Non-monophyly of *Buglossoides* (Boraginaceae: Lithospermeae): Phylogenetic and morphological evidence for the expansion of *Glandora* and reappraisal of *Aegonychon*

Lorenzo Cecchi,[1] Andrea Coppi,[2] Hartmut H. Hilger[3] & Federico Selvi[2]

1 *Università degli Studi di Firenze, Museo di Storia Naturale, sezione botanica "Filippo Parlatore", Via G. La Pira 4, 50121 Florence, Italy*

2 *Università degli Studi di Firenze, Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente (DISPAA), Laboratori di Botanica, Piazzale delle Cascine 28, 50144 Florence, Italy*

3 *Freie Universität Berlin – Institut für Biologie – Systematische Botanik und Pflanzengeographie, Altensteinstr. 6, 14195 Berlin, Germany*

Author for correspondence: *Federico Selvi, federico.selvi@unifi.it*

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Abstract The phylogeny of the small Old World genus *Buglossoides* and its position in tribe Lithospermeae was investigated using nrDNA and cpDNA sequences and morphology. Maximum parsimony and Bayesian analyses of ITS-5.8S and *trnL-trnF* IGS datasets consistently show that this group is close to *Glandora* and *Lithospermum* but not monophyletic. Of the seven species usually included, two were retrieved in the genus *Glandora*, i.e., *B. goulandrisiorum* from northern Greece and *B. gastonii* from the western Pyrenees. Based also on morphology and ecology, the placement of these two rare, rupicolous endemics in *Glandora* is here advocated and new combinations are made. The rest of *Buglossoides* includes two early-diverging clades, one with annual taxa of section *Buglossoides* and one with the three perennials of section *Margarospermum*. Morphological, palynological and ecological data support the separation of these two groups in distinct genera, *Buglossoides* s.str. and the old but largely neglected *Aegonychon*. Within *Buglossoides*, two main clades correspond to the *B. arvensis* and *B. incrassata* complexes. These show a largely sympatric distribution from the south Mediterranean to central and northern Europe. Combined with their strong phenotypic polymorphism, this causes difficulties in the distinction between taxa of the two clades, especially without characteristic cotyledons or fruiting material. Molecular and morphological evidence clearly support the transfer of the west Mediterranean *B. arvensis* subsp. *permixta* to the *B. incrassata* complex.

Keywords *Aegonychon*; Boraginaceae; *Buglossoides*; *Glandora*; ITS; morphology; pollen; phylogeny; *trnL-trnF* IGS

Supplementary Material Electronic Supplement (Table S1) and alignment are available in the Supplementary Data section of the online version of this article at http://www.ingentaconnect.com/content/iapt/tax

■ INTRODUCTION

Buglossoides Moench is a small genus originally based on *B. ramosissima* Moench (= *Lithospermum tenuiflorum* L.f.) and then circumscribed by the monographer Johnston (1954) as comprising seven species native to the Old World, mostly concentrated in the Mediterranean region. They are distinct from those of the closely related Linnaean genus *Lithospermum* by mainly the shortly apiculate anthers and the arrangement of glanduliferous hairs in longitudinal bands or inflexed pleats within the corolla tube vs. the gibbose, invaginated faucal scales present in most taxa of *Lithospermum*, including the type *L. officinale* L. In the absence of clear phylogenetic evidence for its distinctiveness, however, *Buglossoides* has remained a poorly defined generic unit with controversial limits (Al-Shehbaz, 1991) and was often included in a broadly defined *Lithospermum* (Greuter & al., 1984; Zhu & al., 1995; Jeanmonod & Gamisans, 2007).

In Johnston's (1954) opinion *Buglossoides* comprised two groups that he ranked as sections. The first is *B.* sect. "*Eu*"-*Buglossoides*, and contains three annual species including the type (*B. tenuiflora* (L.f.) I.M.Johnst.), with five longitudinal bands of hairs inside the corolla and trigonous-pyriform nutlets with a strongly tuberculate-verrucose surface. *Buglossoides* sect. *Margarospermum* (Rchb.) I.M.Johnst., the second group originally established within *Lithospermum* (Reichenbach, 1830–1832: 336–337), comprises four perennial species with larger corollas showing strongly inflexed pleats and filaments with glanduliferous hairs, as well as globose-ovoid nutlets with a smooth, whitish surface resembling a droplet-shaped piece of porcelaine. These are *L. purpurocaeruleum* L. (syn. *Buglossoides purpurocaerulea* (L.) I.M.Johnst.), *L. zollingeri* A.DC. (syn. *B. zollingeri* (A.DC.) I.M.Johnst.), *L. calabrum* Ten. (syn. *B. calabra* (Ten.) I.M.Johnst.) and *L. gastonii* Benth. ("*gastoni*"; syn. *B. gastonii* (Benth.) I.M.Johnst.). The Greek endemic *L. goulandrisiorum* Rech.f. ("*goulandriorum*"),

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described later (Rechinger, 1971) and then transferred to *Buglossoides* (Govaerts, 1996), was also placed in this latter group. However, the general resemblance in habit and fruit morphology of *L. goulandrisiorum* and the other taxa of *B.* sect. *Margarospermum* with species of *Lithospermum* induced some authors to retain them within the latter genus, therefore restricting the limits of *Buglossoides* to only the annual species of *B.* sect. *Buglossoides* (Edmondson, 1979; Meikle, 1985; Strid & Tan, 1991).

A different view was expressed by Holub (1973), who transferred the perennial species of *B.* sect. *Margarospermum* to *Aegonychon* Gray, therefore considering them generically distinct from both *Lithospermum* and *Buglossoides*. This view has recently been adopted in the Boraginaceae treatment of *Flora Iberica* (Pastor, 2012), but not in other works. Consequently, these species still move among *Lithospermum*, *Buglossoides* and *Aegonychon*, while the annual species are still alternatively placed in *Lithospermum* or *Buglossoides*. Uncertainties result from the somewhat reticulate variation of morphological characters considered to be of taxonomic value and the lack of a comprehensive phylogenetic study of the whole group. Recent papers on Lithospermeae using molecular data have consistently suggested that *Buglossoides* should be a genus separate from a monophyletic *Lithospermum* also including most generic segregates from North America, such as *Nomosa* I.M.Johnst., *Macromeria* D.Don and *Onosmodium* Michx. (Thomas & al., 2008; Cecchi & Selvi, 2009; Ferrero & al., 2009; Weigend & al., 2009). These studies have therefore supported Johnston's (1954) opinion and the phylogenetic significance of the characters of the corolla that he used to distinguish the two genera. They also suggested generic status for *Glandora* D.C.Thomas & al., a monophyletic group recently separated from *Lithodora* Griseb., and have shown its position in the *Lithospermum* s.l. clade close to *Buglossoides* (Thomas & al., 2008), therefore adding a third element to the problem of relationships in this group. On the other hand, incomplete taxonomic sampling in these previous studies prevented to address the monophyly of *Buglossoides* and to draw conclusions about the phylogeny and systematics of this group of Lithospermeae. Using morphological and molecular tools, this paper aims at completing previous work by providing a phylogenetic analysis of a complete sample of both sections of *Buglossoides*, plus a representative selection of *Glandora*, *Lithospermum* and other clades of Old World Lithospermeae. Secondly, this paper provides evidence on relationships within *B.* sect. *Buglossoides* that can help to address the unclear taxonomic status of specific and infraspecific taxa in this small but difficult group of the Euro-Mediterranean flora.

■ MATERIALS AND METHODS

Plant material and taxon sampling. — Most of the taxa/accessions included in this study were sampled by the authors from native populations during field trips across Mediterranean countries. Herbarium vouchers, silica-gel-dried portions of leaf tissue and glutaraldehyde-fixed samples of reproductive structures were collected for each accession. Vouchers are kept in the Boraginaceae herbarium collection of the authors in FIAF, and indicated as FI-HB with relative number. Material of the missing taxa and additional accessions for morphological observations were obtained from herbarium collections in ATH, B, C, FI, HCT, KUN, P and VER. As a result, all specific and most infraspecific taxa of *Buglossoides* s.l. as recognized in the main Euro-Mediterranean floristic and taxonomic literature, especially *Flora Europaea* (Fernandes, 1972) and *Med-Checklist* (sub *Lithospermum*; Greuter & al., 1984), were included in this analysis, with the only exception of *B. glandulosa* (Velen.) R.Fern. (*B.* sect. *Buglossoides*), of which it was not possible to obtain material for DNA isolation.

The complete list of taxa included in this investigation is reported in Appendix 1, with vouchers and INSDC (International Nucleotide Sequence Database Collaboration) accession numbers; the geographic distribution of the *Buglossoides* samples is shown in Fig. 1. For a synopsis of the valid names of taxa originally published in *Lithospermum* and later variously combined in *Buglossoides*, *Aegonychon*, *Rhytispermum* Link, *Margarospermum* (Rchb.) Opiz and *Glandora* see Table S1 in the Electronic Supplement.

DNA extraction and amplification. — Genomic DNA was extracted from silica-gel-dried samples of leaf tissue following a modified 2×CTAB protocol (Doyle & 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 ITS region of nuclear DNA, including ITS1, 5.8S and ITS2, was done using the primers ITS4 and ITS5 of White & al. (1990), while the plastid *trnL-trnF* IGS region was amplified with the primers "c" and "f" of Taberlet & al. (1991). The IGS of *L. hancockianum* Oliv. could not be amplified because of the low quality of genomic DNA obtained from relatively old herbarium material. Preliminary analysis of sequence variation in the protein-coding plastid region *rpoC1* was also performed on a sample of six species of *Lithospermum* and *B.* sect. *Buglossoides* and sect. *Margarospermum*. This region was tested because it had never been analysed before in Lithospermeae in spite of its potential resolving power of species relationships in angiosperms (Chase & al., 2007). Amplification procedures and primers (rpoC1F, rpoC4R) followed standard protocols retrieved from http://www.kew.org/barcoding/protocols.html

Polymerase chain reactions 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 an MJ PTC-100 thermocycler (Peltier Thermal Cycler; MJ Research, St. Bruno, Quebec, Canada). Fourty amplification cycles were run with annealing temperature 50°C, annealing time 30 s and final extension for 45 s at 72°C. For *trnL-trnF* IGS, the PCR cycling conditions were the same as those followed by Moore & Jansen (2006) for the *rps16* plastid region, and used in Weigend & al. (2013).

