



Arnebia purpurea: a new member of formerly monotypic genus *Huynhia* (Boraginaceae-Lithospermeae)

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Abstract

The taxonomic position and affinities of the rare Turkish endemic *Arnebia purpurea* are analyzed using nuclear and plastid DNA sequence data and morphological characters. Phylogenetic analysis of a wide sample of old-world Lithospermeae consistently retrieved a clade with this species sister to *Huynhia pulchra*, the only member of the genus *Huynhia*. All other members of *Arnebia* s.l. (including *Macrotomia*) formed a separate clade subdivided in two lineages corresponding to the groups of the annual and the perennial species. Consequently, *Arnebia* does not appear monophyletic. Floral and palynological characters support the affinity of *A. purpurea* to *Huynhia pulchra*, in especially the stamens inserted at different heights in the corolla tube and the pollen grains with a single row of endoapertures along the equatorial belt. We therefore advocate the placement of *A. purpurea* in *Huynhia* and propose a new combination, implying that the latter is no longer a monotypic genus but includes two species with a sharply allopatric range in the Middle-East. Further studies with additional markers and a wider taxon sampling will help to elucidate relationships in *Arnebia* s.l..

Key words: *Macrotomia*, molecular phylogeny, micromorphology, taxonomy, