The genus Gymnospermium (Berberidaceae) in Italy: identity and relationships of the populations at the western limit of the genus range

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The genus *Gymnospermium* (Berberidaceae) in Italy: identity and relationships of the populations at the western limit of the genus range

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Q1

ABSTRACT

Gymnospermium is a small genus of 7–12 taxa subject to diverging taxonomic treatments and distributed from east China to the Balkans. The recent discovery of *Gymnospermium* in the S-Apennines posed questions about its origin and identity. Accordingly, we performed a systematic investigation by means of morphological, karyological and molecular tools. All populations were diploid with 2n = 14 as for the Balkan *G. scipetarum* (incl. *G. maloi*), and also morphology suggested a close affinity to the latter. However, the Italian populations differed from typical *G. scipetarum* by the lower stamen:petal length and style:carpel length. By including all European and most Asian taxa in a phylogenetic analysis, we shed new light into the species-level relationships of this genus. In the combined ITS-*trn*L-F phylogeny, two major clades were retrieved. It included the central Asian and eastern European taxa plus the Greek endemic *G. peloponnesiacum* sister to *G. odessanum*, and the Balkan and Apennine populations. Such findings further corroborated that the Apennine plant belong to *G. scipetarum*. The native status of the Italian population is supported by exclusive SNPs in both ITS1 and *trn*L-F sequences. Along with morphological evidence, this allows to refer it to the new subspecies *G. scipetarum* subsp. *eddae*.

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Introduction

Gymnospermium Spach (Berberidaceae) is a small genus of early flowering tuberous herbs characterized by fruits with exposed seeds when the membranous pericarp splits (from the Greek "*gymnos*", naked, and "*sperma*", seed). Together with the related genera *Leontice* L. and *Caulophyllum* Michx, it is part of tribe Leonticeae (Spach) Kosenko, which is characterized by succulent staminodia, eureticulate pollen exine, utricular gynoecia, base chromosome number x = 8 (Loconte and Estes 1989; Loconte 1993) and prevalence of quinolizidine alkaloids and β-amyrin triterpenoids (Peng et al. 2006). Recently, a new alkaloid (maloine) was discovered in *Gymnospermium* by Çela et al. (2017). Based on molecular and morphological evidence, *Gymnospermium* and *Caulophyllum* are sistergroups to genus *Nandina* Thunb. and belong with the latter to subfamily Nandinoideae Heintze (Wang et al. 2009; Liu et al. 2017). However, in the APG website versus 13 (Stevens 2001 onwards; http://www.mobot.org/MOBOT/research/APweb/), both genera are placed in subfamily Podophylloideae Eaton.

Species of *Gymnospermium* are mostly allopatric endemics forming four distinct geographic groups in semi-arid steppes, montane shrublands and mesic forests in east and central Asia, east Europe and east Mediterranean (Loconte and Estes 1989; Figure. 1). Most of them are rare and endangered (Loconte 1993), appearing in various national or international Red Lists (e.g. Kim et al. 2016). Despite this vast and fragmented distribution range, the degree of morphological differentiation in the group is surprisingly low and this has caused contrasting interpretations of the limits, status and number of taxa recognized by past authors (Kosenko 1980; Loconte 1993; Wang et al. 2007; Ying et al. 2011).

Even the limits of the generic type species *G. altaicum* (Pallas) Spach (bas.: *Leontice altaica* Pallas), described from the Altai mountains, have been subject to various interpretations. According to Stearn and Webb (1964, 1993), *G. altaicum* occurs from central Asia to eastern Europe in Ukraine and Romania, while Takhtajan (1970) separated the populations from the Black Sea area as *G. odessanum* (DC.) Takht. (bas. *Leontice altaica* var. *odessana* DC.). Isolated populations from the Peloponnese (Greece) were also included in the latter species by Takhtajan (1970), but were later separated as *G. altaicum* subsp. *peloponnesiacum* by Phitos (2003) and finally elevated to species rank by Strid (in Karl and Strid 2009).

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In addition, two other taxa morphologically close to *G. altaicum* have been described from the Balkans, that is, *G. scipetarum* E. Mayer et Pulević and *G. maloi* Kit Tan et Shuka (Tan et al. 2011). Using a morphometric approach, Barina et al. (2016) showed substantial overlap in some quantitative characters of leaves, stems and seeds and suggested the conspecific status of the two Balkan taxa. In addition, lack of genetic divergence between them was shown in a recent molecular investigation of the European species (Barina et al. 2017), and it was concluded that only three species should be recognized in the continent, *G. odessanum*, *G. peloponnesiacum* and *G. scipetarum*, the latter including *G. maloi*.

During phytogeographical researches in southern Italy (Rosati et al. 2014, 2017), we discovered plants of *Gymnospermium* in the understory of a restricted forest area of Monti della Maddalena, a sector of the southern Apennines in the Salerno province. This remarkable finding allowed to place the western distribution limit of *Gymnospermium* beyond the Adriatic sea (Figure 1) and to add a new genus to the Italian flora. At the same time, however, it raised relevant questions about the identity, origin and phylogenetic relationships of these Apennine plants. In the recent checklist of the native Italian flora, in fact, these were referred to *G. scipetarum* but considered as "taxonomically doubtful"; in addition, the latter was considered as "a doubtfully native taxon whose origin of occurrence in Italy is unknown" (Bartolucci et al. 2018). No answers to these doubts were provided in the recent biogeographic analysis of *Gymnospermium* by Barina et al. (2017), which did not include the Italian plants, nor taxa from central Asia such as *G. albertii* (Regel) Takht and *G. darwasicum* (Regel) Takht. To shed light on this issue, we performed morphological, karyological and molecular analyses using two DNA regions from the nuclear and plastid genomes of most species. Results from these analyses provided new evidence on the status of this genus in Italy and suggested implications concerning the biogeographic links between the Italian and Balkan peninsulas.

Materials and methods

Plant material

At the current state of knowledge, *Gymnospermium* occurs in Italy with a single meta-population of six sub-populations found on ridges and karst depressions at 1080–1300 m a.s.l. in the Maddalena mountain chain, Salerno province (Table 1). It mainly grows in the understory of mesophilous forests with *Fagus sylvatica* and *Quercus cerris*, on brown soils developed on siliceous substrata or colluvial materials (Regione 2006; Sgrosso et al. 2010). Other woody species in the area are *Acer cappadocicum* Gled. subsp. *lobelii* (Ten.) A.E.Murray, *A. opalus* Mill. subsp. *obtusatum* (Waldst. et Kit. ex Willd.) Gams, *Castanea sativa* Mill. and *Pyrus communis* L. subsp. *pyraster* (L.) Ehrh.; the shrub layer is mainly formed by *Corylus avellana* L., *Ilex aquifolium* L., *Crataegus monogyna* Jacq., *Euonymus latifolius* (L.) Mill.; the herb layer includes several geophtyes such as *Corydalis cava* (L.) Schweigg. et Körte, *Allium ursinum* L., *Scilla bifolia* L., *Arum maculatum* L., *Anemone apennina* L. and hemicryptophytes such as