Subsequently, 5 µl of each amplification mixture were analysed 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 ABI3l0 sequencer (PE-Applied Biosystems, Norwalk, Connecticut, U.S.A.).

Sequence alignment, datasets and phylogenetic analyses. — Original sequences from the three genomic regions analysed were edited with BioEdit v.7.0 (Hall & al., 1999) and checked for orthology through comparison with GenBank accessions of most closely related taxa. Multiple alignments were performed with Multalin v.5.4.1 (Corpet, 1988) and MAFFT v.5 (Katoh & al., 2005), and then carefully checked for ambiguous positions based on visual inspection of the sequencer output chromatofiles.

For phylogenetic analyses, taxon sampling was expanded with a representative selection of species of the two closely related genera *Glandora* and *Lithospermum*, the sequences of which were retrieved from INSDC. Both Old and New World species of the latter genus were included, as well as the Yunnan endemic *L. hancockianum* Oliv., investigated here for the first time (only ITS-5.8S). Our preliminary analyses of wider datasets also including other North American species of *Lithospermum* and its segregates *Onosmodium*, *Nomosa* and *Macromeria* (*Lithospermum* s.l.) fully confirmed that these species are all included in a single and well-supported monophyletic clade, as demonstrated in previous studies (Weigend & al., 2009; Cohen & Davis, 2009). Our preliminary trees were topologically congruent with those obtained from reduced datasets not including the taxa listed above. In consequence, we finally excluded them from the analyses to avoid redundancy. Outgroups close to the *Lithospermum* clade were selected based on Cecchi & Selvi (2009), and included *Moltkia* Lehm. (clade D), *Arnebia* Forssk. (clade E), *Cerinthe* L. and *Neatostema* I.M.Johnst. (clade B), and *Echium* L. (clade A).

Three single-marker datasets were prepared, ITS-5.8S, *trnL-trnF* IGS and *rpoC1* which included, respectively, 53, 25 and 6 accessions representing 33, 25 and 6 taxa (Appendix 1); the larger size of the first dataset was mainly due to denser

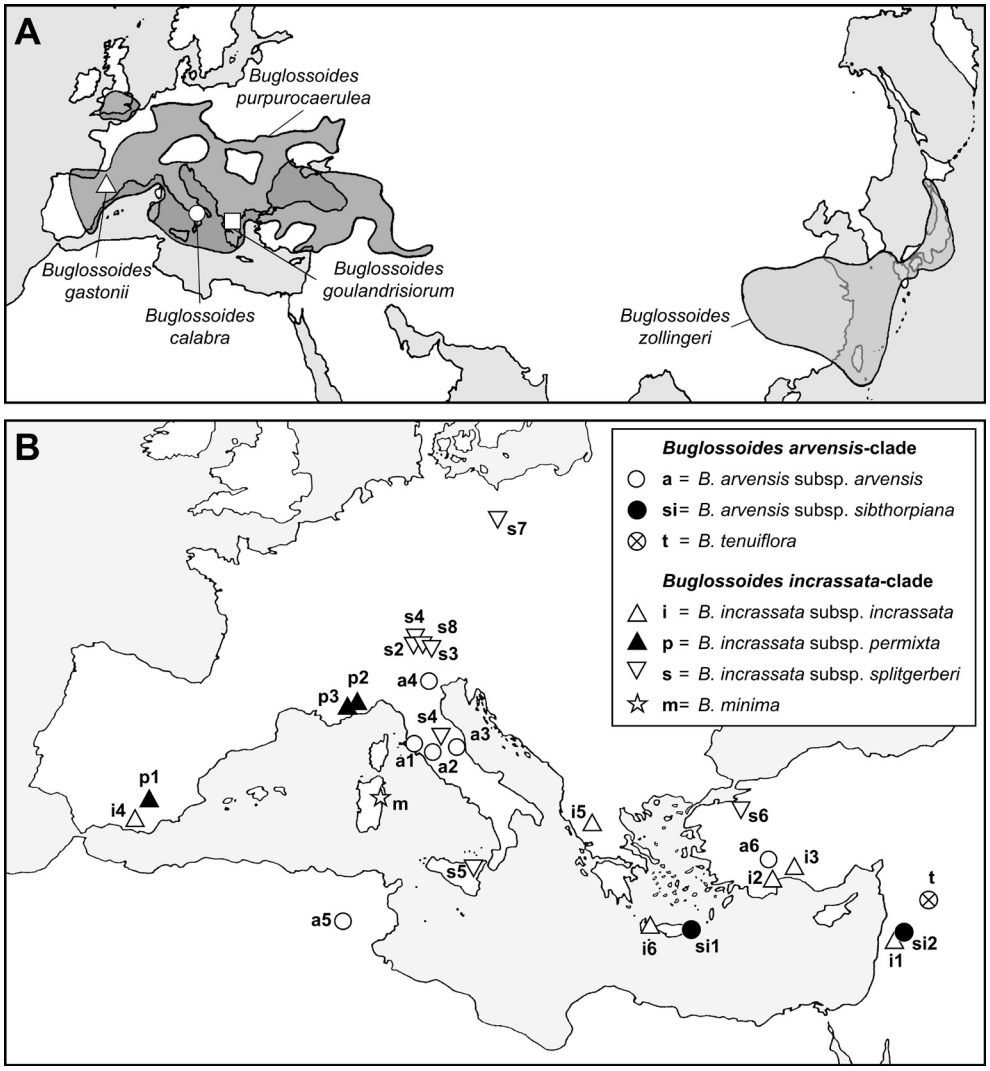

Fig. 1. A, distribution range of the five species of *Buglossoides* sect. *Margarospermum*; **B,** geographic origin of the taxa and accessions of *B.* sect. *Buglossoides* used in this study.

sampling of *B.* sect. *Buglossoides*, that was adopted because of the potential usefulness of ITS-5.8S in solving relationships among closely related species.

Phylogenetic analyses were first carried out for the ITS and IGS alignments (excluding *rpoC1*; see Results), using maximum parsimony and Bayesian methods. Gaps were coded as separate characters according to Simmons & Ochoterena (2000) using FastGap v.1.0.8 (Borchsenius, 2007), and appended at the end of the datasets. Congruence between the two datasets and respective trees was evaluated according to the procedures described by Wiens (1998). Since no conflicting well-supported clade was identified, an additional dataset consisting of concatenated ITS-IGS sequences plus coded gaps was prepared for a combined analysis (25 taxa). Tree construction was first performed using PAUP* v.4.0 (Swofford, 2000), running Heuristic searches with "tree-bisection-reconnection" (TBR) branch-swapping with accelerated transformation (ACCTRAN) optimisation to infer branch (edge) lengths; MULTREES option on, ADDSEQ = random, twenty randomised 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.

The ITS-5.8S and combined ITS-IGS datasets were also analysed using Bayesian inference of phylogeny with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). Based on jModelTest (Posada, 2008), the best-fitting models of nucleotide substitution were GTR for ITS-5.8S, with gamma-distributed rate variation across sites, and GTR+I+Γ for *trnL-trnF* IGS. The analyses were performed using four incrementally heated Markov chains (one cold, three heated) simultaneously started from random trees, and run for one million cycles sampling one tree every ten 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 v.1.6.6 (Page, 1996), with indication of Bayesian posterior probability (PP) values for the internal tree nodes.

Micromorphology (SEM). — Fixed material was dehydrated in an acetone series, critical point-dried with liquid CO_2, mounted on aluminium stubs, coated with gold and observed with an FEI ESEM-QUANTA 200 scanning electron microscope (SEM) working at 30 kV. Pollen grains from dry specimens were first rehydrated in a solution of Aerosol-OT 20% (Bigazzi & Selvi, 1998) and then observed with the SEM.

■ RESULTS

Nuclear ITS-5.8S dataset. — The aligned dataset of ITS1-5.8S-ITS2 sequences used for tree calculation was 748 bp long, including the coded gaps which were appended at the end of the matrix (positions 660–748). In the MP analysis, 344 sites were constant, 211 variable but uninformative and 228 parsimony informative. The most parsimonious trees from the heuristic search had length (L) = 953, consistency index (CI) = 0.61 and retention index (RI) = 0.84. The topology of the resulting strict consensus (not shown) was largely congruent with the 50% majority-rule consensus phylogram from the Bayesian analysis which is described here (Fig. 2); the most relevant difference is described below.

The ingroup, including the members of the *Lithospermum* s.l. clade, was retrieved as a monophyletic assemblage (0.99 PP, 100% BS). This clade was divided in *Lithospermum*/*Glandora* on the one hand, and *Buglossoides* sect. *Margarospermum* and sect. *Buglossoides* on the other hand. Both these clades, however, were poorly supported in the Bayesian phylogram (*Lithospermum*/*Glandora* 0.66 PP, 67% BS; sect. *Margarospermum*/sect. *Buglossoides* 0.73 PP, BS < 50%) and were not found in the MP analysis. Here, the clade of *B.* sect. *Buglossoides* instead was sister to the rest of the ingroup, while *B.* sect. *Margarospermum* was sister to the *Lithospermum*/*Glandora* clade, although with moderate support (76% BS) only. Species of *Lithospermum* were retrieved in a well-supported clade (0.95 PP, 97% BS), with the rare Yunnan endemic *L. hancockianum* and *L. tschimganicum* B.Fedtsch. as successive sisters to the other members of this genus. Species of *Glandora* were also retrieved in a well-supported clade (0.99 PP, 81% BS), which also included *B. goulandrisiorum* and *B. gastonii* of *B.* sect. *Margarospermum*. The former species was sister to the rest of the taxa in this group, while the latter was nested among typical *Glandora* species and sister to *G. oleifolia* (Lapeyr.) D.C.Thomas and *G. nitida* (Ern) D.C.Thomas (0.98 PP, 68% BS). The other three members of *B.* sect. *Margarospermum* were retrieved in a well-supported clade (0.98 PP, 73% BS), with *B. calabra* sister to the *B. zollingeri*/*B. purpurocaerulea* clade (1.0 PP, 99% BS). The annual taxa of *B.* sect. *Buglossoides* were grouped in a strongly divergent clade with a long branch (0.95 PP, 100% BS). This contained two well-supported subclades, one with *B. tenuiflora* and most accessions of the *B. arvensis* (L.) I.M.Johnst. complex (0.96 PP, 96% BS), and one (0.96 PP, 96% BS) with all accessions of the *B. incrassata* (Guss.) I.M.Johnst. complex, including *B. incrassata* s.str. (corresponding to *B. arvensis* subsp. *gasparrinii* (Heldr. ex Guss.) R.Fern. in *Flora Europaea*), *B. incrassata* subsp. *splitgerberi* (Guss.) E.Zippel & Selvi and *B. minima* (Moris) R.Fern.; *B. arvensis* subsp. *permixta* (Jord.) R.Fern. was also included in this second subclade. Relationships within both clades were mostly unresolved and no clear relationships with geographical origin of the accessions could be observed.

