, dehydrated with 96% ethanol for 5 minutes, and air-dried. Chromosomes were counted with a Wild Leitz microscope 020-437-035 (Leitz GmbH, Oberkochen, Germany); images were acquired with a Canon Power Shot S45 camera (Canon, Krefeld, Germany) and processed using Canon Utilities RemoteCapture v.2.7.5.27.

Morphometric analyses

We performed morphometric analyses of 25 individuals from 13 populations of *E. japygica* and eight individuals from four populations of *E. adriatica*, and supplemented them with the morphometric data for 20 individuals from 20 populations of *E. adriatica* from Stojilković *et al.* (2022). In addition, for five individuals of *E. adriatica* from Stojilković *et al.* (2022), we added data for fruit

characters. In total, 34 characters were measured and 15 ratios were calculated (Table 1). Stem and leaf characters were measured manually. All other characters (cyathium, fruit, and seed characters) were measured on images taken with a stereomicroscope Olympus SZX9 (Olympus GmbH, Hamburg, Germany) using the Olympus image analysis software Analysis Pro. Three individuals of *E. japygica*, two of which belong to the same population, had no fruits and two populations had no seeds. In *E. adriatica,* fruit characters were missing for 11 individuals, and seed characters for 16 individuals.

Statistical analyses were performed using SPSS v.24.0 (IBM Corp., Armonk, NY, USA). Correlation among metric characters was tested using Pearson and Spearman correlation coefficients, which exceeded 0.9 in one character pair: length of a middle stem leaf – distance from the base to the widest part of a middle stem leaf. Thus, the latter character was excluded from further analyses. Box plot diagrams were produced for all characters to visualize and show the variation among the four AFLP K-means groups. Since not all morphologically studied populations were included in AFLP analyses, we included those populations in *K*-means groups based on geographic proximity of genetically analysed samples. After standardization to zero mean and one unit variance, principal component analysis (PCA) was performed. Subsequently, discriminant analysis (DA) was performed. The PCA and DA analyses were performed separately for (i) vegetative parts of the plants and cyathium characters, and (ii) for fruit and seed characters.

Based on the morphometric data, we produced taxon descriptions and an identification key. Metric values presented there correspond to the 10th and 90th percentiles, supplemented by extreme values in parentheses.

Elevational distribution

We produced box plots of elevational distribution for both taxa with the visualization package 'ggplot2' in RStudio v.2022.12.0 + 353 (RStudio Team 2022), which was also used for the statistical analyses. Elevational distribution data of *E. japygica* and *E. adriatica* (Supporting Information, Table S1) were tested for normality and homogeneity of variance, after excluding the outlier population 27 of *E. japygica* from Gargano that occurs at 900 m a.s.l. Significance of differences was tested using the Kruskal–Wallis test. The tests were performed for the complete dataset, and separately for the southern populations of *E. adriatica* that occur at similar latitudes as *E. japygica*.

RESULTS

Phylogenetic relationships based on ITS

The trees inferred by parsimony and Bayesian analyses (Supporting Information, Fig. S1) were largely congruent, but the relationships were poorly resolved with low support values. Accessions of *E. adriatica* and *E. japygica* were positioned in a polytomy (PP 0.85) with *E. hercegovina*, *E. nicaeensis*, and one accession of *E. glareosa*, as well as a clade (PP 0.96) with *E. erythrodon* and *E. macroclada*.

The NeighbourNet of *E. adriatica* and *E. japygica* (Fig. 2) showed an unclear geographic structure in the variation of ITS sequences. Most populations were positioned in the centre of the network, with most southern populations (with the exception of population 32) positioned on one side of the mostly linear network, and most northern on the other side. Four populations of *E. japygica* (27, 30, 32, 35) positioned in this central part of the network corresponded to three different ribotypes. Four populations, three of *E. adriatica* (3, 4, 6), and one of *E. japygica* (29) were clearly more divergent.

Phylogenetic relationships based on AFLPs

A total of 251 fragments were scored in 87 individuals; 21 fragments were excluded because they were present or absent in a single individual only. The error rate (Bonin *et al.* 2004), calculated before the exclusion of non-reproducible fragments and based on 12 replicates, was 4.5%.

In the NJ tree (Supporting Information, Fig. S2), most of the clusters had low bootstrap support (BS), with the exception of some terminal clusters mostly including individuals of the same populations that had moderate to high support. In addition, the cluster including *E. adriatica*, *E. hercegovina*, and *E. japygica* had BS 98%, whereas all populations of *E. nicaeensis* were in the outgroup. All accessions of *E. hercegovina* and *E. japygica*, respectively, formed their own clades, but with BS < 50%; only one individual from population 28 of *E. japygica* was nested within *E. adriatica*. The populations of *E. adriatica* were in two main clades with BS < 50%, one including populations 20 and 21, and the other all other populations.

Non-hierarchical *K*-means clustering revealed an optimal separation into two groups (Fig. 3A), one including all populations from Puglia and adjacent Basilicata that pertain to *E. japygica* (green), and the other including all other populations, pertaining to *E. adriatica* (red). The population 27 from Gargano included three individuals that belonged to the former and one to the latter group. With increasing *K*, only the group of *E. adriatica* was further divided. At $K = 3$ (Fig. 3B) northern (Tuscany) and southern (Campania) Apennine populations mostly belonged to one cluster, and those from the central Apennines (Abruzzo) as well as the Southern Limestone Alps (Lombardia, Trentino, Veneto) and Istria (Friuli Venezia Giulia in Italy, Slovenia, Croatia) to the other; different individuals from several populations belonged to divergent clusters. Finally, at $K = 4$ (Fig. 3C) the populations from the southern Apennines (Campania) formed their own cluster.

The NeighbourNet (Fig. 3D) was star-like and reflected the structure indicated by *K*-means clustering. Populations belonging to the blue and green groups were most clearly divergent, whereas those from the yellow and red groups were less differentiated.

Relative genome size and chromosome number

RGS values were continuously distributed and ranged between 1.097 and 1.194 in *E. adriatica*, and between 1.126 and 1.179 in *E. japygica*, with the exception of population 30 that had a divergent RGS of 1.786 (Fig. 4A, B), which was confirmed by the occurrence of double peaks in simultaneously measured samples from populations 28 and 30 (Fig. 4C). This population was thus excluded from statistical analysis. The RGS values of both taxa were normally distributed (Shapiro–Wilk test, $P = 0.9087$ for *E. adriatica* and $P = 0.5757$ for *E. japygica*) and the variance among species was homogeneous (Levene's test, $P = 0.278$). The differences in RGS between both taxa were not significant

Table 1. Characters studied in the morphometric analyses of *Euphorbia adriatica* and *E. japygica.*