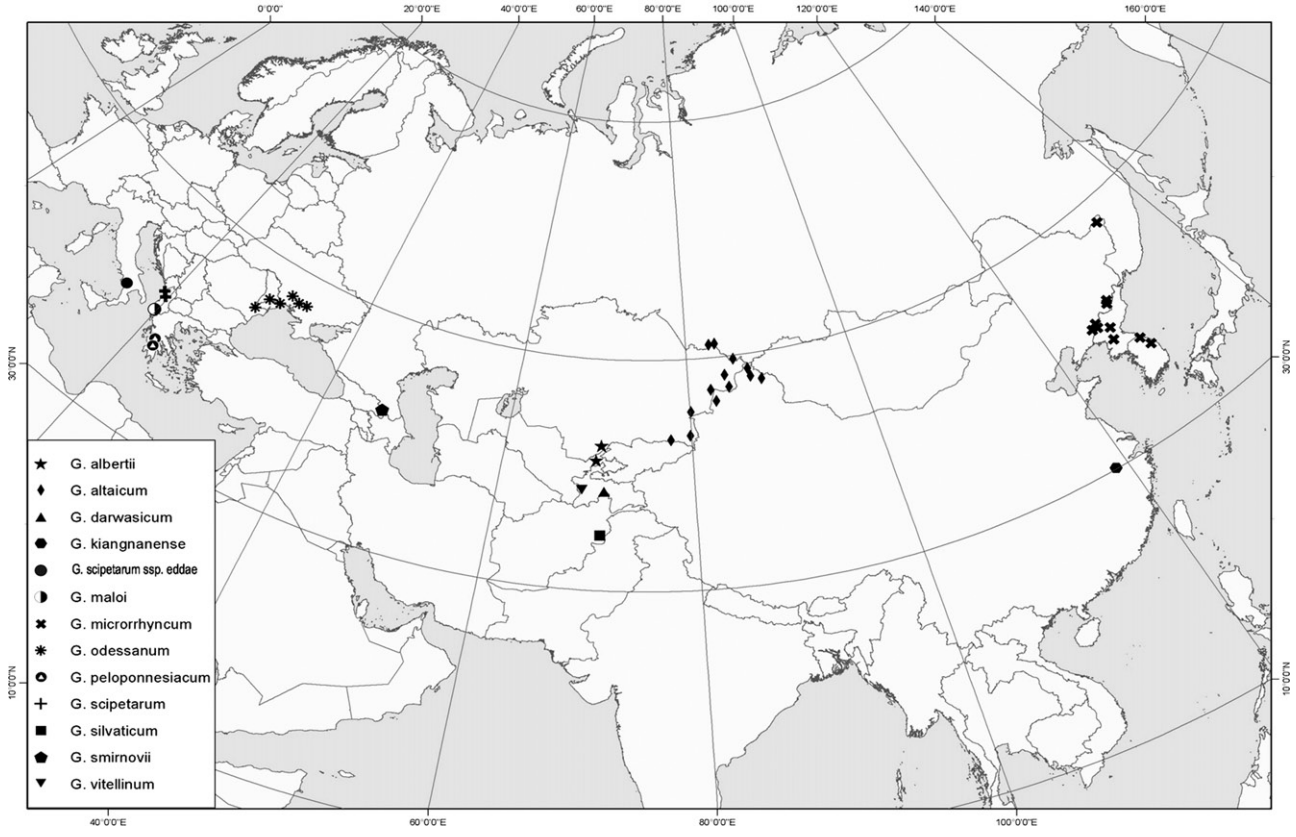

Figure 1. General distribution of all 13 species of *Gymnospermium* described to date, including *G. scipetarum* subsp. *eddae* from southern Italy.

Table 1. Location and main habitat features of the three analyzed subpopulations of *Gymnospermium* in the southern Apennines.

Alt. m a.s.l.	Aspect	Lithology	Habitat	Area of occupancy (m^2)	Locality	Long/Lat
1170	N	Siliceous schists	*Fagus sylvatica-Quercus cerris* forest	~1500	Acqua della Cerasa, Monti della Maddalena, Salerno Province, Italy	15.6960E 40.3667N
1280	N	Siliceous schists	*Fagus sylvatica-Quercus cerris* forest /Forest edges	~2000	Campitello, Monti della Maddalena, Salerno Province, Italy	15.6947E 40.4870N
1250	NE	Siliceous schists	*Fagus sylvatica-Quercus cerris* forest	~6000	Mandranello, Monti della Maddalena, Salerno Province, Italy	15.6824E 40.3841N

Doronicum orientale Hoffm., *Lamium flexuosum* Ten., *Primula vulgaris* Huds. and others. This forest community can be referred to the association *Anemono apenninae-Fagetum sylvaticae* Brullo 1984, which is widespread in the southern Apennines (Rosati et al. 2010; Fascetti et al. 2013).

The three larger subpopulations in the area were sampled for the morphological, karyological and molecular analyses. For molecular analyses, the DNA dataset was completed with original sequences of *G. albertii*, *G. altaicum*, *G. darwasicum* (C Asia), *G. odessanum* (Black Sea region), *G. peloponnesiacum, G. scipetarum* and one population previously referred to *G. maloi* (Europe). Sequences were obtained from silica-gel dried samples of leaf tissue of these taxa from collections by the authors and from plants cultivated in the Botanical Garden of the University of Copenhagen (Appendix 1).

Morphology

Phenotypic characterization of the three subpopulations was achieved by analyzing 4 qualitative and 24 quantitative characters (Supplementary Table S1) with potential taxonomic value based on previous studies (Loconte and Estes 1989, Tan et al. 2011). These characters were measured in the field on 30 living plants for each subpopulation, except for flower measurements (e.g. petal length, stamen length), which were taken on three flowers for each herbarium specimen (10) kept in HLUC. To avoid differences due to phenophase, characters of vegetative parts and pedicel length were contemporarily measured on fully developed plants (i.e. at the end of the flowering period). The height of the plant was measured from the ground surface to the top of the raceme, and stem diameter was measured at the ground level. Seeds were collected and measured during the early phase of plant senescence. Since tubers are deeply buried in the ground, we measured their diameter in only five flowering plants for each sub-population, in order not to damage them. These tubers were then transplanted and maintained in cultivation *ex situ* at the Campus of University of Basilicata for further studies. Phenology was assessed during 4 years of observations (2014–2017).