Plastid *rpoC1* dataset. — Sequencing of the *rpoC1* coding region was performed for six morphologically distinct species representing the main clades retrieved in the ITS phylogeny: *B. arvensis* subsp. *arvensis* (sample no. 1 in Appendix 1; accession no. HG939446), *B. incrassata* subsp. *incrassata* (sample 5; accession HG939451), *B. gastonii* (accession HG939447), *B. goulandrisiorum* (accession HG939449), *B. calabra* (accession HG939448) and *Lithospermum officinale* (sample 2; accession HG939450); sequences were 413 bp long, and showed only four variable positions, three of which were singletons. This low level of variation precluded use of this marker for further analysis.

Plastid *trnL-trnF* IGS dataset. — The *trnL-trnF* IGS alignment included 713 bp, plus coded gaps from position 714 to 755. In the parsimony analysis 612 characters were constant, 74 variable but non-informative and 69 informative. The strict consensus of most parsimonious trees (L = 171; CI = 0.85; RI = 0.80; not shown) was a large polytomy in which monophyly of *Lithospermum*, *Glandora* and *B.* sect. *Buglossoides* was supported. Relationships of the five perennial taxa of *B.* sect. *Margarospermum* to these clades remained unresolved.

Combined ITS-IGS dataset. — The alignment was 1435 bp long; coded gaps appended at the end of the matrix were in position 1355–1435. In the parsimony analysis, 972 sites were constant, 182 variable but uninformative and 281 parsimony informative. The heuristic search retrieved four most parsimonious trees with L = 797, CI = 0.73 and RI = 0.77; the resulting strict consensus was fully consistent with the Bayesian phylogram described here (Fig. 3).

The *Lithospermum* s.l. clade received strong support (1.00 PP, 100% BS), and *Lithospermum* s.str. (1.00 PP, 100% BS) was sister to the rest of the ingroup. This consisted of two clades which were sister to each other (0.97 PP, 63% BS), one (0.99 PP, 74% BS) with *B. goulandrisiorum* and *B. gastonii* as successive sisters to *Glandora*, and one (1.00 PP, 66% BS) with the other three species of *B.* sect. *Margarospermum* (0.99 PP, 90% BS) sister to *B.* sect. *Buglossoides* (1.00 PP, 100% BS). The latter included *B. arvensis* as sister to a group of four accessions of the *B. incrassata* complex (1.00 PP, 100% BS), also comprising *B. arvensis* subsp. *permixta* as sister to the *B. incrassata*

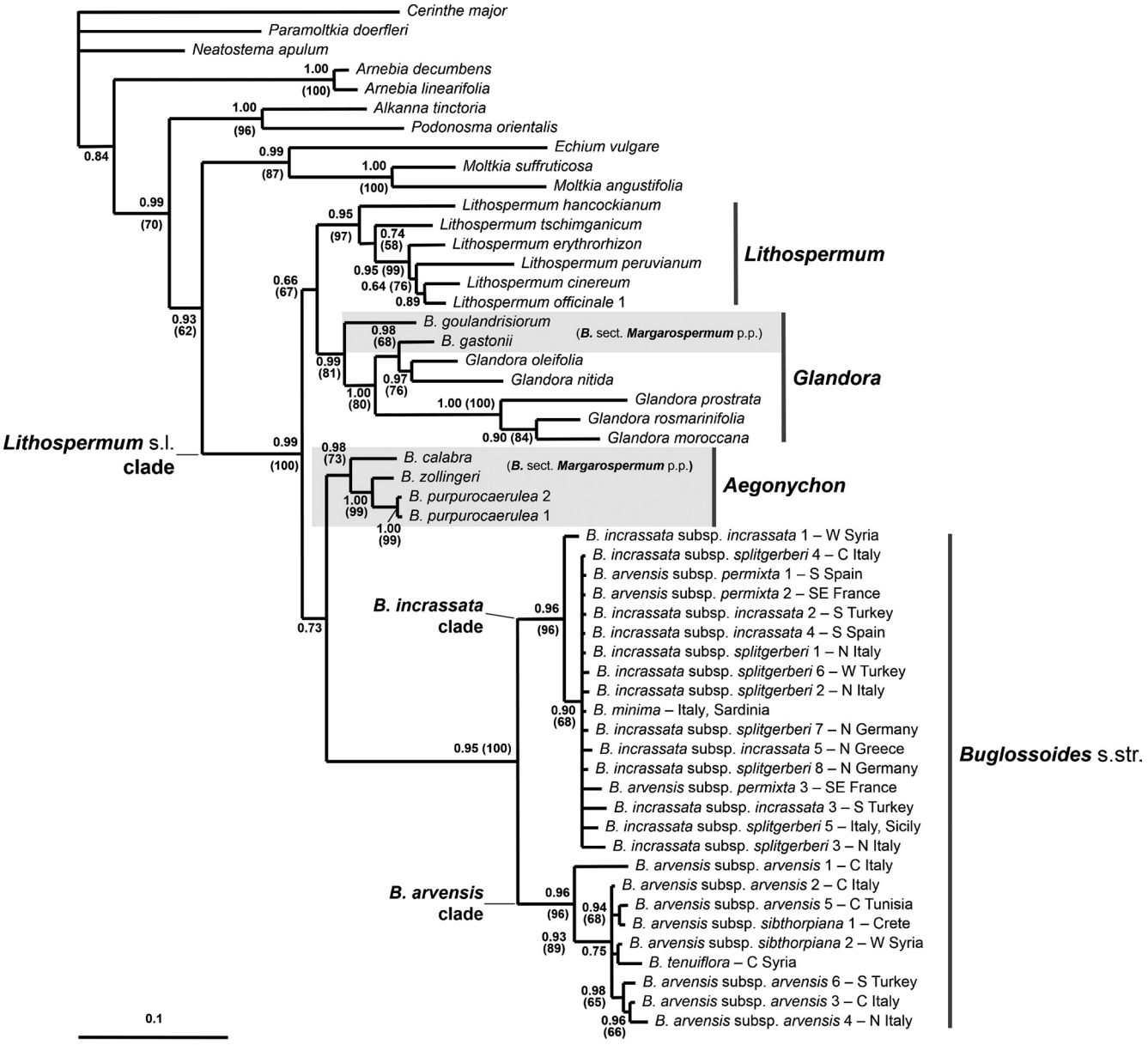

Fig. 2. Bayesian 50% majority-rule consensus phylogram generated by ITS-5.8S sequences showing relationships of *Buglossoides* in the *Lithospermum* s.l. clade; posterior probability values and bootstrap support percentages >50% are shown near nodes.

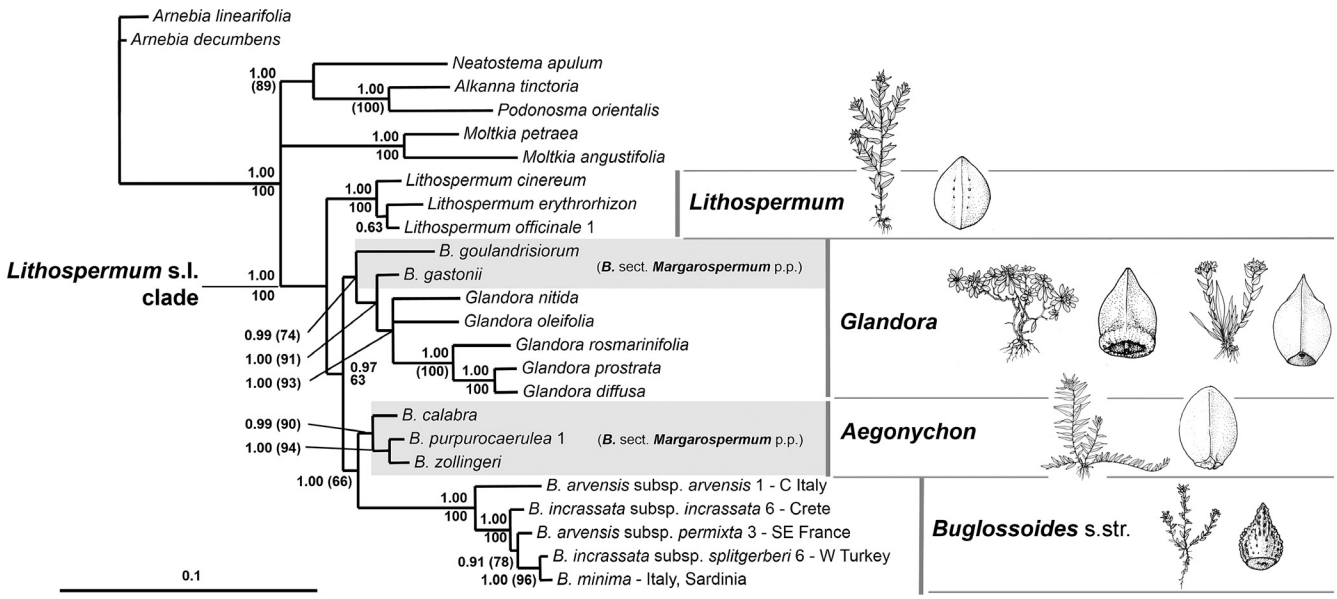

Fig. 3. Bayesian 50% majority-rule consensus phylogram generated by combined ITS-IGS sequences showing position and relationships of *Buglossoides* in the *Lithospermum* s.l. clade; posterior probability values and bootstrap support percentages ≥50% are given near nodes; key characters are shown to the right of the clades (original drawings by L. Cecchi).