Number	Character
	Stem
1	Stem length, cm
2	Stem width, cm
3	Stem glabrous/pubescent
	Pleiochasium
4	Number of terminal rays
5	Length of (the longest) terminal ray, cm
6	Number of branchings of (the longest) terminal ray
	Axillary rays
7	Number of fertile axillary rays
8	Length of (the longest) fertile axillary ray, cm
	Middle stem leaf
9	Length of a middle stem leaf, cm
10	Width of a middle stem leaf, cm
11	Ratio of length of a middle stem leaf:width of a middle stem leaf
12	Distance from the base to the widest part of a middle stem leaf, cm
13	Ratio of distance from the base to the widest part of a middle stem leaf:length of a middle stem leaf
	Ray leaves
14	Length of a ray leaf, cm
15	Width of a ray leaf, cm
16	Ratio of length of a ray leaf:width of a ray leaf
17	Distance from the base to the widest part of a ray leaf, cm
18	Ratio of distance from the base to the widest part of a ray leaf:length of a ray leaf
	Raylet leaves
19	Length of a raylet leaf, cm
20	Width of a raylet leaf, cm
21	Ratio of length of a raylet leaf:width of a raylet leaf
22	Distance from the base to the widest part of a raylet leaf, cm
23	Ratio of distance from the base to the widest part of a raylet leaf:length of a raylet leaf
	Cyathium
24	Length of cyathial involucre, mm
25	Width of cyathial involucre, mm
26	Ratio of length of cyathial involucre:width of cyathial involucre
27	Depth of gland emargination, mm
28	Length of cyathial gland, mm
29	Width of cyathial gland, mm
30	Ratio of depth of gland emargination:length of cyathial gland
31	Ratio of length of cyathial gland:width of cyathial gland
	Fruit
32	Fruit length, mm
33	Fruit width, mm
34	Ratio of fruit length:fruit width
35	Distance from the base to the widest part of the fruit, mm
36	Ratio of distance from the base to the widest part of the fruit:fruit length
37	Style length, mm
38	Fruit glabrous/pubescent/glandular
39	Number of hairs per fruit valve
	Seed
40	Seed length, mm
41	Seed width, mm
42	Ratio of seed length:seed width
43	Distance from the base to the widest part of a seed, mm
44	Ratio of distance from the base to the widest part of a seed:seed length
45	Caruncle length, mm
46	Caruncle width, mm
47	Ratio of caruncle length:caruncle width
48	Distance from the base to the widest part of caruncle, mm
49	Ratio of distance from the base to the widest part of caruncle:caruncle length

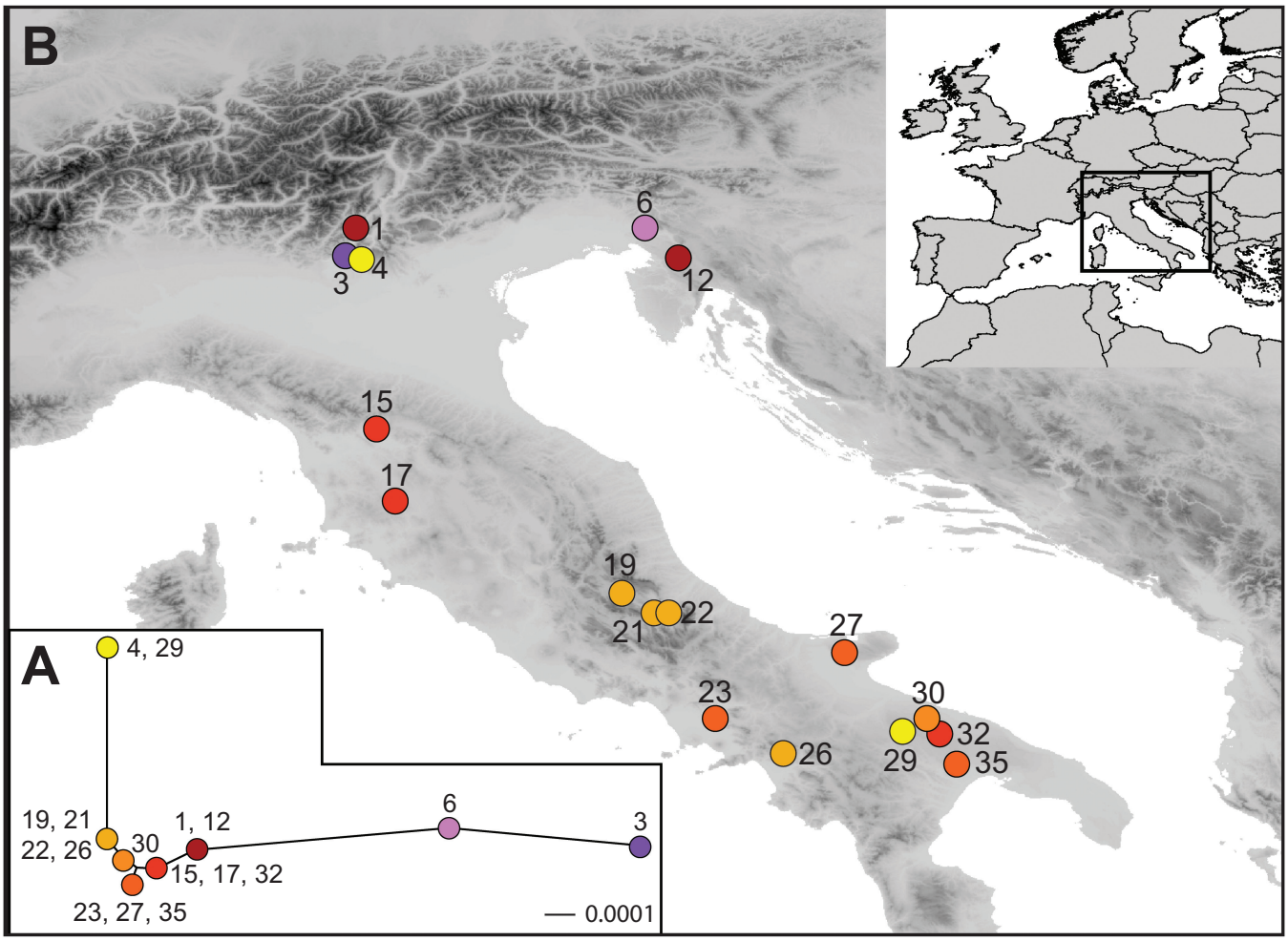

Figure 2. A, NeighbourNet of Internal Transcribed Spacer (ITS) sequences indicating phylogenetic relationships between *Euphorbia japygica* subsp. *prostrata (E. adriatica)* and *E. japygica* subsp. *japygica*. B, geographic distribution of the ribotypes. Population numbers correspond to Supporting Information, Table S1 and Figure 1.

(*t*-test; $P = 0.4743$). We counted 18 chromosomes ($2n = 18$) for population 26 of *E. adriatica* (Fig. 5).

Morphological differentiation

Morphological character states are in Supporting Information, Table S4. For vegetative and cyathium characters, the PCA scatter plot (first three components explaining 26.1, 16.5, and 9.9% of the total variation) showed a separation trend of the green and yellow *K*-means groups from the blue and red groups along the first component and a separation trend of the green and blue groups from the yellow and red groups along the second component, although with strong overlap (Fig. 6A). The characters that contributed most to the separation along the first component, i.e. those having the highest component scores (between 0.65 and 0.90), were length of (the longest) terminal ray, length of (the longest) fertile axillary ray, length of a middle stem leaf, length of a ray leaf, width of a ray leaf, distance from the base to the widest part of a ray leaf, length of a raylet leaf, width of a raylet leaf, and distance from the base to the widest part of a raylet leaf. Along the second component, the characters that contributed most to the separation (scores between 0.58 and 0.79) were width of the stem, number of fertile axillary rays, ratio of the distance from the base to the widest part of a middle stem leaf to the length of a middle stem leaf, width of cyathial involucre, ratio of the length of cyathial involucre to the width of cyathial involucre, depth of gland emargination, and length of cyathial gland. The DA histogram (Fig. 6B) showed a weak overlap between *E. adriatica* and *E. japygica* (Wilks' lambda = 0.13, $\chi^2 = 58.23$, d.f. = 29, $P < 0.001$). Variables with the highest discriminant loadings were width of a raylet leaf, length of cyathial involucre, width of cyathial involucre, ratio of the length of cyathial involucre to the width of cyathial involucre, depth of gland emargination, length of cyathial gland, and the ratio of the length to the width of the cyathial gland. In addition, box plots (Supporting Information, Fig. S3) revealed that *E. adriatica* had longer terminal and axillary rays and bigger ray and raylet leaves as well as cyathial glands. On the other hand, *E. japygica* had more axillary rays.