For morphological data analyses, a homogeneity test of multivariate dispersion within groups (PERMDISP; Anderson, 2004) was carried out before checking for differences in character values between the three subpopulations using non-parametric permutational multivariate analysis of variance (PERMANOVA; Anderson, 2005) based on Euclidean distances. Range, mean and standard deviation values (when available) were compared with those of other taxa analyzed in previous studies (Loconte and Estes 1989, Tan et al. 2011, Barina et al. 2017). Morphological data for *G. scipetarum*, *G. peloponnesiacum* and the population previously referred to *G. maloi* were taken from Tan et al. (2011), while those of *G. odessanum* and *G. smirnovii* were taken from Loconte and Estes (1989) and integrated by original measurements on herbarium specimens kept in KW, KWHU and FI. Non-metric Multidimensional Scaling (NMDS) based on the mean values of 15 quantitative characters (indicated in Supplementary Table S1) was used to summarize and visualize the overall phenotypic relationships between the European taxa and the Italian samples. Unfortunately, no data exist in the literature describing within-population variation of these morphological characters in the above mentioned taxa, so that no statistical comparisons were possible. Statistics was performed with PRIMER v.6.1.11and PERMANOVA + v.1.0.1 (Primer-E Ltd., Luton, UK).

Micromorphology

Pollen grains were collected from herbarium specimens of the Italian plants and from collections of *G. altaicum* and *G. scipetarum* in HLUC. These were then observed with a Scanning Electron Microscope Philips XL 30 Environmental SEM, without previous treatment to avoid alteration of potentially significant traits (Hesse and Waha 1989). The micromorphological characters of the seed testa of Italian plants were observed both on dehydrated and freshly collected seeds.

Karyology

Mitotic chromosome plates of the Italian plants from the three main subpopulations were prepared from actively growing root tips produced by tubers kept in cultivation or seeds germinated in Petri's dishes. Preparation followed the same protocols reported in Tan et al. (2011). Chromosome counts and determination of centromere position (chromosome type) were derived from 10 well-spread metaphase plates of intact cells. The nomenclature of chromosomes

follows Levan et al. (1964). Intra-chromosomal (A) and inter-chromosomal (A_2) asymmetry indexes were calculated according to Watanabe et al. (1999) and Romero Zarco (1986), respectively.

DNA isolation, amplification and sequencing

Genomic DNA of the taxa/accessions taxa mentioned above (Appendix 1) was extracted from silica-gel dried samples of leaf tissue following a modified 2xCTAB protocol (Doyle and Doyle, 1990). The extracted DNA was quantified after agarose gel electrophoresis (0.6% w/v) in TAE buffer (1 mM EDTA, 40 mM Tris-acetate) containing 1 μg/ml of ethidium bromide by comparison with a known mass standard. Amplification of the Internal Transcribed Spacer of nrDNA, including ITS1, 5.8S and ITS2, was done using the primers ITS4 and ITS5 of White et al. (1990), while the primers 'c' and 'f' of Taberlet et al. (1991) were used for the *trn*L-*trn*F Intergenic Spacer (IGS). These two regions were selected for their generally high resolution power of species-level relationships in many angiosperm groups, especially ITS (see also Li et al. 2011), and because already used in previous studies of groups of Berberidaceae (Kim and Jansen 1996; Wang et al. 2007). Polymerase chain reactions (PCRs) were performed in a total volume of 25 μl containing 2.5 μl of reaction buffer (Dynazyme II; Finnzyme, Espoo, Finland), 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM of each dNTP, 1 U of *Taq* DNA polymerase (Dynazyme II; Finnzyme) and 10 ng of template DNA. Reactions were performed in a MJ PTC-100 thermocycler (Peltier Thermal Cycler; MJ Research). After a first denaturation step (5 min at 94 °C), 40 PCR cycles were performed (1 min at 94 °C), primers annealing (50 sec at 52 °C) and extension phase (50 sec at 72 °C). A final extension phase was performed for 10 min at 72 °C. For *trn*L-F IGS, the PCR cycling conditions were the same as those used in Cecchi et al. (2014).

Subsequently, 5 μl of each amplification mixture were analyzed by agarose gel (1.5% w/v) electrophoresis in TAE buffer containing 1 μg/ml ethidium bromide. Excess salts and primer were removed from the PCR reactions with the PCR Purification Kit (Roche, Mannheim, Germany). Automated DNA sequencing was performed directly from the purified PCR products using BigDye Terminator v.2 chemistry and an ABI310 sequencer (PE-Applied Biosystems, Norwalk, CT). In total, 19 original sequences were obtained and deposited in GenBank (accession numbers in Appendix 1), after their checking and editing (BioEdit vs. 7.0; Hall 1999), multiple alignments (available from authors upon request) were performed with MAFFT vs. 7 (Katoh and Standley 2013) and carefully checked for ambiguous positions based on visual inspection of the sequencer output chromatofiles.

Phylogenetic analyses

Taxon sampling in the DNA sequence data matrix was expanded with two species from east Asia, *G. kiangnanense* (Chiu) Loconte and *G. microrrhynchum* (S. Moore) Takht., which were retrieved from GenBank. Accessions of *Caulophyllum* (tribe Leonticeae) and *Nandina* Thunb. were also added as a representative of subfamily Nandinoideae (Wang et al. 2009). Members of other subfamilies of Berberidaceae such as *Berberis* L. of Berberidoideae Kosteletzky and *Epimedium* L. of Podophylloideae Eaton were finally included as additional outgroup representatives. Two single-marker datasets were initially prepared, ITS and IGS which included 17 and 13 accessions, respectively (Appendix 1). Phylogenetic analyses were first carried out for the ITS and IGS alignments separately, using Maximum Parsimony and Bayesian methods. Gaps were coded as separate characters according to Simmons and Ochoterena (2000) using FastGap 1.2 (Borchsenius 2009), and appended at the end of the datasets. Tree construction was first performed using PAUP 4.0 (Swofford 2000), running Heuristic searches with "tree-bisection-reconnection" (TBR) branch-swapping with accelerated transformation (ACCTRAN) optimization to infer branch (edge) lengths; MULTREES option on, ADDSEQ = random, 1000 randomized replicates. All characters were weighted equally, and character state transitions were treated as unordered. Bootstrap support for clades was obtained performing a heuristic search with 1000 replicates, using TBR branch-swapping, 10 random taxon entries per replicate and multrees option on. Bayesian inference of phylogenetic analyses was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best substitution models, based on the Akaike Information Criterion, were identified using FindModel (available at: http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html); these were GTR + Γ for ITS, with gamma-distributed rate variation across sites, and GTR + I + Γ for IGS. Posterior probability (PP) values were estimated with the Markov chain Monte Carlo (MCMC) method using six parallel runs each with four chains (one cold and three heated) simultaneously started from a random tree and run for one million cycles, with parameters sampled every 1000 generations. The stationary phase was reached when the average standard deviation of split frequencies reached 0.01. Trees that preceded the stabilization of the likelihood value (the burn-in) were discarded, and the remaining trees were used to calculate a majority-rule consensus phylogram. The trees were viewed and edited with TreeView (Page 1996), with indication of PPs for the internal tree nodes. Kimura two-parameters pairwise genetic distances (Kimura 1980) were calculated for the two molecular datasets separately, using MEGA 4.1 (Tamura et al. 2007). Finally, an additional dataset consisting of concatenated ITS-IGS sequences for 11 accessions was also prepared and analyzed, since the phylogenies produced by the two single-markers datasets showed no statistically supported topological conflicts (defined as clades with >80% bootstrap support; see Wiens 1998).