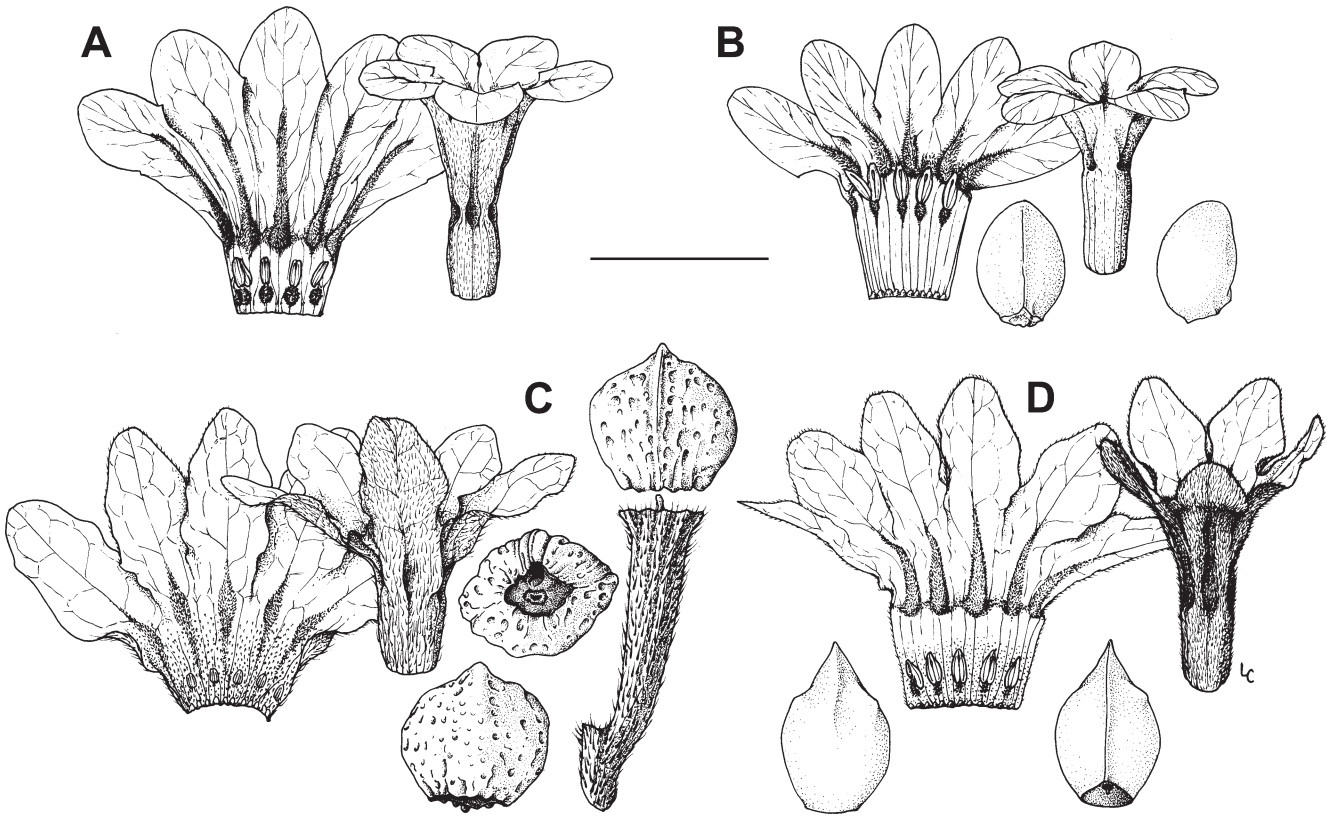

Fig. 4. Floral and fruit morphology of: **A,** *Buglossoides calabra*, opened and intact corolla (*Cecchi & Coppi* FI-HB 07.56); **B,** *B. purpurocaerulea*, opened and intact corolla and mericarpid in lateral and ventral views (*Bigazzi* FI-HB 91.07; *Cecchi, Coppi & Selvi* FI-HB 06.18); **C,** *B. gastonii*, opened and intact corolla, fruiting pedicel with remains of vascular strand, mericarp in ventral and dorsal views and base of mericarpid showing cicatrix (*Boissier & Reuter s.n.*, 1870, FI; *Burnat s.n.*, 1868, FI); **D,** *B. goulandrisiorum* subsp. *goulandrisiorum*, opened and intact corolla and mericarp in dorsal and ventral views (*Cecchi & Selvi* FI-HB 08.38; *Stamatiadou 21217*, ATH). — Scale bar: flowers = 1 cm; nutlets = 0.5 cm. — Original drawings by L. Cecchi.

subsp. *splitgerberi*/*B. minima* group, with good support (0.91 PP, 78 BS).

Inflorescences and flowers. — Since flower and inflorescence morphology of *Lithospermum* and *Glandora* have already been described thoroughly in previous papers and treatments (Johnston, 1954; Weigend & al., 2009; Cohen, 2012) only the most relevant features of *Buglossoides* s.l. are summarized here, with emphasis on the differences between species or groups of species.

Inflorescences are basically frondose-bracteose cincinni ("cymoids") with two to many flowers, usually distinctly elongating in fruit and with well-spaced, nutlet-bearing calyces; however, *B. gastonii* and *B. goulandrisiorum* clearly differ in having short, forked cymes with crowded flowers which remain compact and congested even in fruit.

Floral characters show considerable variation among the taxa included, especially in size and internal structure of the corolla; most of the species are illustrated in Figs. 4–5. Large corollas (up to 25–30 mm in length) with a showy cup-shaped limb of blue to purple colour are typical for species in *B.* sect. *Margarospermum*. Characteristic is the presence of five longitudinal bands on the adaxial (internal) surface of the corolla, which mainly take the form of thickened hairy pleats possibly serving as guides for pollinating insects. These bands are of variable extent and position, and consist of dense trichomes often mixed with sparser and shorter glandular hairs (Fig. 6A). The trichomes are short, enlarged at the apex and obtuse, except for *B. calabra* where hairs are longer and acute at the apex (Fig. 6D). Stamens are always enclosed in the corolla, but filaments are inserted close to the throat in *B. purpurocaerulea* and *B. zollingeri*, while close to the base of the tube (ca. 1.5 mm above) in *B. goulandrisiorum*, *B. calabra* and *B. gastonii*. Very small, oblong anthers (<1 mm) are typical of the last species, which also differs from the others in the very weakly rather than distinctly apiculate anthers. Filaments bear glanduliferous hairs which are also found along longitudinal lines below stamen insertion in *B. purpureocaerulea* and *B. zollingeri*; in *B. calabra*, the filament base takes the form of a rounded bulge and is covered with a congregation of such hairs (Fig. 6C). Glandular trichomes are also scattered over the internal surface of the corolla tube in *B. gastonii* and *B. goulandrisiorum* (Fig. 6B), but not in the other three species. The base of the corolla tube is never distinctly thickened or provided with hairs as usually found in *Lithospermum*.

Small and narrowly infundibular corollas (max. 6 mm in length and 4 mm in diam.) of white, pink or blue colour are typical for taxa in *B.* sect. *Buglossoides*. Five longitudinal bands of short, appressed, papillose trichomes enlarged at the apex run from the throat (base of limb) down to the upper half of the tube (Fig. 6E). In *B. tenuiflora*, the inside of the lower half of the tube has sparse hairs. In all taxa, anthers are inserted on very short filaments just below the base of the bands and close