For the fruit and seeds characters, the PCA (first three components explaining 34.4, 16.2, and 11.7% of the total variation; Fig. 6C) showed a separation trend of the red and yellow groups from the green and blue groups along the first component, but an overlap along the second component. The characters contributing most to the separation along the first component,

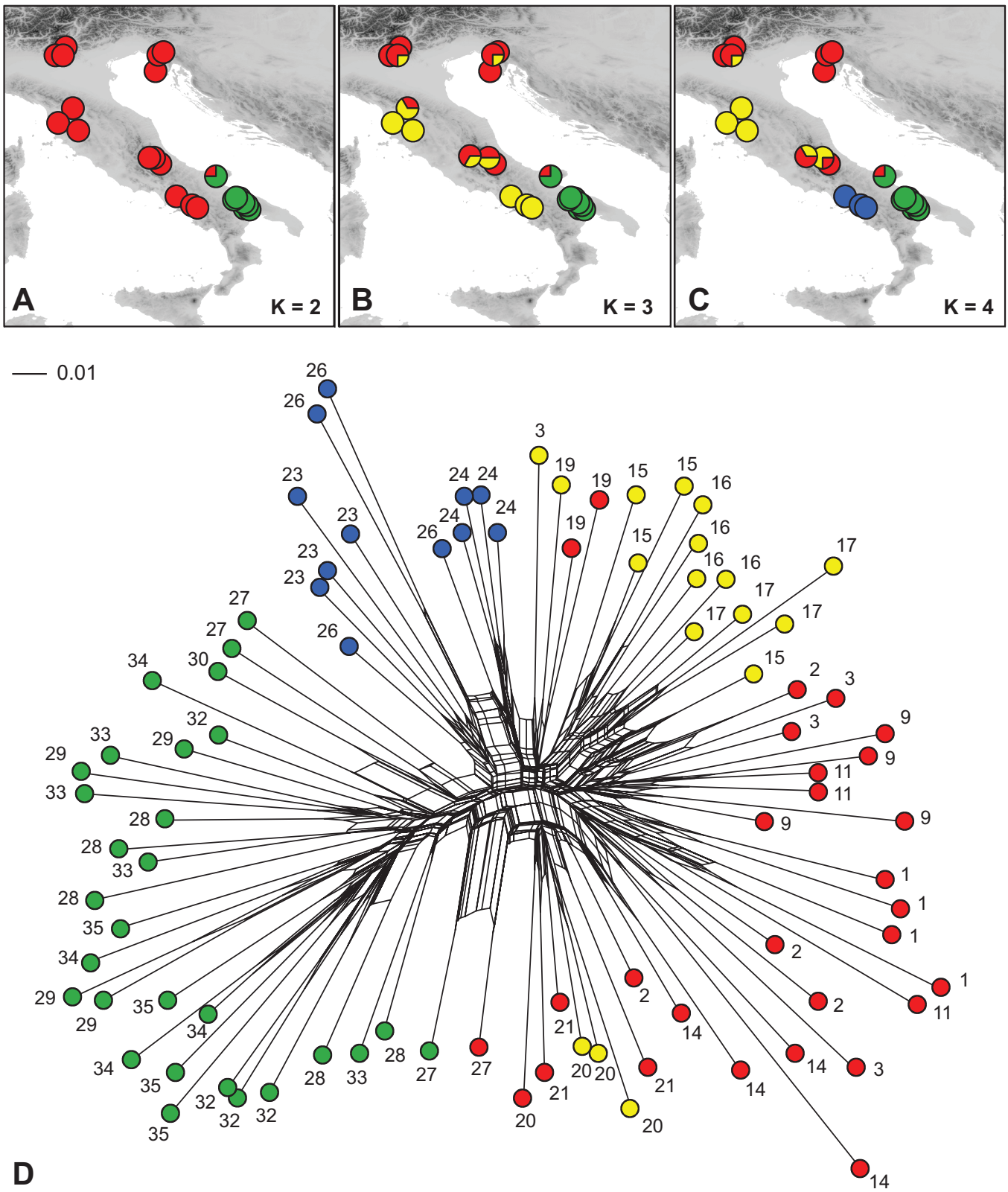

Figure 3. Phylogenetic relationships among populations of *Euphorbia japygica* subsp. *prostrata (E. adriatica)* and *E. japygica* subsp. *japygica* inferred by AFLP fingerprinting. A–C, Geographical distribution of phylogroups inferred by non-hierarchical *K*-means clustering at $K = 2$ (optimal division), $K = 3$ and $K = 4$. D, NeighbourNet based on uncorrected P distances; yellow, red, blue, and green dots indicate the four groups inferred at $K = 4$ (as in C). Population numbers correspond to Supporting Information, Table S1 and Figure 1.

i.e. those having the highest component scores between 0.6 and 0.89, were fruit width, ratio of the fruit length to the fruit width, distance from the base to the widest part of the fruit, fruit indumentum, number of hairs per fruit valve, ratio of the seed length to the seed width, and the ratio of the distance from the base to the widest part of the caruncle to the caruncle length.