Results

Morphology

All examined characters showed a very similar range of variation in the three Italian subpopulations (PERMDISP:

$F = 0.317$; $P = 0.769$) and none was significantly different (PERMANOVA: $F = 1.402$, $P > 0.2$). The plant is described below and illustrated in Figures 2 and 3. In addition, a synoptic table showing variation between the European taxa in the 28 characters analyzed here is given in Supplementary Table S1.

Vegetative characters

The tuber is subglobose, 40–110 mm (mean 60 mm) in diameter and produces 1–12 flowering stems, terete, fleshy, greenish, brown to purplish at base, 3–7 mm in diameter (mean 4 mm), 25–40 (50) cm tall; each stem bears a single (rarely two) cauline leaf inserted below the lowest flower. The 2–10 (–23) basal leaves are compound-ternate, with primary divisions palmately divided into 5–7(–9) leaflets. The leaflets are sessile or with petioles 1–10 (20) mm long (mean 4 mm), unequal, entire, lanceolate to elliptical-oblong, rarely broadly obovate, 36–60 × 15–26 mm; the median leaflet is often larger than the lateral, obtuse or truncate-mucronate at apex (rarely slightly emarginate), thin, glaucous-green above, glaucous beneath; the basal leaflets are larger (1.5–2.0x) than the cauline ones.

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Figure 2. *Gymnospermium scipetarum* subsp. *eddae* in its natural habitat. (A) massive blooming in the understory of an old *Fagus sylvatica* forest stand, 12 April 2015; (B) flowering stems at the anthesis peak, 12 April 2015; (C) basal leaves and flowering stems at the end of anthesis, 21 April 2015; (D) detail of a flowering stem emerging from the forest litter before basal leaves, 23 February 2016; (E) detail of fruit with the characteristically splitting membranous pericarp exposing two seeds, 8 May 2015; (F) seedlings, 14 April 2015. Photos by L.R.

Figure 3. *Gymnospermium scipetarum* subsp. *eddae*. (A1) Habit during flowering, (A2) habit at the beginning of fruiting, (B) dissected flower (two sepals, petals and stamens were removed), (C) carpel at early fruiting stage, (D) dehydrated seed, (E) open carpel with two developing seeds, (F) stamen, (G) sepal and petal (nectary), (H) basal leaf, (I) juvenile plant. Original drawing by Martina Marignani.

Reproductive characters

The inflorescence is a dense, terminal raceme, 90–150 mm long, pendent-deflexed (almost straight and erect during anthesis), bracteate, with (5–)8–13(–16) flowers; the earliest flower is distant from the others. The bracts are suborbicular-reniform, 8–14 × 7–14 mm, with margin often slightly eroded, rarely mucronate at apex. The pedicels are greenish, 12–40 mm long, erecto-patent in flower, recurved-deflexed in fruit. The hypogynous flowers are 17–30 mm in diameter (including sepals). Sepals are unequal, elliptical or oblong-ovate, 10–15 × 4–8 mm, obtuse, petaloid, bright yellow. The six petals (nectaries) are equal, opposite, golden-yellow, nectariferous, cuneiform, 3.5–4.5 mm long, concave at the base, with margins slightly convolute and apex shallowly and irregularly 4–8 toothed, the two lateral teeth longer, recurved. The six stamens are 4.5–6.5 mm long, opposite, enclosed by petals in the lower part; filaments are 3.0–3.5 mm long, anthers are oblong-ovoid, 3 mm, bilocular, dehiscing by apically-hinged flap-like valves. The solitary carpel is 5–7 mm long and bears 2–4 ovules (mean 3) on a basal placenta; the style is 2 mm long and bears a small, truncate stigma with papillate surface. The fruit is a subglobose capsule enclosing 1–4 ovoid-pyriform seeds. These are 6–11.5 mm long (mean 8.6), including white stipe-like strophiole (turning to bright orange-red), green at splitting of the membranous pericarp, reddish-brownish at maturity; the seed surface is smooth, wrinkled when dry, with a finely sculptured testa (Figure 4(E–F)).

Pollen

Non-acetolyzed grains (Figure 4(A,B)) are medium-sized, with polar axis 63–69 μm (mean 64.4 μm) and equatorial diameter

