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Contribution to *Globularia* phylogeny based on nuclear ribosomal spacer and two chloroplast DNA regions

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Abstract

Background and Purpose: *Molecular approach has a major impact on phylogenetic studies of plants, considering that it gives useful information about evolutionary events and relations on all taxonomic levels. The sequence data of the nuclear ITS and of two chloroplast regions,* trn*L-*trn*F spacer and* rbc*L gene, obtained from thirteen* Globularia *L. taxa, including five Anatolian endemics, representing six sections altogether, were analyzed in order to determine the relations between the European and the Anatolian species and get a better insight into the phylogeny of several closely related* Globularia *taxa.*

Materials and Methods: *Total cellular DNA was extracted from fresh or frozen leaf tissue of thirteen* Globularia *samples. The ITS regions of nuclear DNA and two chloroplast DNA regions were amplified and sequenced. Obtained nuclear and combined plastid data matrices were subjected to Maximum Parsimony analyses.*

Results and Conclusions*: Molecular data that were obtained in this study indicate the existence of separate centers of diversification for the European and the Anatolian* Globularia*. The results provide support for relationships among the studied Anatolian endemic species and indications for a redefinition of affinities of some of the European species. The results presented herein are discussed along with available morphological, karyological, phytogeographical and molecular data.*

INTRODUCTION

Globularia L. is a small Angiosperm genus mostly comprised of evergreen perennials and small shrubs, recognizable by blue flowers assembled in globular capitula, which it was named after. The first extensive classifications of *Globularia* were proposed by Schwarz *(1, 2).* They comprised 22-25 taxa, which were divided into nine sections. Subsequently, Schwarz's studies regarding this genus have undergone some modifications by several authors *(3, 4, 5).* The finally accepted classification of *Globularia* separates the genus into eight sections based on morphological characters, cytological data, ecology and distribution. These include *Lytanthus*, *Polycephalium*, *Carradoria*, *Hellenion*, *Globularia*, *Alypum*, *Empetron* and *Gymnocladium (5).*

Since most of the taxa are localized in Central and Southern Europe, Anatolia, Northern Africa and Macaronesia, the Mediterranean basin is viewed as their primary center of diversification, which has started approximately 7.57 million years ago in the Miocene *(6, 7). Globularia vulgaris* L. is the only taxon distributed more northward, reaching the

Baltic region. A total of 15 *Globularia* species are included in the European flora *(8)* and 11 taxa in the flora of Turkey *(9, 10)*. Among and above these taxa there is a considerable number of endemic species, particularly in Macaronesia *(11)*, Morocco *(12)*, Italy *(13)*, Greece *(14)* and Turkey *(9, 15)*. Taxa of *Globularia* are known as outcrossing and mainly exhibit two ploidy levels, diploids (2n=2x=16 chromosomes) and tetraploids (2n=4x=32), whereby autopolyploidy is a proposed mode of genome duplication *(1, 3, 4, 16)*. Cytogeographical relations indicate that endemic taxa and taxa which grow in moderate climate areas are predominantly diploids, while taxa with broader distributions and growing in rigorous habitat conditions are mainly tetraploids *(1, 3)*. All *Globularia* examined by now have the same pollen morphology *(17)*. From an economic point of view, several *Globularia* species are valuable herbal plants, for example *G. alypum* L. and *G. trichosantha* Fisch & C.A. Mey., used in traditional medicine of countries such as Spain, Italy, Tunisia and Turkey *(18, 19, 20)*. Several recent investigations showed that some widespread European taxa also exhibit medicinal potential *(21, 22)*.

In the past few decades, molecular studies have greatly contributed to a better understanding of relations and plant phylogeny at all taxonomic levels. Accordingly, the genus *Globularia* that had traditionally been included in the homonymous family of Globulariaceae, was meanwhile proposed for inclusion into the Plantaginaceae, based on molecular evidence *(23, 24)*. Moreover, the ITS molecular data obtained from 23 *Globularia* taxa proposed interesting conclusions about their Miocene origin and Pleistocene independent development of three European Alpine/montane and two Mediterranean *Globularia* lineages with proposed rate of 0.33 net speciation events per million years *(6, 7, 25, 26)*.