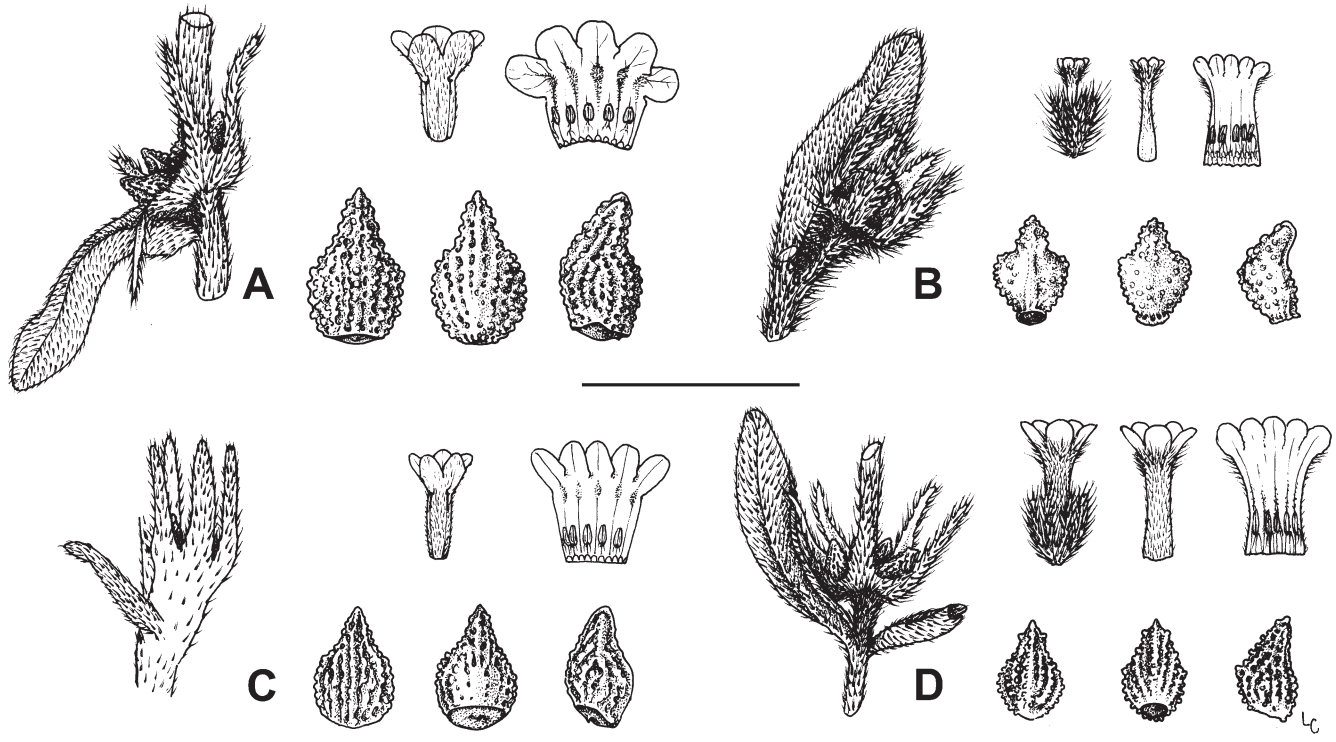

Fig. 5. Floral and fruit morphology of: **A,** *Buglossoides arvensis* subsp. *arvensis*, fruiting calyx with bract and pedicel, opened and intact corolla and mericarpid in dorsal, ventral and lateral views (*Bigazzi* FI-HB 90.03); **B,** *B. tenuiflora*, fruiting calyx with bract and pedicel, opened and intact corolla and mericarpid in dorsal, ventral and lateral views (*Cecchi, Coppi & Selvi* FI-HB 07.13); **C,** *B. incrassata* subsp. *incrassata*, thickened fruiting calyx with bract and pedicel, opened and intact corolla, and mericarpid in dorsal, ventral and lateral views (*Cecchi, Coppi & Selvi* FI-HB 07.40); **D,** *B. minima* (*Sommier s.n.*, 1872, FI), fruiting calyx with bract and pedicel, opened and intact corolla, and mericarpid in dorsal, ventral and lateral views. — Scale bar: flowers = 1 cm; nutlets = 0.5 cm. — Original drawings by L. Cecchi.

to the base of the corolla tube. The anthers are shortly apiculate at the apex. A thickened annulus with sparse, short hairs is found near the base of the corolla tube (Fig. 6F).

Fruits. — Nutlet morphology within *Buglossoides* shows striking variation in terms of size, shape and surface ornamentation. Taxa of *B.* sect. *Buglossoides* all have small (1.7–3.5 × 0.8–1.6 mm) trigonous-pyriform nutlets with a prominently verrucose-tuberculate, brownish surface (Fig. 5A–D, 6H); usually no abortion occurs, resulting in four mature mericarpids per fruit. Species of *B.* sect. *Margarospermum* often have only 1–3 mature mericarpids by abortion, and these are larger and basically ovoid (2.5–4.5 × 1.8–3.5 mm), mostly with a smooth and glossy, whitish to greyish surface and an obtuse-rotundate apex, as in most members of *Lithospermum* (Fig. 4B). Concerning surface ornamentation and apex, however, *B. gastonii* and *B. goulandrisiorum* are exceptions. In the former, the stout, plump nutlet is externally rugose-foveolate and has a short, blunt beak (Figs. 4C, 6G), while the latter has a smooth surface and an acute beak (Fig. 4D). The base of the nutlet of *B. gastonii* and, to a lesser extent, also of *B. goulandrisiorum*, is almost flat and broader than in the other species; the small tubular channel in the ventral position of the cicatrix area is occupied

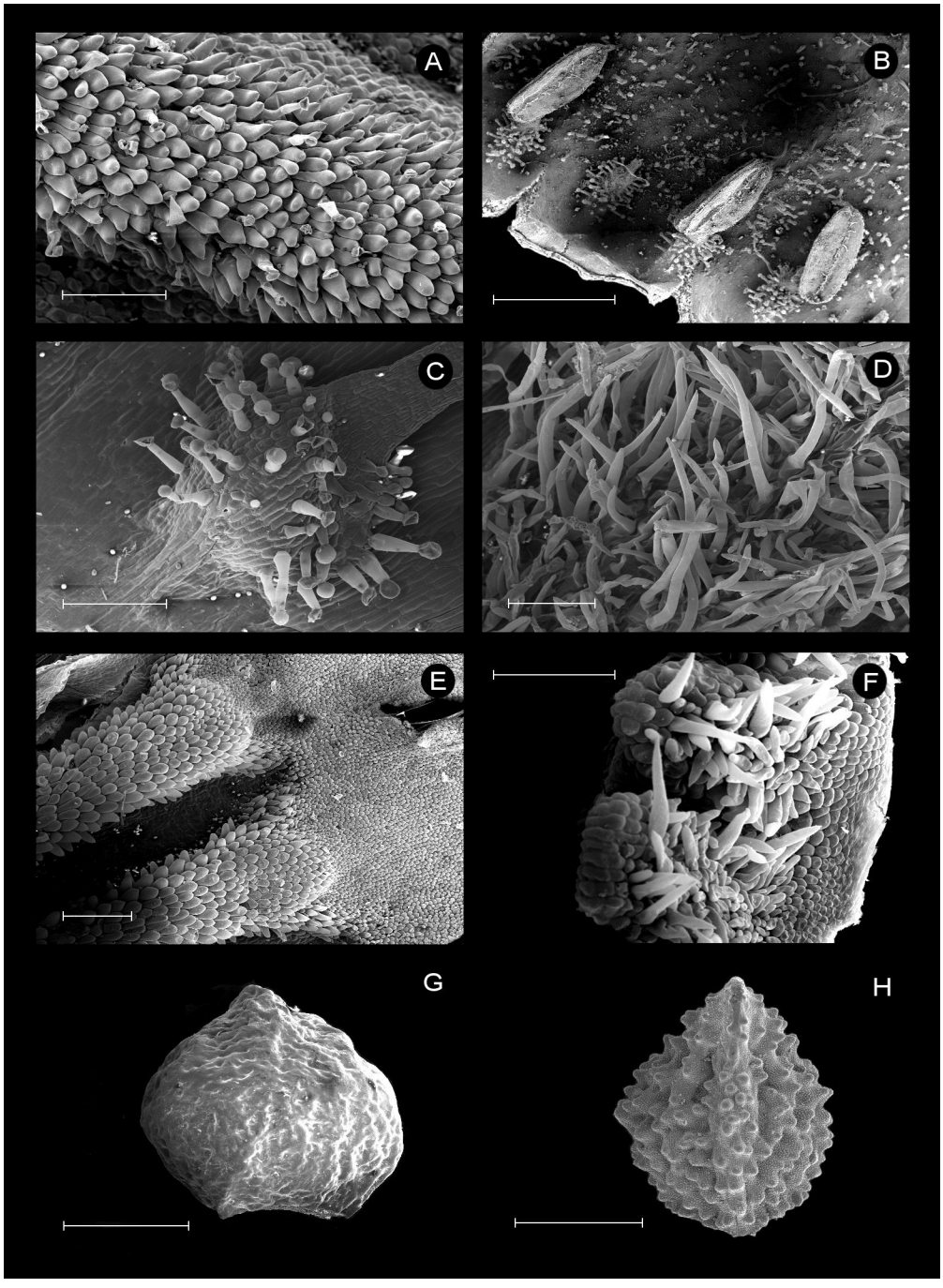

Fig. 6. SEM micrographs of floral and fruit characters. **A–B,** *B. goulandrisiorum* (*Cecchi & Selvi* FI-HB 08.38): **A,** trichomes of the longitudinal hairy bands above throat; **B,** lower part of corolla tube showing stamen position, glanduliferous hairs on filaments and adaxial corolla surface. **C–D,** *B. calabra* (*Cecchi & Coppi* FI-HB 07.56): **C,** gibbous base of stamen filament with glanduliferous hairs; **D,** trichomes of the longitudinal hairy bands above throat. **E–F,** *B. incrassata* subsp. *incrassata* (*Cecchi, Coppi & Selvi* FI-HB 07.42): **E,** abaxial surface of corolla showing upper part of longitudinal hairy bands at throat, with short, obtuse trichomes; **F,** short trichomes of the thickened annulus at the base of corolla tube. **G,** *B. gastonii* (*Döbbeler* FI-HB 07.58), whole mericarpid in lateral view. **H,** *B. incrassata* subsp. *incrassata* (*Cecchi, Coppi & Selvi* FI-HB 07.42), whole mericarpid in lateral view. — Scale bars: A, H = 1 mm; B–E = 200 µm; F = 100; G = 2 mm.

by a strand of vascular tissue the remains of which often arise as a bristle-like protrusion from the almost flat detachment areola of the gynobase (Fig. 4C).

Pollen. — The main stereostructural characters of pollen within *Buglossoides* are summarized in Table 1 and illustrated in Fig. 7. Significant differences exist between the two sections. Grains of taxa of *B.* sect. *Buglossoides* are isopolar, while those of *B.* sect. *Margarospermum* usually show a slight asymmetry in the size of the two poles, and can be defined as subisopolar (Díez & al., 1986) or "slightly heteropolar" (Clarke, 1977). There are usually four apertures in the grains of the *B.* sect. *Margarospermum* species (Fig. 7A–D), but usually six (more rarely up to eight) in those of *B.* sect. *Buglossoides*. In the latter, a microgranulose-spinulose equatorial band connecting the ectoapertures is often visible (Fig. 7F), which is always lacking in *B.* sect. *Margarospermum*. Consequently, the rhombic shape of the well-spaced ectoapertures is more distinct in the taxa of the latter group. The small pore-like endoapertures lie in the

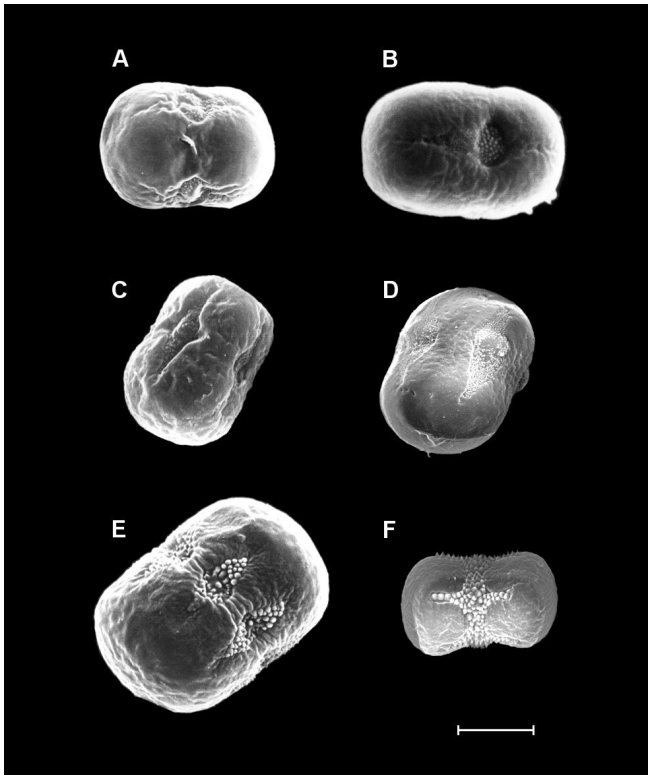

Fig. 7. SEM micrographs of pollen grains of: **A,** *B. zollingeri* (*Silvestri s.n.*, FI); **B,** *B. calabra* (*Cecchi & Coppi* FI-HB 07.56); **C,** *B. gastonii* (*Doassans s.n.*, FI); **D,** *B. goulandrisiorum* (*Cecchi & Selvi* FI-HB 08.38); **E,** *Glandora prostrata* (*Bicknell s.n.*, FI); **F,** *B. tenuiflora* (*Cecchi, Coppi & Selvi* FI-HB 07.15). — Scale bar = 5 μm.