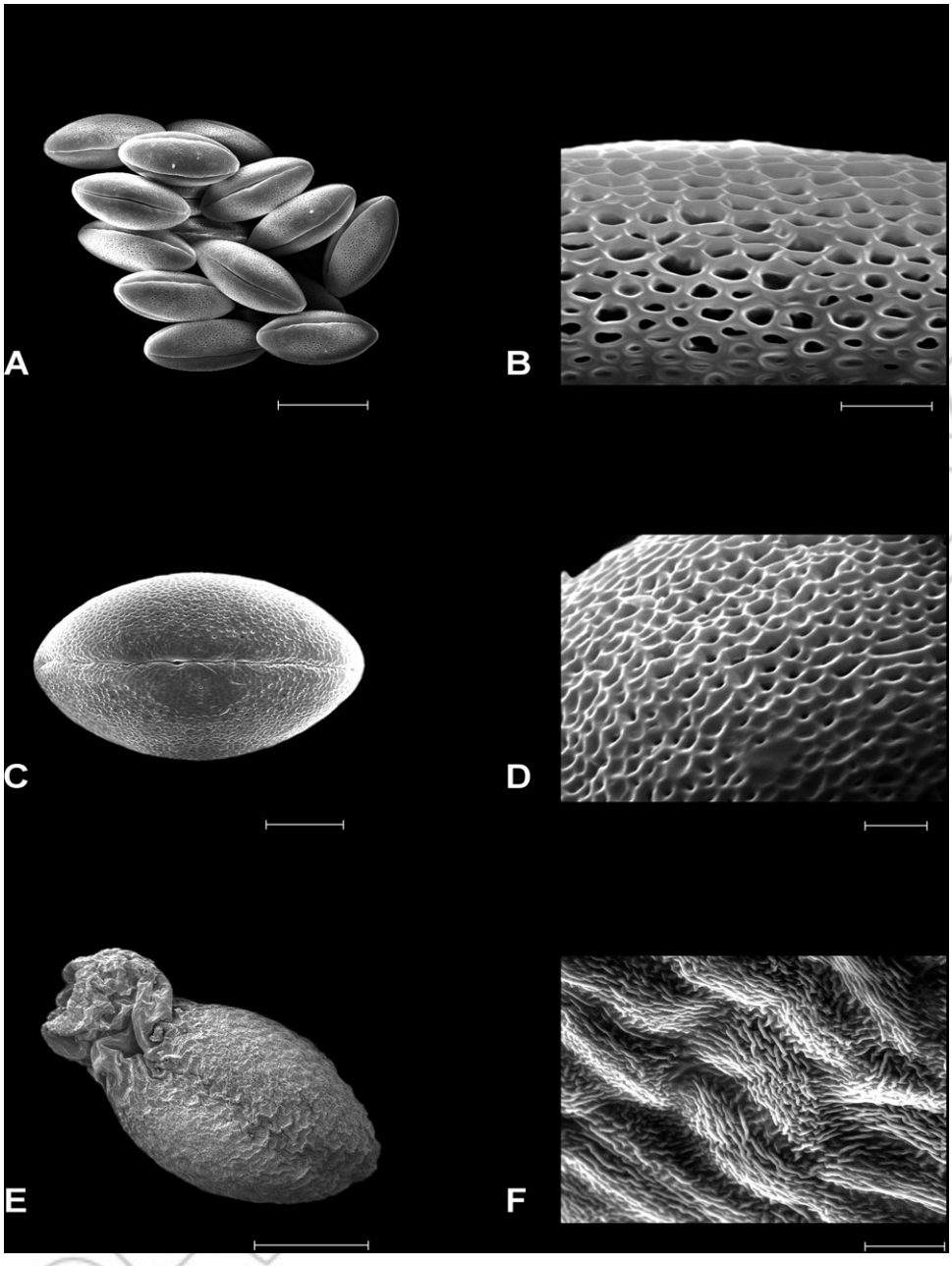

Figure 4. SEM micrographs. (A) *G. scipetarum* subsp. *eddae,* pollen grains; (B) detail of reticulate tectum; (C) *G. altaicum,* pollen grain; (D) *G. altaicum,* detail of reticulate tectum; (E) *G. scipetarum* subsp. *eddae,* dehydrated seed; (F) *G. scipetarum* subsp. *eddae*, detail of the sculpture seed testa. Scale bars: A, F = 50 μm, B = 5 μm, C = 10 μm, D = 2 μm; E = 2 mm.

30–34 μm (mean 32.6 μm); their shape is prolate (P/E 1.97). The three colpi are 60–65 μm long, straight or weakly undulate and granulate at the margins. The tectum is reticulate, with small-sized reticulum cells (1.5–3.1 μm in diameter). This pollen resulted clearly distinct from that described for the east Asian species *G. kiangnanense* and *G. microrrhynchum*, both characterized by an undulate colpial margin (Arkhangelsky and Takhtajan, 1972; Loconte and Estes, 1989). Compared with the eastern European and Asian species, grains of the Italian samples differed for the longer apertures (62.5 vs. 48 μm in *G. darwasicum* and 42 μm in *G. odessanum* and *G. altaicum,* Figure 4(C,D)) and for a longer polar axis (65 μm vs. 50–53 μm in *G. odessanum and G. altaicum*). In addition, cells of the reticulum (approximately 2.1 μm; Figure 4(B)) were larger than in *G. darwasicum* (up to approximately 1.5 μm). With respect to the Apennine plants, the Albanian ones of *G. scipetarum* showed slightly smaller values for polar axis length (63.0 μm), P/E ratio (1.85) and width of the reticulum cells (2.0 μm).

Phenology

Flowering is from early March to late April; fruiting is from April to May.

NMDS analysis

The scattergram from NMDS ordination of the European species showed that the Apennine plants are close to the Balkan populations (Figure 5). All these accessions formed a distinct group from the pair *G. peloponnesiacum-G. odessanum*,

whereas the Caucasian endemic *G. smirnovii* was in a more isolated position. The Italian plants resulted intermediate between *G. scipetarum* and those previously referred to *G. maloi*. At present, however, the latter is considered conspecific with the former (Barina et al. 2017), and this supports the inclusion of all these populations in *G. scipetarum*. The only relevant morphological differences between the Italian and the Balkan plants were the lower ratios stamen:petal length (1.3 vs. 1.5–1.7) and style:carpel length (0.3 vs. 0.5; Supplementary Table S1).

Karyology

All plants from the three subpopulations were diploid with 2n = 14; the karyotype consisted of 1 metacentric and 6 sub-metacentric chromosome pairs (Figure 6). Noteworthy, the two chromosomes of the last pair showed the highest difference in centromere position. Chromosome length ranged from 12.1 to 20.4 μm (Table 2). Index of intra-chromosomal asymmetry (A) was 0.30, while inter-chromosomal asymmetry A_2 was relatively low (0.20).

Phylogenetic relationships

Results from the separate analysis of the three sequence datasets are presented herafter.

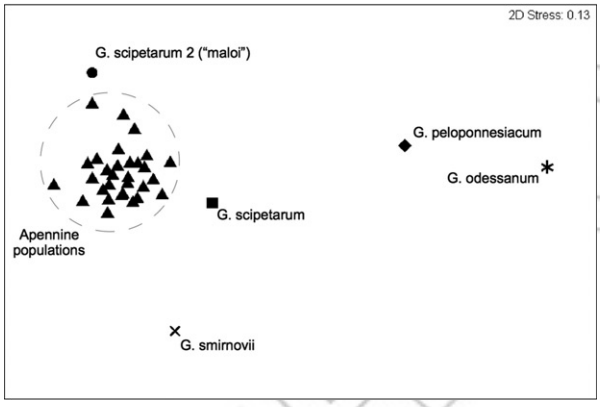

Figure 5. Non-metric multidimensional scaling based on Bray-Curtis similarity comparing Apennine populations of *Gymnospermium* with described European taxa.

Nuclear ITS dataset

The aligned matrix included a total of 686 positions (coded gaps in pos. 648–686), of which 314 were constant, 131 variable but non-informative and 241 (35.1%) variable and parsimony informative. The mean genetic distance within *Gymnospermium* was 0.037. The heuristic search produced two most parsimonious trees with L = 582, consistency index (CI) = 0.854 and retention index (RI) = 0.844. The topology of the resulting strict consensus tree was fully consistent with that of the 50 majority-rule consensus phylogram produced by the Bayesian analysis; this is shown in Figure 7(A) with bootstrap (BS) and PP values. Members of tribe Leonticeae (*Gymnospermium* and *Caulophyllum*) formed a well-supported clade with 1.00 PP and 100% BS. Bayesian support to *Gymnospermium*, including *G. kiagnanense*, was weak (0.70 PP) but this group was more strongly corroborated in the bootstrap analysis (93% BS); *G. kiangnanense* was sister to the group including all the other species, which was strongly supported (1.00 PP; 98% BS). This was divided in two major subclades. The first subclade (0.94 PP; BS <50%) consisted of all the central Asian and E European taxa plus *G. peloponnesiacum* from the S Balkans; in turn, this included the two groups of *G. albertii* sister to *G. darwasicum* (1.00 PP; 85% BS) and of *G. altaicum* possibly sister (0.84 PP; BS <50%) to the pair *G. odessanum* + *G. peloponnesiacum*;

Table 2. Chromosome morphology of *Gymnospermium scipetarum* subsp. *eddae*.