In order to provide better understanding of intrageneric relationships among *Globularia*, Maximum Parsimony analyses were conducted on three DNA regions from seven European, five Anatolian and one Mediterranean *Globularia* taxon. Taking into account the above mentioned studies that comprise most of the *Globularia* species, including Macaronesian endemic species, our study is focused on the comparison of several European and Anatolian species, among which five are endemics. Phylogenetic relationships were reconstructed using the internal transcribed spacer (ITS) of the nuclear rDNA repeats and two chloroplast regions, nontranscribed *trn*L-*trn*F spacer and plastid gene *rbc*L.

MATERIALS AND METHODS

Plant Material and DNA Extraction

Samples of 13 *Globularia* taxa belonging to six sections were studied. The plants were collected from their natural habitats or obtained from botanical gardens. Voucher specimens were deposited in the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Table 1 lists all the data about plant samples, including the geographical distribution of investigated taxa and GenBank accession numbers of the analyzed sequences. Total cellular DNA was extracted from 100–140g dry, fresh or frozen leaf tissue following the procedure of Doyle and Doyle *(27)*, modified as reported in Petit et al *(28)*.

Amplification and Sequencing

The ITS regions of nuclear DNA and two chloroplast DNA regions were amplified via the polymerase chain reaction (PCR) using the primer pairs designed by White et al. *(29)* for ITS, Taberlet et al. *(30)* for *trn*L-*trn*F and Hasebe et al. *(31)* for *rbc*L. Amplification reactions were performed in volumes of 50µL containing 0.5µM of each primer, 200µM of each nucleotide, 2mM $MgCl_2$ and 1.25 units of *TaKaRa Taq* HS polymerase (Takara Bio Inc, Japan). Amplifications were performed using a Biorad MyCycler 1065 under the following conditions: first denaturation at 93°C for 3 min, followed by 35 cycles of template denaturation at 93°C for 1 min, primer annealing at 50°C for 1 min, elongation at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were verified by electrophoresis on 0.7% agarose gels containing ethidium bromide in Tris-acetate EDTA (TAE) buffer and detected under UV light. The size of fragments was estimated by comparison with a molecular size standard (GeneRuler^{TM} DNA Ladder Mix, Fermentas). The PCR products were purified by using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, USA) in accordance with the protocol of the manufacturer. DNA sequencing was performed by Macrogen (Seoul, South Korea) on an ABI3730XL DNA sequencer, using the same primer set as for PCR. Double-stranded sequencing was performed for at least 75% of their total length. All sequences have been deposited in the GenBank Sequence Database (Table 1).

Sequence Alignment and Phylogenetic Reconstruction

Nucleotide sequence fragments were manually edited and aligned using ClustalX.2.1 *(32)*. The alignment is available from the author upon request. The data set contained a total of 13 *Globularia* taxa sequenced in this work, and the sequences of *Plantago major* L. (*trn*L-*trn*F, *rbc*L), *P. lanceolata* L. (ITS), *Veronica officinalis* L. (ITS, *trn*L-*trn*F, *rbc*L), *Digitalis lanata* Ehrh. (ITS, *trn*L-*trn*F) and *D. lutea* L. (*rbc*L), which were retrieved from GenBank. GenBank accession numbers of outgroup taxa are listed in Table 2. We analyzed the nuclear and combined plastid data sets independently and thus two sequence data matrices were generated: (1) an ITS data matrix and (2) a *trn*L-*trn*F combined with a *rbc*L data matrix, all comprised of 13 *Globularia* taxa and 3 outgroup taxa.

Sequence data matrices were subjected to phylogenetic analyses using the Maximum Parsimony Method in

TABLE 1 *Plant material, voucher information, geographical distribution and GenBank accession numbers.*