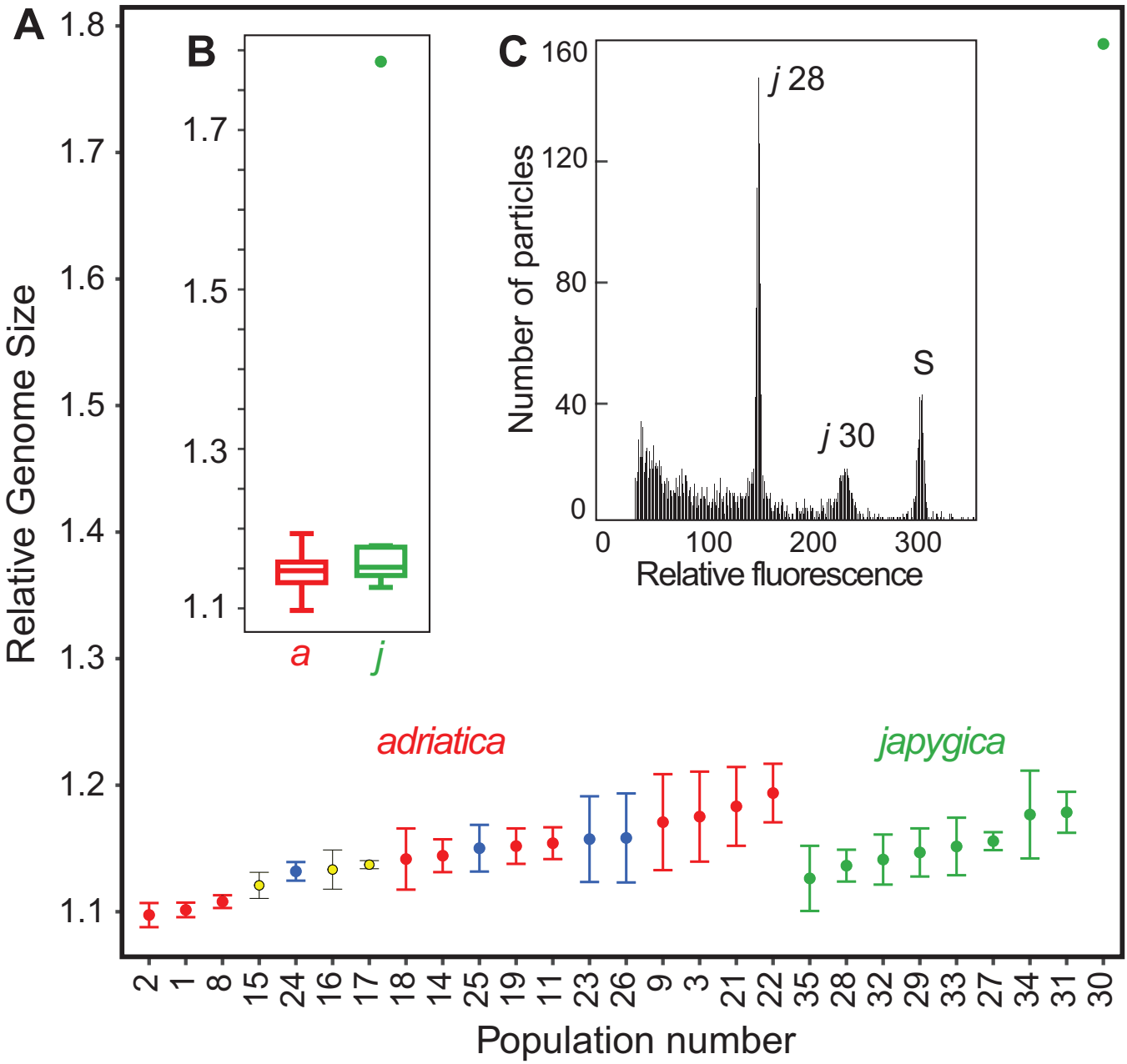

Figure 4. Relative genome size (RGS) variation in *Euphorbia japygica* subsp. *prostrata (E. adriatica)* and *E. japygica* subsp. *japygica*. A, RGS variation in all populations showing means ± standard deviation; colours correspond to the groups inferred by non-hierarchical *K*-means clustering at $K = 4$ (as in Fig. 3C). B, RGS variation per taxon; *a*, *E. j.* subsp. *prostrata*, *b*, *E. j.* subsp. *japygica*. C, Flow cytometry histogram of simultaneous measurement of RGS of pooled samples from populations 28 and 30 of *E. japygica* measured together with *Pisum sativum* as an internal standard (S). Population numbers correspond to Supporting Information, Table S1 and Figure 1.

Along the second component, the characters that contributed most to the separation (scores between 0.5 and 0.88), were seed length, seed width, distance from the base to the widest part of a seed, caruncle length, caruncle width, and the ratio of the caruncle length to the caruncle width. The DA histogram (Fig. 6D) showed an overlap between the two taxa (Wilks' lambda = 0.257, $\chi^2 = 47.502$, d.f. = 16, $P < 0.001$). Variables with the highest discriminant loadings were seed width, ratio of seed length to seed width, distance from the base to the widest part of a seed, ratio of the distance from the base to the widest part of a seed to the seed length, caruncle length, and caruncle width.

In addition, box plots (Supporting Information, Fig. S3) revealed that *E. japygica* had wider fruits, and consequently, a lower ratio of fruit length to fruit width. All populations of *E. japygica* had hairy fruits, whereas in *E. adriatica* fruits were hairy in all populations from Campania as well as in one population from Abruzzo. Lengths of the seeds and consequently the ratio of the seed length to seed width were higher in *E. adriatica*, while *E. japygica* had higher values in caruncle length and width.

Elevational distribution

Euphorbia adriatica was distributed between 109 and 1535 m a.s.l., whereas *E. japygica* was between 368 and 552 m a.s.l.,

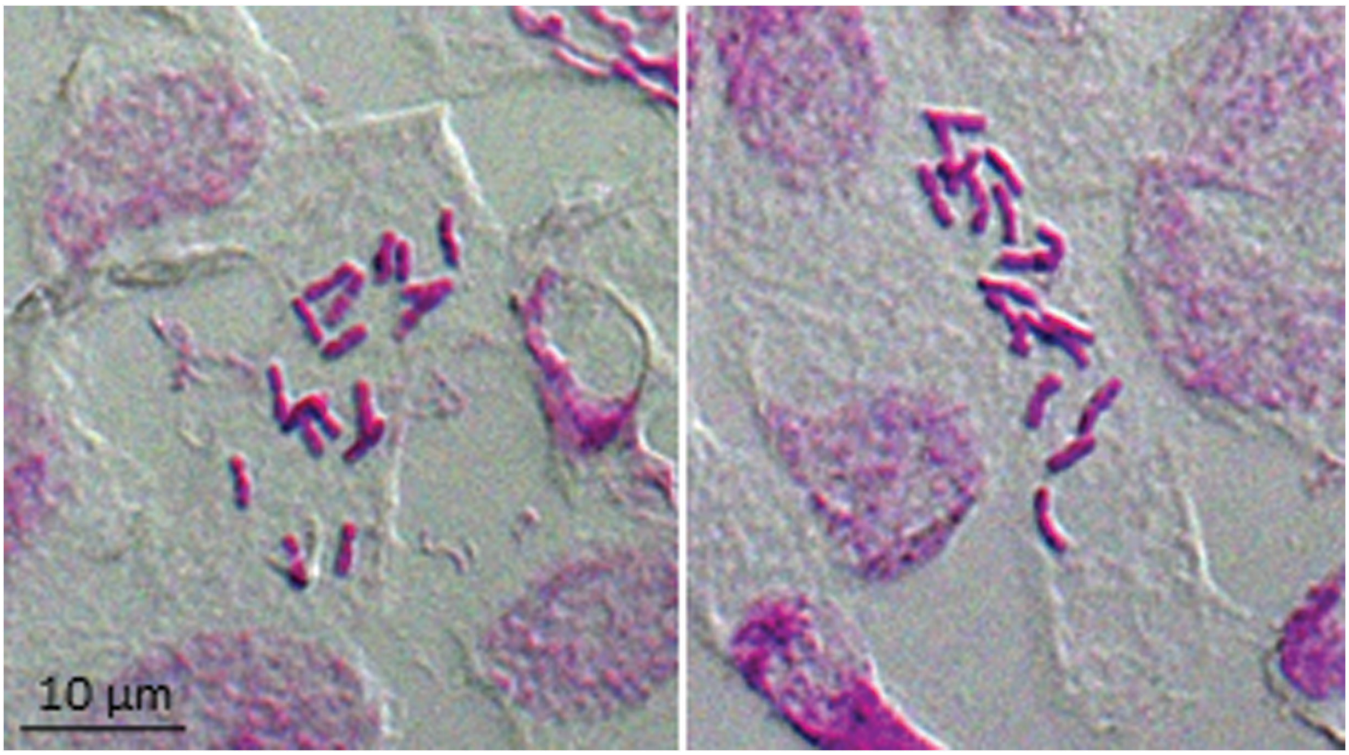

Figure 5. Metaphase plates of *Euphorbia japygica* subsp. *prostrata (E. adriatica)* from population 26 with $2n = 18$.

with the exception of population 27 from Gargano that occurs at 900 m a.s.l. (Fig. 7). This outlying population was thus excluded from statistical analyses. The elevation values of both taxa were normally distributed (Shapiro–Wilk test, $P = 0.1003$ for *E. adriatica* and $P = 0.5327$ for *E. japygica*) and the variance among species was not homogeneous (Levene's test, $P = 0.005663$). The differences in elevation between both taxa were not significant (Kruskal–Wallis test; $P = 0.4982$), although the elevational distribution was much wider in *E. adriatica* compared to *E. japygica*. On the contrary, when considering only the southern populations of *E. adriatica* from Abruzzo and Campania, their elevational distribution was significantly different from that of *E. japygica* that occurs at similar latitudes (Kruskal–Wallis test; $P = 0.0007518$). *Southern* populations of *E. adriatica* were recorded at higher elevations, between 505 and 1535 m a.s.l. Also in this case, the elevation vales were normally distributed (Shapiro–Wilk test, $P = 0.8847$) and the variance among species was not homogeneous (Levene's test, $P = 0.04605$).