Table 1. Main pollen characters of members of *Buglossoides* sect. Buglossoides and sect. *Margarospermum*.

Taxon	Polar diam. (P) [μm]	Equator. diam. (E) [μm]	P/E	Shape	Apertures	Ectoaperture shape	Colpus length [μm]	Endoaperture position	Colpus membrane	Tectum	Origin of material examined (vouchers in FI)
B. sect. *Buglossoides*											
B. arvensis subsp. *arvensis*	13.1	8.5	1.53	isopolar	6(–7)	rhombic	7.1	median	granular	psilate	Italy
B. incrassata subsp. *incrassata*	11.2	6.2	1.80	isopolar	6(–7)	rhombic	5.1	median	granular	psilate	Israel
B. minima	13.1	9.1	1.44	isopolar	6	rhombic	7.3	median	granular	psilate	Italy
B. tenuiflora	14.0	9.8	1.43	isopolar	6	rhombic	6.5	median	granular	psilate	Syria
B. sect. *Margarospermum*											
B. calabra	11.9	8.5	1.40	subisopolar	4	rhombic	6.9	median	granular	psilate	Italy
B. purpurocaeruleum	12.6	9.5	1.32	subisopolar	4	rhombic	9.6	polar	granular	psilate	Italy
B. zollingeri	12.5	9.4	1.33	subisopolar	4	rhombic	8.3	median	granular	psilate	Taiwan
B. gastonii	11.6	8.1	1.40	isopolar	4	rhombic	7.5	median	granular	psilate	France
B. goulandrisiorum	12.0	7.9	1.40	subisopolar	4	rhombic	7.5	median	granular	psilate	Greece

centre of the ectoapertures in the taxa of *B.* sect. *Buglossoides* but closer to the larger pole in those of *B.* sect. *Margarospermum*, in line with their slightly heteropolar structure.

■ DISCUSSION

Evidence for the placement of *Buglossoides gastonii* and *B. goulandrisiorum* in *Glandora*. — Unlike previous broad-scale phylogenetic studies of Boraginaceae (e.g., Långström & Chase, 2002; Thomas & al., 2008; Cecchi & Selvi, 2009; Weigend & al., 2009, 2013; Cohen, 2014), this work includes a nearly complete taxonomic sampling of taxa described under *Buglossoides*, allowing to better understand the relationships in this Old World group of Lithospermeae.

First, all species of this genus as circumscribed by Johnston (1954) were confirmed to be outside *Lithospermum* s.str., implying that the taxonomic treatments in some reference Floras are not in line with phylogenetic evidence (Edmondson, 1979; Meikle, 1985; Strid & Tan, 1991; Jeanmonod & Gamisans, 2007). *Lithospermum* is a morphologically diverse monophyletic group (Weigend & al., 2009, 2010), here shown to include also *L. hancockianum* from the Yunnan region in south China. Pollen and fruit characters had already suggested its position in this genus (Seibert, 1978; Riedl, 1993; Zhu & al., 1995), and the present work suggests it is sister to all other species, including *L. tschimganicum* (≡ *Ulugbekia tschmiganica* (B.Fedtsch.) Zakirov) also from central Asia and of similar morphology (Johnston, 1954).

On the other hand, evidence is provided that *Buglossoides* is not monophyletic (or "holophyletic" sensu Hörandl & Stuessy, 2010). This confirms results by Weigend & al. (2009), where the members of *Buglossoides* investigated were sister to *Glandora* and *Lithospermum* and did not form a single clade. Combined cpDNA and nrDNA sequence data showed that the majority of species of *Buglossoides* form a well-supported group, but also that the two rare endemics *B. goulandrisiorum* (Greece) and *B. gastonii* (France and Spain) are outside this group and sister to members of the central-western Mediterranean genus *Glandora*. This implies a noteworthy east–west Mediterranean disjunction, suggesting that the ancestor of *Glandora* may have been a rupicolous species formerly more widely distributed across the Mediterranean mountains, including the southern Balkans. ITS sequence data alone retrieved *B. gastonii* as sister to *G. oleifolia* and *G. nitida*, both restricted to the mountains of eastern and southern Spain, respectively (Pastor, 2012). Ecological characters support a relationship of *B. goulandrisiorum* and *B. gastonii* to *Glandora*, as they are the only two species of *Buglossoides* that live in open, rocky habitats similar to species of *Glandora*.

Although morphological traits such as the shortly apiculate anther tips and the longitudinal hairy bands in the corolla would support placement of these two endemics in *B.* sect. *Margarospermum* as proposed by previous authors (Johnston, 1954; Rechinger, 1971; Aldén, 1976), other characters show their relationship to *Glandora*. This is particularly true for *B. gastonii* and *G. nitida*, one of the most differentiated species of the genus. In both these Pyrenean endemics the fruit surface is not smooth but slightly tumulose (*G. nitida*) or foveolate-rugose (*B. gastonii*), and the broad basal cicatrix is almost flat and without the peg-like appendage (elaiosome) found in most members of *Glandora*; as a consequence, the detachment areola has only a weakly developed depression instead of being cup-shaped as in the rest of the latter genus (see Thomas & al., 2008). Unlike in the other members of *B.* sect. *Margarospermum*, the remains of the vascular strand which enter the funicular canal in the cicatrix during development of the mericarpid arise bristle-like in a ventral position from the areole in *B. gastonii*. Although these characters are less evident in *B. goulandrisiorum*, this species shares with *B. gastonii* and *G. nitida* the acute, nearly beaked apex of the nutlet, and with most other *Glandora* species the lack of slender, long-procumbent stems (typical of *B. purpurocaerulea*, *B. calabra* and *B. zollingeri*), cymes which do not elongate in fruit and the scattered glandular hairs on the adaxial surface of the corolla (lacking in the other taxa of *B.* sect. *Margarospermum*). In addition, the slight pollen heteropolarity of *G. nitida* (Díez & al., 1986) provides another connection to species of *B.* sect. *Margarospermum*. Altogether, this suggests *B. gastonii* and *B. goulandrisiorum* to be palaeoendemics with plesiomorphic characters that have been partly retained in species of *B.* sect. *Margarospermum* and partly in species of *Glandora*, especially *G. nitida* and *G. diffusa* (which also has vertical hairy bands inside the corolla; Thomas & al., 2008). However, the two species are clearly early-diverging members in the *Glandora* clade.

Reappraisal of *Aegonychon*. — The three remaining species of *B.* sect. *Margarospermum* form a well-supported group with *B. calabra* sister to the *B. purpurocaerulea*/*B. zollingeri* clade. This fits with the morphological distinctiveness of this narrow endemic of the southern Apennines, which has longitudinal bands of long, acute trichomes in the corolla, stamens inserted in the lower half of the tube (as in *B. goulandrisiorum* and *B. gastonii*) and a rounded bulge with stipitate glands at the base of the filaments. Accordingly, *B. calabra* is possibly the closest relative to the ancestor of the other two wide-ranging but sharply allopatric Eurasian species, which are clearly close to each other also in view of their common morphological traits (Popov, 1953; Johnston, 1954).

Previous investigations did not clearly resolve relationships between members of *B.* sect. *Margarospermum* and those of sect. *Buglossoides* or other groups in the *Lithospermum* s.l. clade (Thomas & al., 2008; Weigend & al., 2009; Cohen, 2014). This study shows a deep phylogenetic divergence between the three species discussed above and those of *B.* sect. *Buglossoides*, which finds support in their morphological, palynological, and ecological differentiation. Here, we largely corroborate the observations by Johnston (1954) and provide additional palynological evidence for their separation. The shift from subisopolar, usually 4-aperturate grains to isopolar, 5–8-aperturate grains has likely been a major change from *B.* sect. *Margarospermum* to *B.* sect. *Buglossoides* (see also Díez & al., 1986). Furthermore, habit, fruit and flower characters allow an even more immediate distinction between the species of the two sections. While *B. calabra*, *B. purpurocaerulea* and

B. zollingeri have conserved the plesiomorphic fruit structure of most Old World *Lithospermum* s.str., apomorphic traits have originated in the annual taxa of *B.* sect. *Buglossoides*, such as the smaller size, the trigonous-pyriform shape, the strongly tuberculate-verrucose mericarpid surface and the frequent synaptospermic dispersal (L. Cecchi & F. Selvi, pers. obs.). These three species also have smaller flowers, as well as a broader range of corolla colours (e.g., white) and an internal rearrangement consisting mainly in the loss of glanduliferous hairs on the stamen filaments and the development of a thickened annulus at the base of the corolla tube. Reduction has also occurred in plant size, habit and life-cycle, leading to the shift from robust, often rhizomatous perennials to small, strictly therophytic (winter annual) plants. Most likely, this has proceeded in parallel with the ecological shift from mesophilous forest habitats to xerophilous, open environments such as fields (*B.* sect. *Buglossoides*).