Taxon	Source of material1)	Voucher number	Geographical distribution2)	GenBank accession number	Section4)
Globularia alypum L.	42°30′ N 18°19′ E, 1	16025_1	Mediterranean	ITS KP278477 LF3) KT853048 *rbc*L KT853061	*Alypum*
G. anatolica Duran, Çetin, Öztürk	37°44′ N 29°20′ E, 2	16030_1	Anatolia endemic	ITS KT757362 LF KT853049 *rbc*L KT853062	*Polycephalium*
G. cordifolia L.	44°43′ N 14°58′ E, 3	16035_1	Central / SE Europe	ITS KP278478 LF KT853050 *rbc*L KT853063	*Empetron*
G. davisiana O. Schwarz	36°44′ N 30°32′ E, 2	16040_1	Anatolia endemic	ITS KT757363 LF KT853051 *rbc*L KT853064	*Polycephalium*
G. dumulosa O. Schwarz	mc, 4	16042_1	Anatolia endemic	ITS KT757364 LF KT853052 *rbc*L KT853065	*Hellenion*
G. hedgei H. Duman	37°27′ N 30°54′ E, 2	16043_1	Anatolia endemic	ITS KT757365 LF KT853053 *rbc*L KT853066	*Polycephalium*
G. meridionalis (Podp.) O. Schwarz	45°22′ N 14°30′ E, 3	16045_1	Central / SE Europe	ITS KP278479 LF KT853054 *rbc*L KT853067	*Empetron*
G. nudicaulis L.	45°45′ N 10°36′ E, 5	16050_1	Central Europe	ITS KT757366 LF KT853055 *rbc*L KT853068	*Gymnocladium*
G. punctata Lapeyr.	45°22′ N 14°30′ E, 3	16059_1	Europe	ITS KP278480 LF KT853056 *rbc*L KT853069	*Globularia*
G. repens Lam.	mc, 5	16060_1	SW Europe	ITS KT757367 LF KT853057 *rbc*L KT853070	*Empetron*
G. sintenisii Hausskn. & Wettst.	37°42′ N 41°24′ E, 2	16065_1	Anatolia endemic	ITS KT757368 LF KT853058 *rbc*L KT853071	*Polycephalium*
G. trichosantha Fisch. & C.A.Mey.	mc, 6	16070_1	E Europe, Anatolia, Crimea	ITS KT757369 LF KT853059 *rbc*L KT853072	*Globularia*
G. vulgaris L.	56°58′ N 16°46′ E, 7	16072_1	N Iberia, NW Baltic	ITS KT757370 LF KT853060 *rbc*L KT853073	*Globularia*

1) 1 – Collected by R. Garić from natural stands in Croatia, 2 – Collected by A. Duran from natural stands in Turkey, 3 – Collected by K. Hazler Pilepić from natural stands in Croatia, 4 – Botanische Gärten der Christian-Albrechts-Universität zu Kiel, Germany; 5 – Botanische Gärten der Universität Bonn, Germany; 6 – Botanische Gärten der Philipps-Universität, Marburg, Germany; 7 – Collected by S. Maslo from natural stands in Sweden. mc – missing coordinates. 2) Tutin et al *(3)*; Duran et al *(9); Schwarz (1)*; Duman *(15)*; Wettstein *(40)*.3) LF – *trn*L-*trn*F intergenic spacer. 4) Schwarz *(1)*; Duman *(15)*; Duran et al *(9)*.

MEGA 6 *(33)*. Three outgroups were used to root the trees. Phylogenetic reconstructions were carried out via heuristic searches using the Subtree-Pruning-Regrafting (SPR) algorithm *(34)* with search level 1 in which the initial trees were obtained by random addition of sequences (10 replicates). Gaps and missing data were excluded from the dataset. The bootstrap method *(35)* was employed to examine the robustness of various clades revealed in the trees.

RESULTS

Analysis of the ITS Data Set

The aligned length of the ITS data set was 863 base pairs, 468 of which were constant, while 255 were variable characters and 111 (13%) were potentially parsimony-informative characters. Six equally parsimonious trees (consistency index 0.763, retention index 0.794) were

TABLE 2 *Outgroup samples' sequences retrieved from GenBank.*

Taxon	Sequence	Accesion number
Plantago lanceolata L.	ITS	AY101898.1
P. major L.	*trn*L-*trn*F	FJ490807.1
P. major L.	*rbc*L	KF602240.1
Veronica officinalis L.	ITS	DQ534900.1
V. officinalis L.	*trn*L-*trn*F	AF486391.1
V. officinalis L.	*rbc*L	HQ590322.1
Digitalis lanata L.	ITS	AY591284.1
D. lanata L.	*trn*L-*trn*F	AY591318.1
D. lutea L.	*rbc*L	FM207428.1

found by Maximum Parsimony analysis of the ITS region of the 13 taxa studied. The 50% consensus tree is presented in Figure 1. Branches corresponding to partitions reproduced in less than 50% of the trees were collapsed.