DISCUSSION

Our study revealed a pronounced genetic differentiation among the populations of the *E. nicaeensis* alliance from the Apennine Peninsula, the southern margins of the Eastern Alps and the north-western Balkan Peninsula, but did not confirm the hypothesis of Stojilkovič *et al.* (2022) that the southern Italian populations from Puglia and Basilicata are of polyploid origin. On the contrary, with the exception of population 30 that was the only population of *E. japygica* studied by Stojilkovič *et al.* (2022) and that exhibits higher RGS, all other populations had uniformly lower RGS similar to RGS of *E. adriatica* (Fig. 4). This suggests that they are all diploid with 18 chromosomes as estimated here for population 26 of *E. adriatica* (Fig. 5) and previously for closely related *E. nicaeensis* (Perry 1943, Löve 1978). The deviating RGS of the single studied individual of population 30 remains intriguing and might be due to local polyploidization detected also in single populations of *E. spinosa* L. (Stevanoski *et al.* 2020) and *E. gasparrinii* (Peruzzi *et al.* 2018) and in some populations of *E. montenegrina* (Caković *et al.* 2021). In addition, accumulation of retrotransposons and other repetitive elements is considered a main driver of genome size increase in different angiosperms alongside polyploidy (Pellicer *et al.* 2018), for instance leading to a 2-fold increase in GS in the wild rice relative *Oryza australiensis* (Piegu *et al.* 2006), and could also be responsible for the increased RGS in the population 30.

Phylogeographic patterns within the Apennine Peninsula

Our AFLP data (Fig. 3) indicated that the populations from Puglia and Basilicata in southern Italy are most divergent in respect to other areas. These populations pertain to *E. japygica* described by Tenore (1830) from this area and point to the phylogeographic peculiarity of this region. Distinct phylogeographic lineages in central and southern Puglia (Murge, Salento and adjacent areas) have been also revealed in different animals and some plants (Cozzolino *et al.* 2003, Schmitt *et al.* 2021) and this area appears to be one of the most distinct phylogeographic regions in Italy (Schmitt *et al.* 2021) that harbours several endemic taxa (Bianco *et al.* 1994, Perrino *et al.* 2006, Wagensommer *et al.* 2014, 2020), including *E. japygica*. Similarly to Murge, also the Gargano Peninsula in northern Puglia is renowned for its high endemism (Sbordoni and Cobolli-Sbordoni 1973, Tornadore *et al.* 2023 Sbordoni and Cobolli-Sbordoni 1973, Brullo *et al.* 2009) and was suggested to be one of the 52 putative refugia within the Mediterranean region (Médail and Diadema 2009). However,

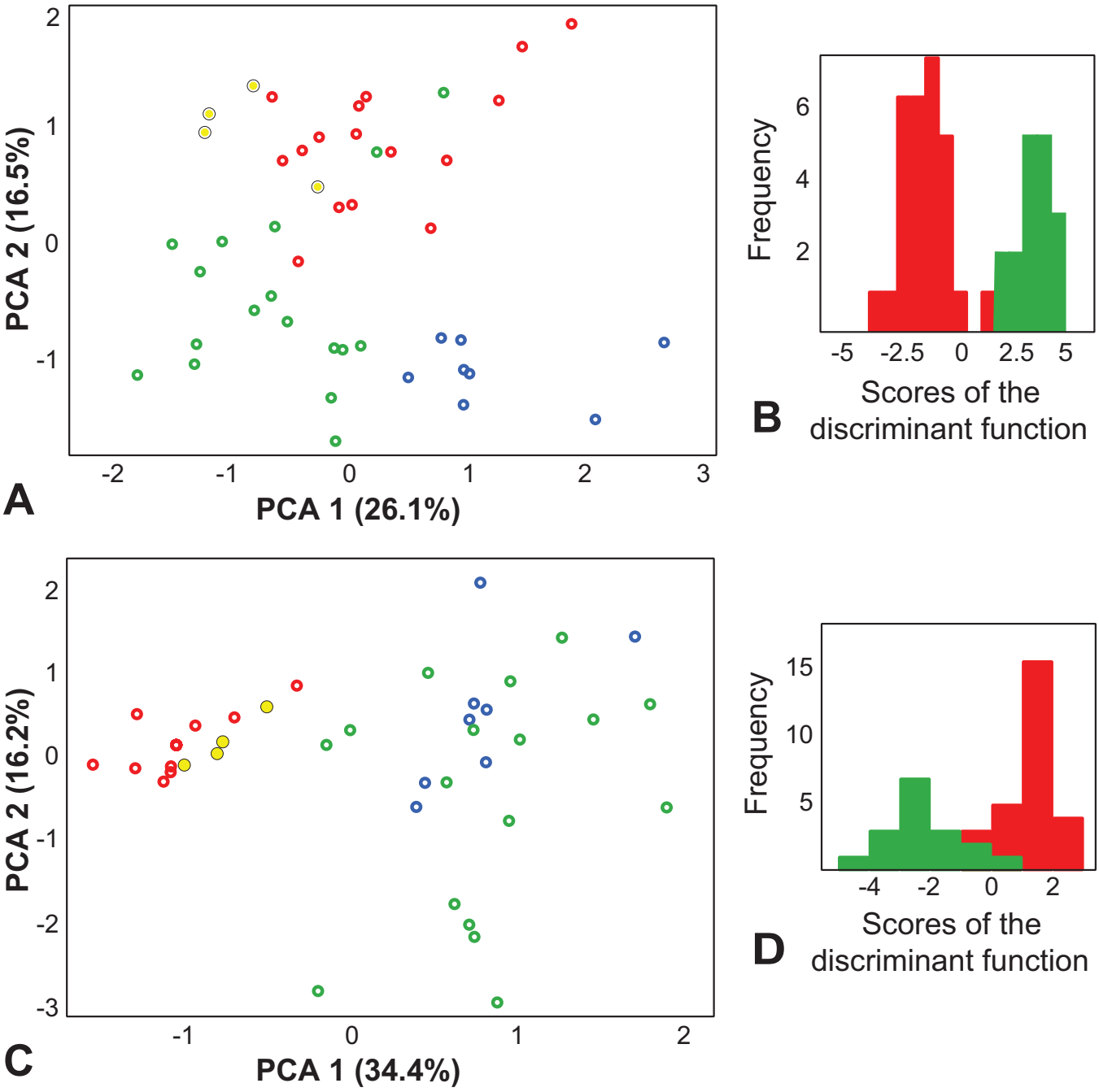

Figure 6. Morphological differentiation between *Euphorbia japygica* subsp. *prostrata (E. adriatica)* and *E. japygica* subsp. *japygica* based on 33 metric characters and 15 ratios. A, Principal component analysis (PCA) scatterplot and B, discriminant analysis (DA) histogram of vegetative and cyathium characters. C, PCA scatterplot and D, DA histogram of fruit and seed characters. Colours in A and C correspond to groups inferred by non-hierarchical *K*-means clustering at $K = 4$ (as in Fig. 3C), whereas in B and D they correspond to *E. j.* subsp. *prostrata* (*E. adriatica;* red) and *E. j.* subsp. *japygica* (green).

contrary to several other studies that inferred deep genetic divergence of Gargano populations (Lumaret *et al.* 2002, Cozzolino *et al.* 2003, Ansell *et al.* 2008, Španiel *et al.* 2011, Schmitt *et al.* 2021), our study identified their similarity to the population from Murge in the case of *E. japygica*. Nevertheless, the Gargano population appears to be genetically mixed, as one individual grouped with the populations of *E. adriatica* by *K*-means clustering (Fig. 3), which indicates gene flow between Gargano populations and the geographically closest populations of *E. adriatica* in Abruzzo, similarly as shown for *Fagus sylvatica* L. (Vettori *et al.* 2004). Interestingly, the Gargano population of *E. japygica* is growing at much higher altitude (900 m) than all other populations of *E. japygica* that grow in lowlands of Murge. On the contrary, all southern populations of *E. adriatica* from Abruzzo and Campania grow at much higher elevations in mountainous areas (Fig. 7), which suggests that genetic differentiation revealed by AFLPs is also accompanied by ecological differentiation between *E. japygica* and *E. adriatica* in the south of its distribution.