In view of the strong correlation between our phylogenetic findings, morphology and ecology, we advocate separation of *B.* sect. *Buglossoides* from *B. calabra*, *B. zollingeri* and *B. purpurocaerulea* into two different genera distinct from *Lithospermum* and *Glandora*. Previous authors had already restricted *Buglossoides* to only the annual species, but retained the perennial species in *Lithospermum* as originally described (Edmondson, 1979; Meikle, 1985; Strid & Tan, 1991). Generic separation of the two sections was proposed by Berchtold & Opiz (1839: 73–74) who placed *L. purpurocaeruleum* L. in *Margarospermum* (Rchb.) Opiz (see also Pouzar, 1964), although Gray (1821) had already described *Aegonychon* based on both *L. repens* Stokes (= *L. purpurocaeruleum* L.) and *L. arvense* L. This problem was resolved by Holub (1973) who showed that *L. purpurocaeruleum*, later typified by Selvi (in Cafferty & Jarvis, 2004), has to be accepted as the type of *Aegonychon*. Holub (1973) transferred all five species of *B.* sect. *Margarospermum* to *Aegonychon* so that the necessary nomenclatural combinations are already available: *A. calabrum* ("*calabricum*") (Ten.) Holub, *A. gastonii* (Benth. ex A.DC) Holub, *A. goulandrisiorum* (Rech.f.) Holub, *A. purpurocaeruleum* (L.) Holub and *A. zollingeri* (A.DC.) Holub. In recent treatments, Holub's (1973) taxonomy has been adopted in the *Flora Iberica* treatment (Pastor, 2012) for *A. purpurocaeruleum* and *A. gastonii*. As described above, however, we propose to place *B. gastonii* and *B. goulandrisiorum* in *Glandora*, therefore restricting *Aegonychon* to the three woodland species which form a well-supported monophyletic clade.

Such treatment formally recognizes phylogenetic relationships and the major patterns of morphological diversity in the group.

Relationships within *Buglossoides* s.str. — This work includes a broad geographical-taxonomic sampling of *B.* sect. *Buglossoides* to better understand relationships in this small but polymorphic and difficult group of annual species that are commonly found especially in dry, synanthropic habitats of the Mediterranean area and Europe (Fernandes, 1972, 1973). In line with two previous studies of this group in Alto Adige/South Tyrol (Zippel & Wilhalm, 2003) and central Europe (Clermont & al., 2003), both based on ITS1 sequences and morphology, our analysis retrieved two well-supported sister clades: one with accessions of the *B. arvensis* complex and one with accessions of the *B. incrassata* complex. The two clades differ in 14 and 5 positions in the ITS and *trnL-trnF* IGS regions, respectively (see also Clermont & al., 2003). Notably, there is no geographic separation of the two clades which largely overlap in most of their ranges from North Africa to Central Europe (see Fig. 1B). The *B. arvensis* clade includes the typical *B. arvensis* subsp. *arvensis* and *B. arvensis* subsp. *sibthorpiana* (Griseb.) R.Fern., a weakly distinct race from mainly SE Europe and the Middle East (Strid, 2000). *Buglossoides tenuiflora* from arid habitats of the southeast Mediterranean is also nested in this clade, but morphological characters such as yellowish hairs on the calyx and distinctly 2-gibbous nutlets with very fragile pericarp show that this is a separate species. The *B. incrassata* clade includes typical *B. incrassata* and subsp. *splitgerberi*, both originally described from Sicily (Selvi & Cecchi, 2009). The two subspecies are widely distributed and largely sympatric in the Mediterranean region and the southern Alpine area (Zippel & Wilhalm, 2003), but subsp. *splitgerberi* extends more to the north and also occurs as a weed in Central Europe (Clermont & al., 2003, sub *B. arvensis* subsp. *sibthorpiana*). In addition, *B. arvensis* subsp. *permixta* from the western Mediterranean and *B. minima* endemic to Sardinia, Sicily and, possibly, south Italy, were also retrieved in this group. This clear phylogenetic result is partly matched by morphological evidence. The two synapomorphic traits for the *B. incrassata* complex are the circular cotyledons without secondary venation and the obliquely thickened fruit pedicel (Fig. 5C), whereas the *B. arvensis* complex is characterized by oblong cotyledons with distinct secondary venation and the pedicel remaining thin in fruit (Fig. 5B; Clermont & al., 2003; Zippel & Wilhalm, 2003). In *B. incrassata* subsp. *splitgerberi*, however, thickening of fruit pedicels is only partial, causing considerable difficulty in the distinction from *B. arvensis* s.l. (Selvi & Cecchi, 2009). Distinctly thickened fruit pedicels and circular cotyledons can instead be easily observed in material of *B. arvensis* subsp. *permixta* from southern France and Spain (L. Cecchi & F. Selvi, pers. obs. in cultivated material from the French Maritime Alps; see also Pastor, 2012), which nicely fits the position of this taxon in the *B. incrassata* clade. Notably, Jordan (1855: 344–346) himself noticed that the fruit pedicel of the specimens he used to describe the species was shorter and more distinctly thickened than in typical *L. arvense*. This character is also visible in one of the specimens (WAG 323, the only specimen with fruits that we could trace) that Jordan grew, after description of the species in 1855, from seeds of the type collection from the Hautes Alpes. This further corroborates the placement of this taxon in the *B. incrassata* complex, rather than in the *B. arvensis* complex as proposed by Fernandes (1971, 1972).

Partial discrepancy between morphology and phylogenetic relationships is caused instead by the rare *B. minima*, which has circular cotyledons without secondary venation (L. Cecchi & F. Selvi, pers. obs. on cultivated material from Sardinia) but non-thickened fruiting pedicels (Fig. 5D), perhaps as a consequence of character loss or reversal. The presence of three 1-bp insertions in the ITS1 sequence of this taxon and its peculiar

combination of characters (type depicted in Selvi & Cecchi, 2009) suggest to keep it as a separate species, at least until more data on population variation are available.

The strongly polymorphic *B. incrassata* subsp. *splitgerberi* resembles *B. arvensis* (both subspecies) in habit and flower characters, and can only be distinguished by the partially thickened fruiting pedicels or the circular, unveined cotyledons (Zippel & Wilhalm, 2003). These characters cannot be observed in many herbarium specimens, which therefore often are virtually impossible to identify correctly. Although the distribution range of *B. incrassata* subsp. *splitgerberi* includes large parts of the east and central Mediterranean, the Middle East and Central Europe as shown here, this taxon still remains imperfectly known and deserves further investigation.

■ NEW COMBINATIONS

Based on the discussion above, the following new combinations are made:

Glandora gastonii (Benth.) L.Cecchi & Selvi, **comb. nov.** ≡ *Lithospermum gastonii* Benth. in Candolle, Prodr. 10: 83. 1846 ("*gastoni*"; correction of the epithet's original spelling mandated by *ICN* Art. 60.12) ≡ *Buglossoides gastonii* (Benth.) I.M.Johnst. in J. Arnold Arbor. 35: 45. 1954 ≡ *Aegonychon gastonii* (Benth.) Holub in Folia Geobot. Phytotax. 8: 164. 1973 – Holotype: [FRANCE]. "Rochers de Balourde en montant des Eaux-Bonnes au Pic de Gers", *Gaston* (G-DC barcode G00148824!; isotype: "Rochers de Balourde en montant Pic de Gers", Aug 1839, FI-W No. 130145!).

Glandora goulandrisiorum (Rech.f.) L.Cecchi & Selvi, **comb. nov.** ≡ *Lithospermum goulandrisiorum* Rech.f. in Bot. Not. 124: 355. 1971 ("*goulandriorum*"; correction of the epithet's original spelling mandated by *ICN* Art. 60.12). ≡ *Aegonychon goulandrisiorum* (Rech.f.) Holub in Folia Geobot. Phytotax. 8: 165. 1973 ≡ *Buglossoides goulandrisiorum* (Rech.f.) Govaerts, World Checkl. Seed Pl. 2: 14. 1996 – Holotype: "Graecia, Epirus: Montes Tymphi, in praeruptis calc. ad austro-orientem lacus Drakolimni, 1900–2000 m", 12 Aug 1969, *Stamatiadou 7244* (W!; isotype: ATH!).

Glandora goulandrisiorum subsp. ***thessalica*** (Aldén) L.Cecchi & Selvi, **comb. nov.** ≡ *Lithospermum goulandrisiorum* subsp. *thessalicum* Aldén in Bot. Not. 129: 305. 1976 ≡ *Aegonychon thessalicum* (Aldén) Holub in Preslia 58: 301. 1986 ≡ *Buglossoides goulandrisiorum* subsp. *thessalica* (Aldén) Govaerts, World Checkl. Seed Pl. 2: 14. 1996 ≡ *Aegonychon goulandrisiorum* subsp. *thessalicum* (Aldén) Valdés in Willdenowia 34: 61. 2004 – Holotype: "Graecia, Thessalia: Mons Koziakas, supra pagum Elati, in praeruptis calcareis, 1900 m", 7 Jul 1972, *Aldén 151* (LD!).

Paratypes: "[Graecia, Thessalia:] Mt. Koziakas, 11 km NW of Pili (near Elati), ca. 1800 m", *A*[*ldén*] *1202*; "[Graecia, Thessalia:] 5 km NE of Pertoulion, 1750–1900 m", *A*[*ldén*] *1205*.

Buglossoides incrassata subsp. ***permixta*** (Jord.) L.Cecchi & Selvi, **comb. nov.** ≡ *Lithospermum permixtum* Jord. in Schultz, Arch. Fl. France Allem.: 344. 1855 ≡ *Buglossoides arvensis* subsp. *permixta* (Jord.) R.Fern. in Bot. J. Linn. Soc. 64: 374. 1971 ≡ *Buglossoides permixta* (Jord.) Holub in Preslia 58: 301. 1986 – Type not designated, Ind. loc.: [France] "Gap (Hautes Alpes)".

Material grown by Jordan himself after 1855 from seeds of the type collection that was sent to him by Blanc from the Hautes Alpes is kept under "*Lithospermum permixtum* Jord." in the herbaria of Montpellier and Wageningen (MPU019782!, MPU019783!, WAG0000323!; http://plants.jstor.org); one of these specimens could be designated as a neotype in case no original material collected before publication in 1855 is found. The thickened fruit pedicels are visible in especially WAG0000323, which includes fruiting material collected in June 1858.