The topology of the ITS consensus tree indicates that *Globularia* is separated into two major clades with high bootstrap support (BS = 83%/83%). Within clade I a sister relationship between all the European taxa is apparent, while within clade II all the Anatolian taxa are clustered together with the Mediterranean taxon *G. alypum*. The taxa within the European clade are separated into two well-supported subclades (BS = 100%). The first subclade comprises representatives of the section *Globularia* along with one from the section *Gymnocladium*, while all taxa in the second subclade belong to the section *Empetron*. In the Anatolian clade, the taxa from sections *Polycephalium* and *Alypum* form a subclade (BS = 66%) divided into two branches (BS = 66% and 50%), while *G. dumulosa* is supported as a sister to this alliance (BS = 83%).

Analysis of Combined *trn*L-*trn*F and rbcL Data Set

The final alignment of the combined *trn*L-*trn*F and *rbc*L data sets had 2217 positions, 1659 of which were constant, while 501 characters were variable and 81 characters (4%) were potentially parsimony-informative. Maximum Parsimony analysis of the combined *trn*L-*trn*F and *rbc*L regions of the 16 taxa found two most parsimonious trees (consistency index 0.809, retention index 0.827). The 50% majority rule consensus tree is shown in Figure 2.

The main topology of the consensus tree produced from combined *trn*L-*trn*F/*rbc*L sequence data was not much different from that of the ITS consensus tree. However, lower resolution was noticed for some taxa in the plastid sequences-based tree. All *Globularia* taxa were grouped into two major clusters. The extensive one (BS = 100%) comprises all European taxa along with the Anatolian *G. dumulosa,* while the Mediterranean *G. alypum* forms a weakly supported sister clade (BS = 50%). The second clade contains three representatives from the Anatolian section *Polycephalium* (BS = 100%), while *G. sintenisii* is separated without support.

DISCUSSION

The ITS sequence data, which are widely used for phylogenetic reconstruction of closely related species, showed an appropriate level of variation in the genus *Globularia*. Applied cpDNA regions were found to be less informative for this genus. Slower evolutionary rates of chloroplast genome may cause difficulties in finding the appropriate phylogenetic signal in some plant taxa *(36).*

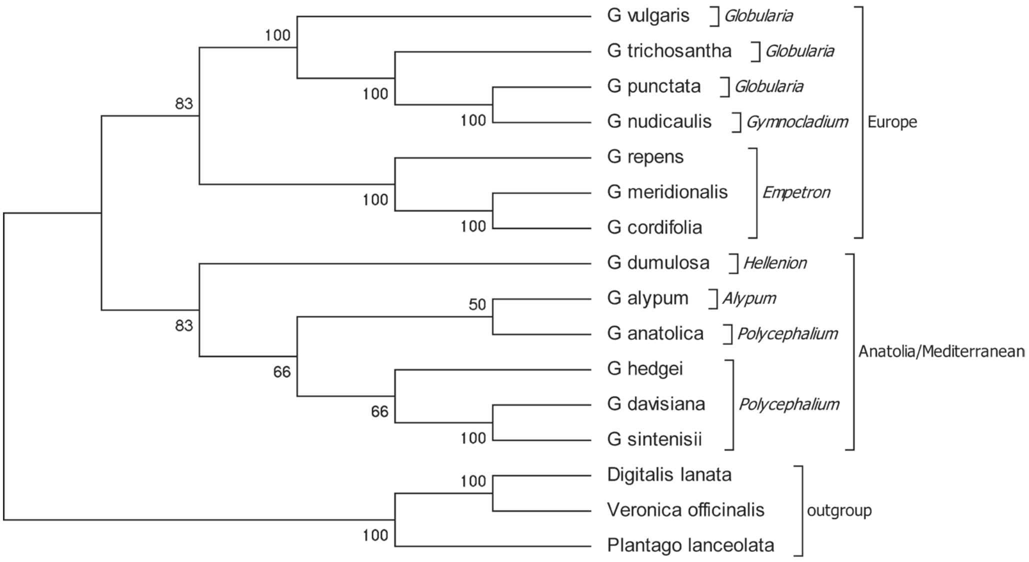

Figure 1. *The 50% consensus tree of 6 most parsimonious trees (CI = 0.763; RI = 0.794) from the Maximum Parsimony analysis of nrDNA ITS sequences obtained from* Globularia *taxa. Numbers above the branches are bootstrap values. Brackets indicate sections discussed in the text.*