Chromosome	Group	L (μm)	S (μm)	L + S	L/S	Type
1	1	10.84	9.61	20.44	1.13	m
2		10.14	9.23	19.36	1.10	m
3	2	13.44	6.79	20.23	1.98	sm
4		12.59	6.79	19.38	1.86	sm
5	3	12.29	5.95	18.24	2.07	sm
6		11.91	5.74	17.65	2.08	sm
7	4	10.33	5.51	15.84	1.87	sm
8		10.04	5.23	15.27	1.92	sm
9	5	9.68	4.81	14.49	2.01	sm
10		9.53	4.69	14.21	2.03	sm
11	6	8.63	3.69	12.32	2.34	sm
12		8.27	3.83	12.10	2.16	sm
13	7	10.16	5.85	16.00	1.74	sm
14		11.89	5.55	17.44	2.14	sm

Chromosome nomenclature according to Levan et al. (1964); L: long arm; S: short arm; centromere position: m = metacentric; sm = submetacentric. Chromosomes sequence and group labels according to Figure 6.

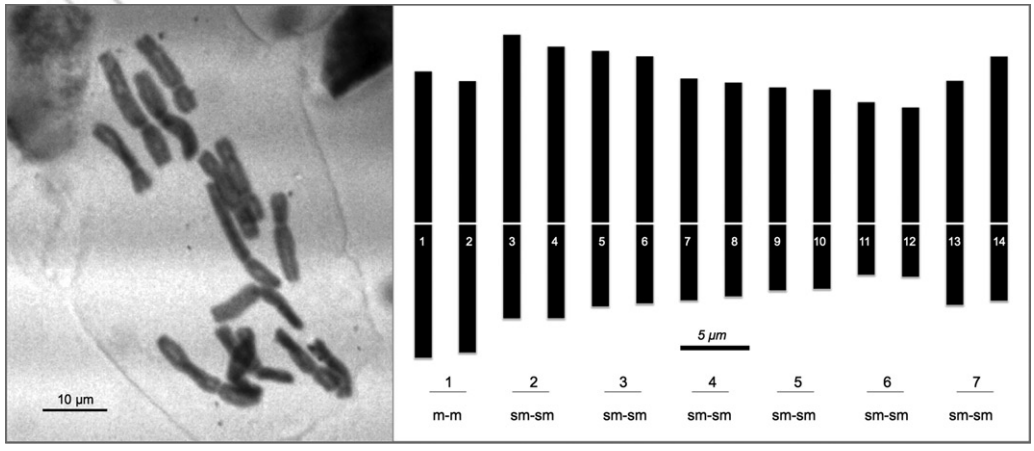

Figure 6. Chromosome metaphase plate and idiogram of *G. scipetarum* subsp. *eddae,* showing, 2*n* = 2*x* = 14 and chromosome morphology.

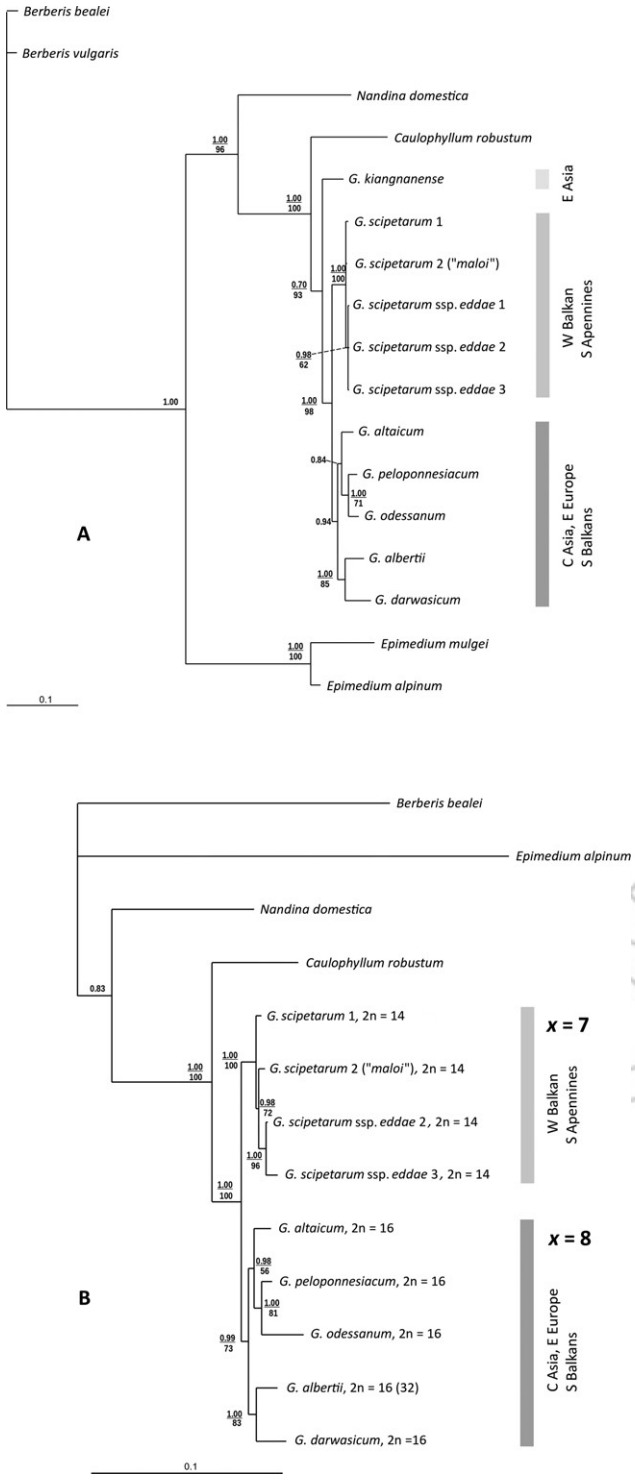

Figure 7. Phylogenetic trees of *Gymnospermium*. (A) Majority-rule consensus phylogram from Bayesian analysis of ITS sequences; PP values and bootstrap support >50% are shown above and below nodes, respectively. The major clades are indicated to the right; (B) Majority-rule consensus phylogram from Bayesian analysis of concatenated ITS-IGS sequences; PP values and bootstrap support >50% are shown above and below nodes, respectively. Chromosome numbers are reported for each investigated species (sources in the text), and the major clades with the respective chromosome base number are indicated to the right.

the affinity between the two latter taxa was corroborated by 1.00 PP and 71% BS. The position of *G. altaicum* remained unresolved in the bootstrap tree. The second subclade was well supported and included all the accessions from Albania and Italy (1.00 PP; 100% BS), which shared five substitutions and 1-bp insertion in the ITS1 region. Relationships between the Italian and Balkan populations were not resolved, and genetic distance between them was the same (0.002). However, the Italian accessions clustered together with 0.98 PP and 62% BS and were characterized by a unique 1-bp substitution (A/C transversion) in the ITS1 region.