The second main AFLP cluster included most of the investigated populations pertaining to *E. adriatica*. It is characterized

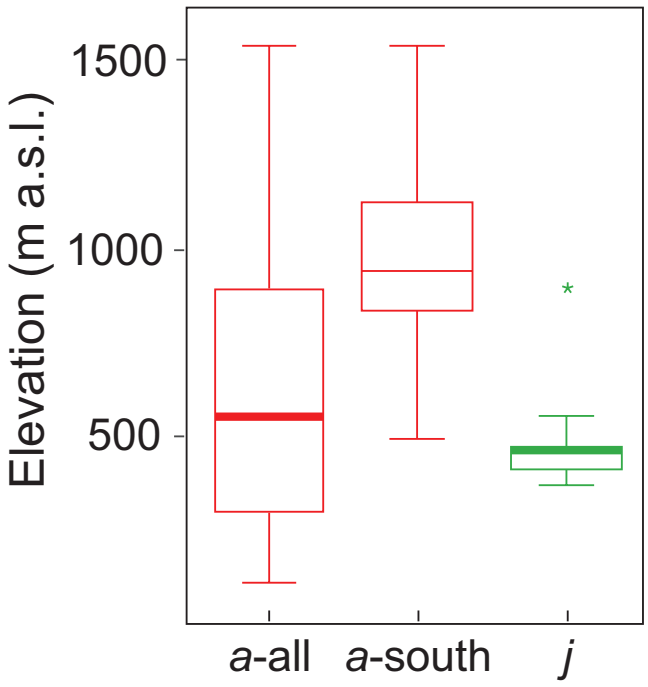

Figure 7. Elevational distribution of *Euphorbia japygica* subsp. *prostrata (E. adriatica, a*, red) and *E. japygica* subsp. *japygica* (*j*, green). For *E. j.* subsp. *prostrata (E. adriatica)* the elevational distribution of all populations (*a*, all) is shown, as well as separately for the southern populations from Abruzzo and Campania (*a*, south).

by a weak genetic differentiation among groups of populations from disjunct areas across Italy and the north-western Balkan Peninsula. Even if divergence of the populations from Campania was indicated only at $K = 4$, these populations were distinct and connected by several shared splits in the NeighbourNet, where they had an intermediate position between the Apulian *E. japygica* and the more northern populations of *E. adriatica* (Fig. 3D). Also in *Alyssum diffusum*, the tetraploid populations from the southern Apennines (Calabria, Basilicata) were clearly divergent and probably the source for the colonization of the central Apennines (Španiel *et al.* 2011). On the contrary, in other studied plants, the southern Apennine populations rather appeared more closely related to the Apulian populations and the rest of southern Italy, whereas the more northern populations were more divergent (Leonardi and Menozzi 1995, Fineschi *et al.* 2002, Heuertz *et al.* 2006, Grassi *et al.* 2009, Schmitt *et al.* 2021).

Traces of genetic admixture between the red and yellow AFLP clusters and their clustering on one side of the NeighbourNet (Fig. 3) indicate strong phylogeographic connections among the central and northern Apennines (including Tuscany), the southern margin of the Eastern Alps and the north-western Balkan Peninsula, suggesting that all these areas shared a common Pleistocene refugium. Alternatively, two refugia, one possibly positioned west of the Apennine main chain in Tuscany, and the other to the north-east of the Apennines, giving rise to the central Apennine, Alpine and Balkan populations with subsequent contacts and gene flow between both groups in the Holocene could also explain the observed pattern. The divergence among *E. adriatica*, *E. hercegovina*, and *E. nicaeensis* was dated to the late Pleistocene, 0.4–0.6 Mya (95% HPD 0.1–1.1 Mya; Stojilkovič *et al.* 2022) and the pattern revealed in our study is thus a bit younger, probably pertaining to one of the last glaciations.

Also, the ITS data, with exception of populations 4 and 29 that shared a divergent ribotype, showed a geographic pattern of genetic differentiation, although less clear (Fig. 2). In this case, especially the northern populations 3 and 6 from the southern margin of the Eastern Alps and the Balkan Peninsula were most divergent, whereas all other populations had more similar ribotypes. The divergence of more northern populations might be the result of genetic drift that more strongly affects single markers than genome-wide derived AFLPs, possibly as a result of founder effects during colonization of these areas from glacial refugia. Also in other closely related species the weak genetic differentiation indicated by ITS only partly corresponded to phylogenomic patterns indicated by RAD sequencing data (Stojilkovič *et al.* 2022).

In summary, our phylogeographic data revealed a clear divergence of the southern Italian populations, especially those from Puglia and neighbouring Basilicata, and to a lesser extent those from the southern Apennines in Campania. This partly corresponds to patterns previously revealed for some plant species (e.g. Cozzolino *et al.* 2003, Španiel *et al.* 2011), but also contrasts with some studies that indicated more pronounced genetic differentiation of northern Italian populations (e.g. Cozzolino *et al.* 2003, Ansell *et al.*; 2008, Grassi *et al.* 2009). The complexity that was revealed on one hand and the paucity of phylogeographic studies of the Apennine plants on the other underlines the necessity of similar studies ranging across the Apennine Peninsula and beyond.

Taxonomic considerations: one or two species?

Stojilkovič *et al.* (2022) recently separated the central and northern Italian as well as the north-western Balkan populations as a new species, *E. adriatica*, from the western Mediterranean *E. nicaeensis* and suggested that the southern Italian populations might pertain to a distinct polyploid species, *E. japygica*. However, our phylogeographic and RGS data based on more complete geographic sampling, especially in the south of the Apennine Peninsula, indicated only a weak genetic differentiation of the populations from Puglia and adjacent Basilicata with traces of admixture in the Gargano population. Therefore, more comprehensive data do not support separation of the south-eastern Italian populations as a distinct species *E. japygica*. In addition, the genetic patterns do not fully overlap with the geographic distribution of the diagnostic morphological character, i.e. the fruit indumentum. Namely, not only did the genetically most divergent south-eastern Italian populations have hairy capsules, but also those from westerly adjacent Campania and also one population from the central Apennines, in which individuals both with glabrous as well as hairy fruits were found (Supporting Information, Fig. S3; Fig. 8). This variation in indumentum probably triggered the discussions regarding the presence or absence of *E. japygica* in Abruzzo and Campania in Italian floristic literature (Tammaro 1995, Conti 2007, Del Guacchio 2010, Fenu *et al.* 2016). The discrepancy between genetic divergence and fruit indumentum indicates that this character alone is not sufficient to discriminate between both taxa; indeed, there are other characters revealed by our morphometric study that additionally contribute to