Nevertheless, both trees showed similar topology and several resulting conclusions were derived.

The genus *Globularia* in this paper is represented by 13 taxa. Seven of them originate from Europe, five are Anatolian, while one is a Mediterranean species. The taxa investigated in our study belong to six sections and the resulting parsimony analysis mainly corroborates the current classification and interspecific affinities. The results of our analyses provide strong evidence for an early split within the genus and further diversification of two lineages in both the European and the Anatolian area.

According to the ITS 50% consensus tree, *G. vulgaris*, *G. punctata* and *G. trichosantha*, representatives of the section *Globularia* in our data set, form a well-supported clade (BS = 100%) including the *G. nudicaulis*, the single representative of the section *Gymnocladium*. By contrast, the cpDNA tree shows two well supported dichotomous nodes (BS = 100%), the first consisted of *G. vulgaris* and *G. punctata,* while the other one includes *G. nudicaulis* and *G. trichosantha.* The aforementioned taxa are evergreen perennials: *G. vulgaris*, *G. punctata* and *G. nudicaulis* form pleiocormous rosettes, while *G. trichosantha* develops stolons and has an espalier like growth form *(5). G. vulgaris* is a tetraploid *(1, 3, 13, 16)* showing two disjunct distribution areas, one on the Iberian peninsula and in Southern France, and the other on the Baltic islands of Öland and Gotland *(8).* This could indicate that *G. vulgaris* used to be more widespread than it is today. With respect to the estimated origin of *Globularia* in the Late Miocene *(7)*, it is possible that these populations separated during the Quaternary and that the polyploidization occured due to unfavorable conditions of glaciation periods. It has been proven that effective 2n gametes, which may lead to polyploidization, are induced by abiotic stress such as temperature *(37).* Interestingly, no morphological difference has been noticed between the separated populations *(16). G. vulgaris* shares a common cpDNA haplotype with *G. punctata,* which is a diploid taxon *(1, 3, 5, 16)* and the most widespread European *Globularia*, suggesting the possibility of a common maternal ancestor and overlapping areas in the past. Nevertheless, the ITS data indicates a close relationship of *G. punctata* with *G. nudicaulis,* a montane European taxon distributed in the Alps, the Pyrenees and mountains of Northern Spain. This diploid taxon, which shows great uniformity throughout the geographical range of its distribution (from Northern Spain to the Alps, *8, 13*), belongs to the section *Gymnocladium*, along with two taxa: *G. gracilis* Rouy et Richt., which is restricted to the Pyrenees and *G. liouvillei* Jah. et Maire, an endemic taxon of High Atlas in Morocco. According to Schwarz *(1)*, affiliation to the section *Gymnocladium* is based on the development of short stolons. However, *G. nudicaulis* was found to grow without stolons *(5).* Whereas earlier obtained ITS data *(26)* have put *G. nudicaulis* in a separate clade along with *G. gracilis* from the same section, the results of our ITS analysis that indicate a sister-relationship between *G. nudicaulis* and *G. punctata* are somewhat unexpected. Moreover, in the cpDNA tree, *G. nudicaulis* was positioned with *G. trichosantha,* another member of the section *Globularia.* These results may be a consequence of incomplete taxon sampling. *G. trichosantha* is a diploid taxon distributed from the Eastern Balkans to the Crimea. Comes and Kadereit *(26)* have considered this species an early branching taxon of the Asia Minor clade together with *G. punctata*, but our molecular data clearly support separation of these European samples from those of Asia Minor.