Revised key to Old World genera of the *Lithospermum* s.l. clade

1. Annual herbs, usually small. Corolla ≤7 mm long. Mericarpids trigonous-pyriform, strongly verrucose-tuberculate, up to 3.5 mm long, usually 4 per fruit …… ***Buglossoides***
1. Herbaceous perennials, subshrubs or shrubs (rarely biennials). Corolla ≥8 mm long. Mericarpids mainly ovoid, up to 4.5 mm long, frequently smooth and shiny, rarely slightly rugose-foveolate, tumulose or minutely tuberculate, usually 1–3 through abortion …… 2
2. Corolla frequently with gibbose, invaginated faucal scales, always without longitudinal hairy bands. Hairy annulus at the base of tube often present. Mericarpids usually smooth and shiny, often with sparse, punctate, pit-like depressions, scattered or along ventral keel, rarely rugose …… …… ***Lithospermum***
2. Corolla without faucal scales, with or without longitudinal hairy bands. Hairy basal annulus always absent. Mericarpids smooth and shiny or rarely slightly tumulose to tuberculate, always without pit-like depressions …… 3
3. Dwarf shrubs or cespitose perennials of open, rocky habitats, without long-procumbent, slender stems. Fruiting cymes contracted, with calyces closely appressed to nutlets. Often heterostylous. Vertical hairy bands inside corolla present or absent. Glanduliferous hairs on adaxial side of corolla tube always present. Cicatrix with a peg-like appendage, rarely flat with a minutely protruding channel (*G. gastonii*, *G. goulandrisiorum*, *G. nitida*); areole a cup-shaped depression in the flat gynobase, or areole oblique to nearly planar with only a weakly developed depression in ventral position (*G. gastonii*, *G. nitida*) …… ***Glandora***
3. Perennial herbs of forest habitats, with numerous slender, long-procumbent and leafy stems. Fruiting cymes strongly elongated with well-spaced calyces. Homostylous. Vertical bands of trichomes in corolla and patches of glanduliferous hairs on stamen filaments always present. Glanduliferous hairs inside corolla tube absent. Mericarpid cicatrix without peg-like appendages; detachment areoles flat …… …… ***Aegonychon***

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Appendix 1. List of taxa and INSDC (International Nucleotide Sequence Database Collaboration) accession numbers for DNA sequences (ITS, *trnL-trnF* IGS when available) used in this study. Vouchers are kept in the Boraginaceae herbarium collection of the authors in FIAF, and indicated as FI-HB with relative number. Voucher information is given only for specimens originally analysed here (marked with an asterisk) or in Cecchi & Selvi (2009) (marked with [a]); INSDC numbers of taxa analysed for the *rpoC1* region are given in the "Results" section.

OUTGROUP: ***Alkanna tinctoria*** (L.) Tausch: FJ763250, FJ763304; ***Arnebia decumbens*** (Vent.) Coss. & Kralik: Syria around Al Qaryatayn, *Cecchi & al.* (FI-HB 07.05), KJ394991*, HG939444*; ***Arnebia linearifolia*** DC.: Syria, around An-Nasyriah, *Cecchi & al.* (FI-HB 07.03), EU919580[a], HG939445*; ***Cerinthe major*** L. subsp. ***major***: Italy, Sardinia, near Osilo, *Cecchi & Coppi* (FI-HB 08.01), EU919583[a]; ***Echium vulgare*** L.: FJ763247; ***Moltkia angustifolia*** DC.: Syria, around Deir-ez-Zor, *Cecchi & al.* (FI-HB 07.18), EU919593[a], FJ763306; ***Moltkia petraea*** (Tratt.) Griseb.: FJ763194, FJ763258; ***Moltkia suffruticosa*** (L.) Brand subsp. ***suffruticosa*****:** Italy, Veneto, Mt. Summano, *Cecchi & Coppi* (Herb. Cecchi 624), KJ394993*; ***Neatostema apulum*** (L.) I.M.Johnst.: FJ763198, FJ763262; ***Paramoltkia doerfleri*** (Wettst.) Greuter & Burdet: Albania, Kukes, Mt. Pastrik, *Cecchi & al.* (FI-HB 06.20), EU919605[a]; ***Podonosma orientalis*** (L.) Feinbrun: Syria near Palmyra, Jebel-et-Tar, *Cecchi & al.* (FI-HB 07.16), EU919607[a], FJ763307. — INGROUP: ***Buglossoides arvensis*** (L.) I.M.Johnst. subsp. ***arvensis***, **1:** Italy, Tuscany, Marina di Grosseto, *Selvi* (FI-HB 07.64), KJ394968*, HG939439*; **2:** Italy, Latium, around Viterbo, *Cecchi & Selvi* (FI-HB 06.42), KJ394965*; **3:** Italy, Umbria, Castelluccio di Norcia, *Dal Lago* (MNAV-Dal Lago), KJ394961*; **4:** Italy, Veneto, Piane di Schio, *Scortegagna* (MNAV), KJ394962*; **5:** Tunisia, around Feriana, *Selvi & Bigazzi* (FI-HB 04.48), KJ394963*; **6:** Turkey, around Burdur, *Cecchi & Selvi* (FI-HB 13.50), KJ394960*; ***B. arvensis*** subsp. ***permixta*** (Jord.) R.Fern., **1:** Spain, Jaén, Sierra de la Sagra, *Cecchi & al.* (FI-HB 11.13), KJ394970*; **2:** France, Maritime Alps, Valle Roya, *Andrieu* (FI-HB 10.94), KJ394971*; **3:** France, Maritime Alps, Caussols, *Tison* (FI-HB 13.63), KJ394982*, HG939442*; ***B. arvensis*** subsp. ***sibthorpiana*** (Griseb.) R.Fern., **1:** Greece, Crete, Thripti, *Hilger* (FI-HB, 13.84), KJ394964*; **2:** Syria, around Yabroud, *Cecchi & al.* (FI-HB 07.37), KJ394966*; ***B. calabra*** (Ten.) I.M.Johnst.: Italy, Calabria, Villaggio Mancuso, *Cecchi & Coppi* (FI-HB 07.56), KJ394986*, FJ763305; ***B. gastonii*** (Benth.) I.M.Johnst.: Germany, Schachen Alpine Garden (culta), *Döbbeler* (FI-HB 07.58), KJ394988*, HG939437*; ***B. goulandrisiorum*** (Rech.f.) Govaerts subsp. ***goulandrisiorum*****:** Greece, Ipiros, Mt. Timfi around lake Drakolimni, *Cecchi & Selvi* (FI-HB 08.38), KJ394989*, HG939443*; ***B. incrassata*** (Guss.) I.M. Johnst. subsp. ***incrassata*****, 1:** Syria, Damascus, around Zebdani, *Cecchi & al.* (FI-HB 07.42), KJ394981*; **2:** Turkey, Antalya, Termessos, *Cecchi & Selvi* (FI-HB 10.12), KJ394972*; **3:** Turkey, Antalya-Akseki, Gembos Yayla, *Cecchi & Selvi* (FI-HB 10.03), KJ394983*; **4:** Spain, Granada, Sierra Nevada; *Cecchi & al.* (FI-HB 11.16), KJ394973*; **5:** Greece, Ipiros, Mt. Timfi near Astraka, *Cecchi & Selvi* (FI-HB 08.37), KJ394979*; **6:** FJ763191, FJ763255; ***B. incrassata*** subsp. ***splitgerberi*** (Guss.) E.Zippel & Selvi, **1:** Italy, South Tyrol, Kompatsch, *Wilhalm* (BOZ PVASC5518), KJ394974*; **2:** Italy, South Tyrol, Paulsner Feld, *Wilhalm* (BOZ PVASC5526), KJ394976*; **3:** Italy, South Tyrol, Faslar, *Wilhalm* (BOZ PVASC5524), KJ394985*; **4:** Italy, Umbria, Mt. Subasio, *Selvi* (FI-HB 06.01), KJ394969*; **5:** Italy, Sicily, Mt. Etna, *Bianchini 11499* & *Di Carlo* (VER), KJ394984*; **6:** Turkey, Bursa, Uludag, *Cecchi & Selvi* (FI-HB 10.44), KJ394975*, HG939440*; **7:** Germany, Brandenburg, Neutornow, *Hand 5773* (B), KJ394978*; **8**: Italy, South Tyrol, Mals, *Hand 5633* (B), KJ394980*; ***B. minima*** (Moris) R.Fern.: Italy, Sardinia, Mt. Tului, *Coppi & Selvi* (FI-HB 09.22), KJ394977*, HG939441*; ***B. purpurocaerulea*** (L.) I.M.Johnst., **1:** FJ789859, FJ763308; **2:** AJ555897; ***B. tenuiflora*** (L.f.) I.M.Johnst.: Syria, ruins of Palmyra, *Cecchi & al.* (FI-HB 07.15), KJ394967*; ***B. zollingeri*** (A.DC.) I.M.Johnst.: Taiwan, Yehai, *s.coll.* (HCT, TFRI 80) , KJ394987*, HG939438*; ***Glandora diffusa*** (Lag.) D.C.Thomas: FJ763246, FJ763300; ***G. moroccana*** (I.M.Johnst.) D.C.Thomas: FJ789867; ***Glandora nitida*** (Ern) D.C.Thomas: FJ763245, FJ763299; ***G. oleifolia*** (Lapeyr.) D.C.Thomas: FJ789869, FJ789887; ***G. prostrata*** (Loisel.) D.C.Thomas: Japan, cultivated (commercial material), *Fukunaga* (FI-HB 08.62bis), KJ394992*, FJ763277; ***G. rosmarinifolia*** (Ten.) D.C.Thomas: FJ763236, FJ763291; ***Lithospermum cinereum*** DC.: FJ763240, FJ763295; ***L. erythrorhizon*** Siebold & Zucc.: EF199861, FJ763309; ***L. hancockianum*** Oliv.: China, Yunnan, Yunnanfu, *Handel-Mazzetti 6058* (KUN), KJ394990*; ***L. officinale*** L., **1:** FJ763189, FJ763254; **2:** Italy, Abruzzo, Gran Sasso-Laga, *Bigazzi & Selvi* (FI-HB 03.03) [*rpoC1* sequence accession no. in Materials and Methods]; ***L. peruvianum*** DC.: FJ763216; ***L. tschimganicum*** B.Fedtsch.: FJ763220.