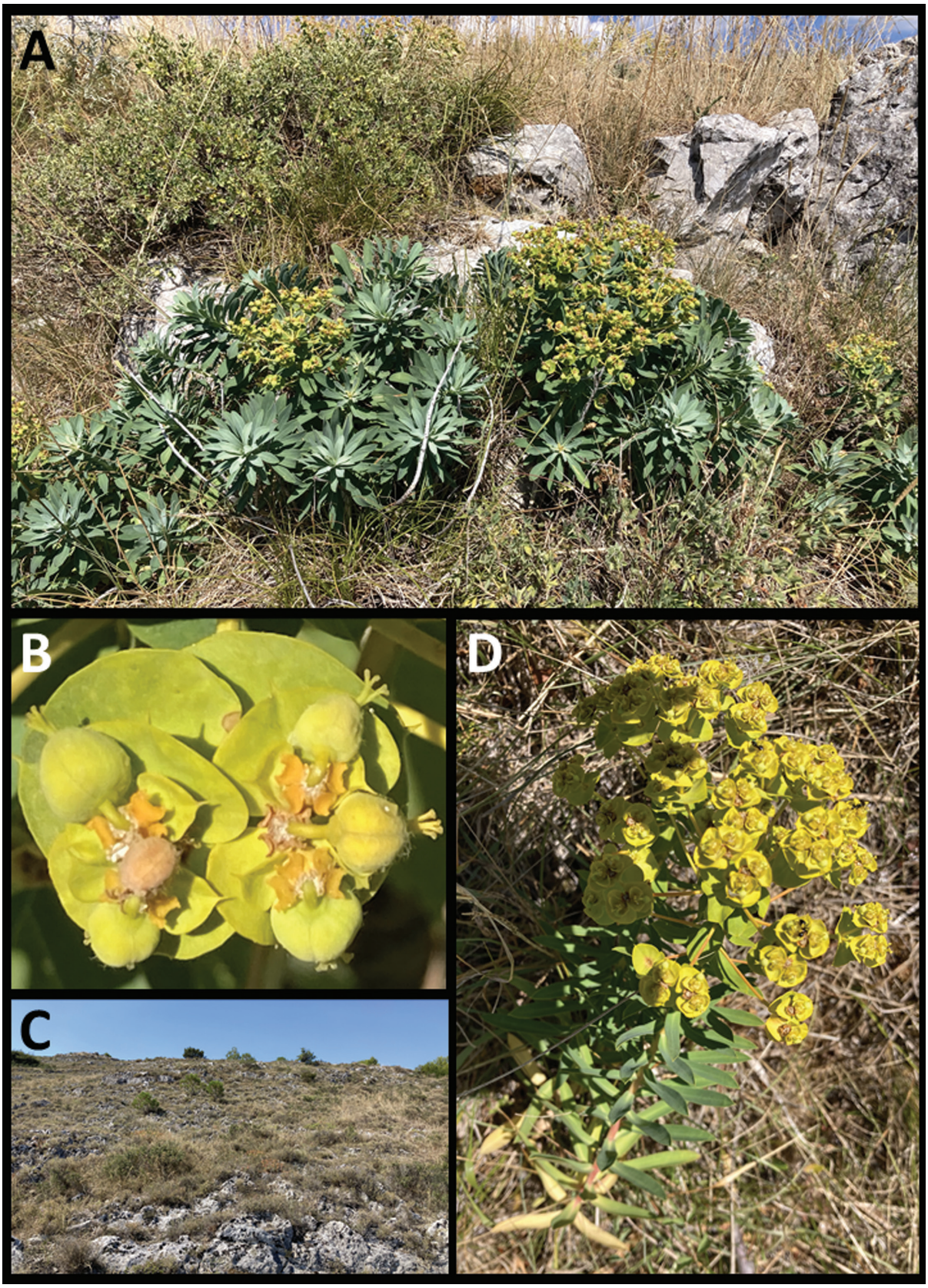

Figure 8. *Euphorbia japygica* in its native environments. A, *E. japygica* subsp. *prostrata (E. adriatica)* from Punta di Tormine in Monti Picentini (Campania, Italy), indicating variability in fruit indumentum within the same plant (B). C, Typical habitat of *E. japygica* subsp. *japygica* (Murgia Timone east of Matera, Basilicata, Italy). D, *E. japygica* subsp. *prostrata (E. adriatica;* at *locus classicus* Senožeče, Slovenia). Photographs: B. Frajman.

discrimination between the two main genetic groups (Fig. 6; see taxon descriptions next). In the light of these considerations, we propose to treat the populations belonging to the two main genetic groups as subspecies, *E. japygica* subsp. *japygica* in Puglia and Basilicata and *E. japygica* subsp. *prostrata (E. adriatica)* in the rest of the area. Whereas the former occurs mostly in lower elevations, but reaching 900 m in Gargano, the latter has much wider elevational span (Fig. 7), but can be found only in the mountains in the southern part of its distribution (Abruzzo, Campania), which indicates ecological differentiation of these populations from *E. japygica* subsp. *japygica* at similar latitudes.

Taxonomic treatment

Identification key

1a. Longest terminal ray (1.4)1.5–3.8(5.2) cm long. The longest fertile axillary ray (1.4)1.7–4.7(5.1) cm long. Ray leaves (0.8)0.9–1.4(1.5) × (0.5)0.6–0.9(1.3) cm. Raylet leaves (0.4)0.5–0.8(0.9) × (0.7)0.8–1.1(1.2) cm. Cyathial involucre (1.6)2.0–2.6(2.8) × (1.3)1.5–2.2(2.5) mm. Fruits pubescent, (2.0)2.3–4.0(4.5) mm wide, 0.9–1.1 times longer that wide. Seeds (1.9)2.0–2.7 mm long, 1.2–1.5 times longer than wide. *Altopiano delle Murge and Gargano in the southern Apennine Peninsula (Basilicata, Puglia)*..... ***E. japygica* subsp. *japygica***

1b. Longest terminal ray (2)2.5–5.5(6.7) cm long. The longest fertile axillary ray (2)2.7–5.8(7.2) cm long. Ray leaves (0.7)0.9–2.0(2.5) × (0.5)0.6–1.2(1.5) cm. Raylet leaves (0.5)0.6–1.1(1.4) × 0.8–1.5(1.8) cm. Cyathial involucre (1.6)1.8–2.6(3.3) × (1.4)1.7–2.5(3.2) mm. Fruits glabrous or pubescent, (0.7)1.2–3.2(4.6) mm wide, 1.0–1.6(1.8) times longer that wide. Seeds (2.4)2.6–2.7(2.8) mm long, (1.2)1.3–1.5(1.7) times longer than wide. *Throughout the Apennine Peninsula (Abruzzo, Campania, Toscana), southern margin of the Alps (Friuli-Venezia Giulia, Lombardia, Trentino-Alto Adige, Veneto), north-western Balkan Peninsula* ..... ***E. japygica* subsp. *prostrata (E. adriatica)***

Euphorbia japygica* Ten. subsp. *japygica, Fl. Napol. 4: 266. 1830 ≡ *E. nicaeensis* var. *japygica* (Ten.) Nyman, Consp. Fl. Eur.: 653. 1881 ≡ *E. nicaeensis* subsp. *japygica* (Ten.) Arcang., Comp. Fl. Ital.: 620. 1882 ≡ *E. seguieriana* var. *japygica* (Ten.) Fiori, Fl. Italia 2: 286. 1901 ≡ *Tithymalus nicaeensis* subsp. *japygicus* (Ten.) Soják, Cas. Nár. Mus., Odd. Prír. 140: 174. 1972.—Neotype (designated here): '*Euphorbia niceaensis* W. Bert. Fl. Ital. | 5. p. n. 76. [in Bertoloni's handwriting] | *Euphorbia japygica* [unknown hand] | Involucellis viridibus | primu [sic!] intuitu ab *E. ni-* | *censi* discrimi- | natur [Tenore's hand] | Lecce [unknown hand] | Misit Tenore 1842 [Bertoloni's hand]' (BO *s.n.*!) (Translation: '*Euphorbia niceaensis* Willd. [treated by] Bertoloni in *Flora italica* vol. 5, p. 31 \ *Euphorbia japygica*\ Can be distinguished at first sight from *E. nicaensis* on account of the green floral involucres \ [from] Lecce \ Sent by Tenore in 1842').