The position of three taxa in our study that belong to the European section of *Empetron* (*G. cordifolia*, *G. meridionalis* and *G. repens*) was in good agreement with the current intrageneric classification. Close relationship was confirmed by both of our trees (BS = 100). These taxa are morphologically very similar dwarf shrubs, distributed in mountainous regions. *G. repens* is a diploid taxon *(1, 3)*, slightly smaller than the other two, commonly found in mountainous regions of South-Western Europe. Comes and Kadereit *(25, 26)*, proposed that *G. cordifolia* and *G. repens,* along with other Apennines/Balkan *Globularia* taxa (*G. neapolitana* O. Schwarz and *G. stygia* Orph. ex Boiss.), originated in the Pleistocene. Origin of many montane taxa could be explained by the ecological niche concept.

G. cordifolia and *G. meridionalis* form a supported (BS = 100) subcluster indicating their common ancestor and encouraging existing debates about their taxonomic affiliation. These two taxa are widespread in the mountains of Central and Southern Europe, from the Pyrenees to the Carpathians and show high degree of morphological and karyological polymorphism (2n=16 or 32, *4, 16, 38, 39*). In his early work, Wettstein *(40)* had noticed the existence of an intermediate form between these two taxa (*G. cordifolia* var. *intermedia*). In spite of that, Schwarz *(2)* persisted in the division of these taxa, giving distinguishing features and precise geographical distribution for each taxon. Nonetheless, a further comprehensive comparison of these two *Globularia* throughout their distribution area confirmed a high level of variability *(38).* Therefore, differentiation based on their morphological features, clearly described by Schwarz, is not always possible, as both of the taxa display mixed characteristics finally leading to their questionable separation *(4, 38).* Even though our results indicate common genealogy, at this level of research it could not be assessed with certainty whether the taxa should be divided or considered as one. Molecular data could suggest that *G. cordifolia* and *G. meridionalis* are evolutionary young, sympatric taxa, among which total reproductive isolation cannot be confirmed, especially taking into account their mixed characteristics, particularly seen in the populations from overlapping areas, such as the Dinaric Alps *(38, 41).* Tetraploid forms, which are mostly found in the Apennines and in the Dinaric regions, could be an effective way of

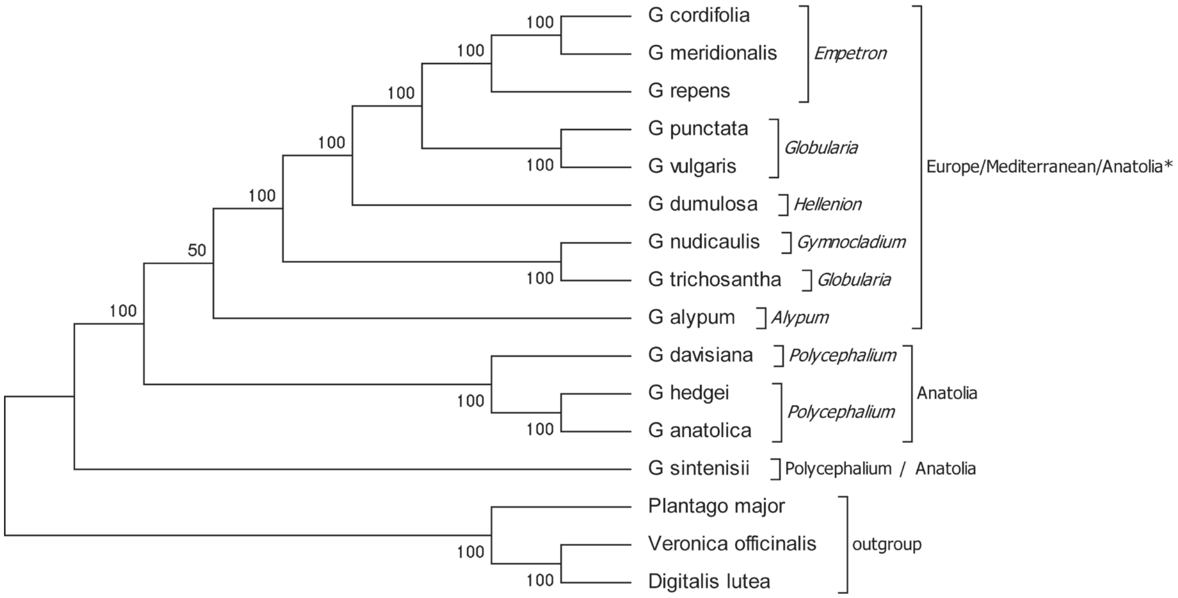

Figure 2. *The 50% consensus tree of 2 most parsimonious trees (CI = 0.809; RI = 0.827) from the analysis of combined* trn*L*-trn*F* *and* rbc*L* *sequences obtained from* Globularia *taxa. Numbers above the branches are bootstrap values. Brackets indicate sections discussed in the text.*
*G. dumulosa