Plastid IGS dataset

The aligned matrix included 1017 positions, of which 705 constant, 252 variable but parsimony non-informative and 60 (5.9%) parsimony informative. Hence, this region showed a much lower rate of variation than nuclear ITS and provided a weak phylogenetic signal. The mean genetic distance within *Gymnospermium* was 0.015 and the highest distance was in the pair *G. microrrhynchum-G. odessanum* (0.039). The heuristic search produced 84 most parsimonious trees with L = 352, CI =0.935 and RI =0.758. Both the strict consensus and the 50% majority-rule consensus phylogram from Bayesian analysis retrieved *Caulophyllum* (1.00 PP; 98% BS) sister to *Gymnospermium* (1.00 PP, 90% BS) in a well-supported group (1.00 PP; 100% BS). However, relationships within *Gymnospermium* remained almost completely unresolved in the Maximum Parsimony analysis, where only the affinity between the Italian populations emerged (83% BS); these were characterized by one 1-bp substitution in position 16 and 1-bp insertion in position 43. This affinity also emerged in the Bayesian analysis (0.99 PP), where the Italian accessions resulted sister to the Balkan populations (0.86 PP); a unique 8-bp insertion in positions 728–735 supported the common ancestry of this Apennine-Balkan group. Genetic distance between the Apennine and Balkan populations (0.017) was considerably higher than the average one between the Balkan populations (0.008). Relationships between the E Asian, C Asian and E European taxa were not resolved, with only a weak support to a group with *G. darwasicum, G. peloponnesiacum* and *G. odessanum* (0.80 PP; BS <50% BS).

Combined ITS-IGS dataset

The aligned matrix included a total of 1686 positions, of which 1044 were constant, 477 variable but non-informative and 165 variable and parsimony informative. The heuristic search produced a single tree with L = 843, CI =0.91 and RI =0.73. The topology of this tree was largely consistent with that of the 50% majority-rule consensus phylogram from Bayesian analysis (Figure 7(B)). *Caulophyllum* was sister to *Gymnospermium* (1.00 PP; 100% BS), which showed a split in two major clades. The first clade (0.99 PP; 73% BS) included all species from C Asia and E Europe, plus *G. peloponnesiacum* from S Greece. The latter was sister to *G. odessanum* (1.00 PP; 81% BS) and both were in turn sister to *G. altaicum* (0.98 PP; 56% BS); a second group (1.00 PP, 83% BS) was formed by *G. albertii* sister to *G. darwasicum*. In the second main clade (1.00 PP, 100% BS), *G. scipetarum* was sister to the Italian accessions (0.98PP; 72% BS), that clustered together with 1.00 PP and 96%BS.

Taxonomy

The Italian plant can be referred to a new subspecies of *G. scipetarum* based on slight but convergent morphological and genetic distinguishing characters, a likely result of its long time of isolation in the southern Apennines (see Discussion).

Gymnospermium scipetarum subsp. eddae Rosati, Farris, Fascetti et Selvi, *subsp. nov.*

Type. ITALY: Campania Region, Salerno Province, Maddalena Mts., Padula, in *Fagus sylvatica* forest, 1300 m a.s.l., UTM 33 T 558 E; 4470 N, *Rosati and Fascetti,* 22 April 2015. Holo- HLUC 6183!, Iso- FI 050306!, SS 2000/2963!).

Paratypes: Ibid., 9 April 2013, *Farris and Rosati*, 3550 HLUC!; ibid., 1 April 2015, *Rosati*, HLUC 6185!; ibid., 17 May 2015, *Rosati* HLUC 6178!.

English Diagnosis. Differs from *G. scipetarum* by the lower ratio stamen to petal (1.3 vs. 1.5-1.7) and the lower ratio style to carpel (0.3 vs. 0.5).

Etymology. The new species is dedicated to Mrs. Edda Lattanzi, an eminent student of the Italian flora who strongly encouraged the botanists of University of Basilicata to organize, in 2013, the botanical excursion of the Italian Botanical Society on the Maddalena mountains. The discovery of *Gymnospermium* in Italy was done during the preparation of this excursion.

Conservation. Following the IUCN (2012) criteria for red listing, *G. scipetarum* subsp. *eddae* should be considered as Endangered (EN) in Italy, based on the severely fragmented and restricted occupancy area ($< 5\,km^2$) and the small number of subpopulations (six). Overall population trend is unknown and is currently under investigation.

Discussion

This study provides the first in-depth insights into the morphology, karyology and phylogenetic relationships of the Italian population of *Gymnospermium* found in April 2013 (Rosati et al. 2014, 2017).

Morphology largely supports its close similarity to the populations from Albania and Montenegro, all of which were considered to belong to *G. scipetarum* (Barina et al. 2016) also based on genetic evidence (Barina et al. 2017). Both morphometric and molecular data presupposed that *G. maloi*, previously recognized as a separate species endemic to south Albania (Tan et al. 2011), should be considered a heterotypic synonym of *G. scipetarum* because not sufficiently distinct. Close relationship between the Balkan and Apennine populations of the latter species emerged from also our molecular phylogenetic analyses. In spite of the relatively low rates of sequence variation in the two regions analyzed here, our results support that these populations form a clade with remarkable molecular auto-apomorphies such as the 8-bp insertion in the IGS region. This was lacking in the taxa of the second major clade retrieved in our combined ITS-IGS phylogeny, that included all the taxa from mainly central Asia and Eastern Europe, plus the Greek endemic *G. peloponnesiacum*. Interestingly, a common 6-bp insertion in the IGS region underscores the close affinity between the latter and *G. odessanum* already indicated by Takhtajan (1970) and Phitos (2003) on morphological grounds. In addition, our molecular data also supported the affinity of both latter species to *G. altaicum*, as also found by Barina et al. (2017). Hence, *G. peloponnesiacum* is more closely related to the geographically distant east European and central Asian species than to the only other Mediterranean taxon *G. scipetarum* in Albania, Montenegro and Italy. According to Barina et al. (2017), *G. scipetarum* is more closely related to the Caucasian endemic *Gymospermium smirnovii*. Interestingly, *G. albertii* and *G. darwasicum*, not analyzed in previous studies on Berberidaceae (e.g. Kim and Jansen 1996; Kim et al. 2004, Wang et al. 2007, Barina et al. 2017), resulted also included in this second major clade but they formed a distinct subclade, in line with their phenetic similarity and parapatric distribution in central Asia (Loconte and Estes 1989).

Remarkably, the phylogenetic coherence and distinctiveness of the *G. scipetarum* s.l. clade is supported by karyological evidence. As for the Italian plants, the Albanian populations previously referred to G. *maloi* have 2n =14 (Tan et al. 2011), however, chromosome number for typical *G. scipetarum* is still unclear. Both 2n = 14 and 16 have been observed in *G. scipetarum* (Z. Zekaj, unpublished data) but genome size is very similar to that of *G. maloi* ($2\,C = 29.55 \pm 1.35$ pg. vs. 29.44 ± 0.47 pg., respectively; Tan et al. 2011). All other *Gymnospermium* species investigated to date are instead characterized by 2n = 16 (rarely some populations by 2n = 32; Kosenko 1977, 1978, 1979; Loconte 1993; Kim et al. 2004), including *G. peloponnesiacum* (Figure 7(B)). The same number was reported for species of *Leontice* (Kosenko, 1977, 1978, 1979), suggesting that $x = 8$ is the primary chromosome number in the two genera (Wang et al. 2007). A trend of descending chromosome number has likely occurred in the westernmost part of the *Gymnospermium* range, probably acting as a major speciation driver of *G. scipetarum*. Therefore, the base number $x = 7$ is a significant synapomorphic trait of the Balkan-Apennine populations of the latter species, underscoring their common ancestry.