Description: Glabrous and glaucous perennial, (12)20–31(38) cm high, with (1.3)2.1–3.5(3.8) mm thick stems. Terminal rays 5–10(11), the longest (1.4)1.5–3.8(5.2) cm long, 1–2 times dichotomously branched. Fertile axillary rays (2)3–13(15), the longest (1.4)1.7–4.7(5.1) cm long. All leaves with entire margin. Cauline leaves (narrowly) oblanceolate, (1.7)2.1–3.2(3.7) × 0.4–0.7(0.9) cm, (2.7)3.3–5.6(6.6) times longer than wide, widest at (0.5)0.6–0.7 of their length, with cuneate base and acute apex. Ray leaves broadly ovate, (0.8)0.9–1.4(1.5) × (0.5)0.6–0.9(1.3) cm, (1.0)1.1–2.0(2.6) times longer than wide, widest at (0.2)0.3–0.6(0.7) of their length. Raylet leaves broadly ovate to reniform, (0.4)0.5–0.8(0.9) × (0.7)0.8–1.1(1.2) cm, 0.5–0.8(1.0) times longer than wide, widest at 0.1–0.3 of their length, with cordate base and obtuse apex. Cyathial involucre campanulate, (1.6)2.0–2.6(2.8) × (1.3)1.5–2.2(2.5) mm, (0.8)1.0–1.5(1.7) times longer than wide. Cyathial lobes usually pubescent. Cyathial glands obovate-truncate, 0.5–1.2 × (1.2)1.3–1.6(1.7) mm, (0.3)0.4–0.8(0.9) times longer than wide, with 0.0–0.3(0.5) mm deep emargination. Fruits pubescent, with (7)10–53(130) hair per fruit valve, pruinose-papillose, broadly ovate, (1.9)2.6–4.1(4.9) × (2.0)2.3–4.0(4.5) mm, 0.9–1.1 times longer than wide, styles (0.5)1.0–2.1(2.2) mm long. Seeds ovoid, smooth, yellowish, brownish, or greyish, (1.9)2.0–2.7 × (1.4)1.6–1.9(2.0) mm, 1.2–1.5 times longer than wide. Caruncle conical, 0.5–0.8 × (0.6)0.7–1.1(1.2) mm, 0.6–0.8 times longer than wide.

Distribution: Altopiano delle Murge and Gargano in the southern Apennine Peninsula (Italy: Basilicata, Puglia).

Habitat: arid grasslands, scrublands, and open forests up to 900 m (Fig. 8).

***Euphorbia japygica* subsp. *prostrata* (Fiori) Del Guacchio & Frajman, comb. nova** ≡ *Euphorbia seguieriana* var. *prostrata* Fiori, Nuov. Fl. Italia 2: 183. 1926 ≡ *E. nicaeensis* subsp. *prostrata* (Fiori) Arrigoni, Inform. Bot. Ital. 12: 140. 1980 (publ. 1981).—Type: Flora Italica—Herbarium Adr. Fiori: 'Prov. di Firenze, Impruneta ai Sassi neri, solo serpentinoso, 315 m' 4 Jun 1911, *Adr[iano] Fiori s.n.* (FI002664!).

= *Euphorbia adriatica* Stojilkovič, Zaveska & Frajman in Front. Plant Sci. 13:815379: p. 18–19 (2022). – Type: Flora of Slovenia, Primorska, Kras: south of the road Senožeče – Senadole, 1.5 km west of Senožeče; 550 m; 14° 0′ 38′′ E, 45° 43′ 9′′ N; dry meadow. 16 August 2021 *V. Stojilkovič & B. Frajman 16939*. (Holotype: W0164201; https://w.jacq.org/W0164201. Isotypes in IB 113154, LJU, FI018954, ZA 62967 & 62969.)

Description: Glabrous and glaucous perennial, (7)14–39(45) cm high, width (1)2–3.4(4) mm thick stems. Terminal rays (5)6–11(12), the longest (2)2.5–5.5(6.7) cm long, 1–2 times dichotomously branched. Fertile axillary rays 2–10(14), the longest (2)2.7–5.8(7.2) cm long. All leaves with entire margin. Cauline leaves (narrowly) oblanceolate, (1.8)2.0–4.7(5.6) × (0.3)0.4–0.9(1.1) cm, (3.0)4.0–6.2(6.8) times longer than wide, widest at 0.6–0.7(0.8) of their length, with cuneate base and acute apex. Ray leaves broadly ovate, (0.7)0.9–2.0(2.5) × (0.5)0.6–1.2(1.5) cm, (0.9)1.1–2.4(2.8) times longer than wide, widest at 0.4–0.6(0.7) of their length. Raylet leaves broadly ovate to reniform, (0.5)0.6–1.1(1.4) × 0.8–1.5(1.8) cm, 0.6–0.8(0.9) times longer than wide, widest at (0.1)0.2–0.4 of their length, with cordate base and obtuse apex. Cyathial involucre campanulate, (1.6)1.8–2.6(3.3) × (1.4)1.7–2.5(3.2) mm, (0.8)0.9–1.4(1.6) times longer than wide. Cyathial lobes usually pubescent. Cyathial glands obovate-truncate, (0.3)0.5–1.3(1.6) × (0.9)1.0–1.8(2.2) mm, (0.2)0.4–0.8(1.0) times longer than wide, often with two lobate horns, with emargination/horn length 0.0–0.4(0.5) mm. Fruits glabrous or pubescent, with 0–17(47) hair per fruit valve, pruinose-papillose, broadly ovate, (0.8)1.4–4.2(4.6) × (0.7)1.2–3.2(4.6) mm, 1.0–1.6(1.8) times longer that wide, styles (0.7)1.1–1.7(2.1) mm long. Seeds ovoid, smooth, yellowish, brownish or greyish, (2.4)2.6–2.7(2.8) × (1.6)1.7–2.0(2.2) mm, (1.2)1.3–1.5(1.7) times longer than wide. Caruncle conical, (0.5)0.6–0.7(0.9) × 0.8–1.0(1.1) mm, 0.6–0.8 times longer than wide.

Distribution: Southern, central, and northern Apennine Peninsula to the southern margin of the Alps (Italy: Lombardia, Trentino-Alto Adige, Veneto, Friuli-Venezia Giulia, Toscana, Abruzzo, Campania), north-west Balkan Peninsula (Croatia: Istria, Kvarner, northern Dalmacija; western Slovenia: Primorska). Note: the species was up until now not known from northern Dalmacija in Croatia. However, pictures published on iNaturalist from several locations north-west of Šibenik by S. Ćato clearly indicate a disjunct occurrence of this taxon in the area.

Habitat: submediterranean grasslands, scrublands, open forests, and rocky outcrops, mostly over calcareous substrate but also on serpentine up to 1500 m (Fig. 8).

SUPPLEMENTARY DATA

Supplementary data is available at *Botanical Journal of the Linnean Society* online.

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CREDIT STATEMENT

Micol Boschin (sampling, laboratory work, data analyses, writing) Peter Schönswetter (data analyses, writing), and Božo Frajman (conceptualization, sampling, data analyses, writing).

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY

The data underlying this article are available in the GenBank Nucleotide Database at, and can be accessed with accession numbers listed in Supporting Information, Tables S1 and S3. Morphological character states are in Supporting Information,Table S4.

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