adaptation during glaciations and a powerful force for postglacial recolonizations. Existence of a tetraploid form could have some evolutionary advantage, especially in the areas with strong environmental changes *(42)*. However, molecular data that give evidence of close links between *G. cordifolia* and *G. meridionalis* could also suggest that these taxa could be considered as one. The results of phytochemical comparative studies of Croatian populations showed a pronounced similarity between their essential oil composition *(43)* and iridoid content *(44)*. From everything mentioned above, the consideration of these taxa as subspecies, which is very common in older botanical literature, cannot be dismissed *(5, 45, 46, 47, 48, 49, 50)*. However, the use of low-copy nuclear genes or some other molecular markers, which could provide sufficient resolution and further evidence on relations among these two taxa, is necessary.

Five Anatolian endemic taxa, *G. anatolica*, *G. davisiana*, *G. dumulosa, G. hedgei* and *G. sintenisii*, form a clearly resolved clade along with the Mediterranean *G. alypum* on the ITS tree. Based on the chloroplast sequence data, phylogenetic positions of *G. alypum* and *G. sintenisii* were poorly resolved, as demonstrated by weak or no bootstrap support, respectively. According to the same data set, *G. dumulosa,* which belongs to the section *Hellenion***,** was positioned among the European taxa. It is a high-mountainous, long-lived shrub that is morphologically well-characterized by hemispherical cushion-forming growth *(5)*. It probably originated in the Pliocene, as did the majority of *Globularia (7)*. Isolated and well-adapted, it remains to be present until today in the area of high mountains.

The remaining investigated Anatolian endemic taxa (*G. anatolica*, *G. davisiana*, *G. hedgei* and *G. sintenisii*) belong to the section *Polycephalium. G. anatolica* is a new endemic species found recently in the Honaz Mountain National Park *(9)*, an important biogeographical region, very rich in endemic plants. Although this taxon is morphologically most similar to *G. sintenisii (9)*, our ITS data indicate a poorly supported relation with *G. alypum*, which could be a consequence of our incomplete dataset or poor resolution of the ITS alone. By contrast, the cpDNA data confirm its close relation with *G. hedgei* and *G. davisiana*. This result is in accordance with the previous morphological/taxonomic studies which support *G. anatolica* as a member of the section *Polycephalium (9). G. hedgei*, a local Turkish endemic restricted to a single location in Yazili Kanyon National Park *(15)*, is yet another new species included in our analysis. It is a cushion-forming perennial divided into several rosettes that is morphologically very similar to *G. orientalis* L. and *G. sintenisii,* while it differs from all *Globularia* species by densely stellate hairs. Molecular data confirm its relation with other Anatolian taxa, *G. davisiana* and *G. sintenisii*, and especially with *G. anatolica* on the basis of chloroplast markers. Finally, *G. davisiana* and *G. sintenisii* are joined together in the ITS tree confirming their similar geographical range and possible origin from a common lineage.

In conclusion, this study indicates existence of separate centers of diversification for European and Asia Minor *Globularia*. Although Kadereit and Comes *(7)* proposed that evolutionary events in *Globularia* dated earlier than the Quaternary, it could be hypothesized that speciation of some of the European and Anatolian taxa began during the glacial period, having in mind that morphological characteristics are often under severe ecological selection pressure, which may lead to the creation of advantages necessary for adaptation to extreme habitat conditions. Closely related taxa, such as *G. cordifolia* and *G. meridionalis*, as well as the majority of Anatolian endemics, probably developed in such conditions exactly. Nevertheless, conduction of additional research to confirm these assumptions is necessary.

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