On the other hand, the Italian plants showed a slight morphological divergence in two quantitative floral characters and in both genomic regions analyzed here. Remarkably, their genetic distance to the Balkan populations was substantially higher than that between these populations, due to a few unique positions especially in the IGS region. As a result, the Italian accessions formed a terminal clade which strongly supports their native status. In fact, the very low rate of sequence divergence in *Gymnospermium*, even between well distinct, allopatric species as shown here, implies that the divergence of the Italian plants can only be originated by a long time of geographical and reproductive isolation. The alternative hypothesis of deliberate human introduction of *G. scipetarum* to Italy in historical times would have been supported if the Apennine plants were identical to the Balkan ones in their morphological and molecular profiles, which was not the case here. Hence, these results provide an answer to the open question in the recent checklist of the Italian native flora (Bartolucci et al.

2018) and, at the same time, justify the placement of the Italian populations into a new subspecies endemic to the southern Apennines.

From a biogeographic point of view, the presence of *Gymnospermium* in the southern Italian peninsula demonstrates that the westward spread of this genus has been greater than previously thought. In fact, it reached the central Mediterranean region despite the present-day barrier of the Adriatic sea. This pattern of disjunct amphi-Adriatic distribution is not unique to *G. scipetarum* since it is known for a number of other dicot and monocot groups such as those of *Cardamine maritima* (Kučera et al. 2010), *Campanula* series *Garganicae* (Park et al. 2006) and *Sesleria tenuifolia* (Di Pietro et al. 2005). In addition, several species have a similarly disjunct range, such as *Asyneuma limoniifolium* (L.) Janch., *Erica manipuliflora* Salisb., *Inula verbascifolia* (Willd.) Hausskn. and *Cerinthe retorta* Sm. (Wagensommer et al. 2014). These and other possible examples clearly show the phytogeographic affinity between southern Italy and the western Balkans, resulting from either over-sea seed dispersal events or multiple trans-Adriatic terrestrial connections during the pre-glacial periods (Turill 1929; Pignatti 1982; Di Pietro and Misano 2010). Land bridges between these two peninsulas existed repeatedly in the Oligocene and late Miocene, in particular during the Tortonian and Messinian periods, and are well supported by stratigraphical evidence and terrestrial vertebrate fossils (Azzaroli and Guazzone, 1980; Musacchio et al. 2006; Patacca et al. 2008). We assume that the colonization of the present-day southern Apennines by populations of *G. scipetarum* was made possible by these past land bridges, since long-distance dispersal events appear much less probable in view of the peculiar fruit and diaspore morphology in this genus. Mature fruits are often borne on downwardly recurved pedicels and possess a membranous pericarp that splits to expose the seeds when ripe (Loconte 1993; Figure 3(E)). Presence of a stipe-like strophiole on the seed indicates that short-distance dispersal by ants is the main strategy in this genus, as also reported by Beattie (1983) and Lengyel et al. (2009). Indeed, myrmecochory is a major mechanism of dispersal in many herbaceous plants of forest understory communities (Gorb and Gorb 2003) and is supposed to have acted as a major driver of speciation in numerous angiosperm families by enhancing geographical isolation by extremely limited dispersal distances. Remarkably, Lengyel et al. (2009) quote *Gymnospermium* and *Leontice* among the many cases of sister genera where myrmecochory versus non-myrmecochory (*Leontice*) may have been a driver for a higher speciation rate.

Similarly, to most congeneric species that appear in national red lists (Chang et al. 2004; Oprea 2005; Kim et al. 2016), or are subject to special conservation programs (Mikatadze-Pantsulaia et al. 2010), *G. scipetarum* subsp. *eddae* should be considered as "endangered" due to its very narrow distribution. Combined with its early flowering time and fugacious appearance above the ground level, the restricted occupancy area explains why this herb has escaped the observations of the many and illustrious botanists who have explored the southern Apennines in the last two centuries. This shows that field researches can still lead to significant botanical findings even in a relatively well-known area of Europe, with important implications for the conservation of vascular plant diversity at the local and global scale (Marignani et al. 2014).

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Appendix 1. List of taxa and International nucleotide sequence database collaboration (INSDC) accession numbers for DNA sequences (ITS, *trn*L-F IGS when available) used in this study (alphabetical order). Voucher information is given only for specimens of *gymnospermium* originally analyzed here (original accession number marked with an asterisk).

Berberis bealei Fortune: KU221046, FJ626558; ***Berberis vulgaris*** L.: EF488082; ***Caulophyllum robustum*** Maxim.: EU592026, AF325911; ***Caulophyllum thalictroides*** (L.) Michaux: FJ626552; ***Epimedium alpinum*** L.: DQ851480, AY362445; ***Epimedium multiflorum*** T.S. Ying: GQ924950; ***Gymnospermium albertii*** (Regel) Takht.: *K. Tan et G. Vold*, 19 March 2015, Denmark (culta), KX257208*, KX272778*; ***Gymnospermium altaicum*** (Pallas) Spach: *P. Golyakov*, 2 April 2015, Russia, 51,144787N; 82.970832E, [HLUC 6032], KX257207*, KX272777*; ***Gymnospermium darwasicum*** (Regel) Takht.: Kit Tan et G. Vold, 19 March 2015, Denmark (culta), KX257209*, KX272779*; ***Gymnospermium scipetarum*** subsp. ***eddae*** Rosati, Fascetti, Farris et Selvi: 1) *L. Rosati*, 22 april 2015, Italy, 40.3667N 15.6960E, [HLUC 6184], KX257203*, KX272773*; 2) *L. Rosati*, 22 april 2015, Italy, 40.3836N 15.6767E, [HLUC 6182], KX257202*, KX272772*; 3) *L. Rosati*, 17 May 2015, Italy 40.3841N 15.6824E, [HLUC 6171], KX257201*; ***Gymnospermium microrrhynchum*** (S. Moore) Takht.: FJ626556; ***Gymnospermium odessanum*** (DC.) Takht.: *I. Moysiyenko*, 10 april 2015, Ukraine, 47.206873N; 33.152694E, [KHER (without number)], KX257206*, KX272776*; ***Gymnospermium peloponnesiacum*** (Phitos) Strid: *K. Tan et G. Vold*, 26 april 2015, Denmark (culta), KX257205*, KX272775*; ***Gymnospermium scipetarum*** E. Mayer et Pulević: 1) *K. Tan & G. Vold*, 26 march 2015, Denmark (culta), KX257200*, KX272771*; 2) *K. Tan et G. Vold*, 26 March 2015, Denmark (culta sub. **G. *maloi*** Kit Tan et Shuka), KX257204*, KX272774*; ***Nandina domestica*** Thunb.: AY362430, AF335295.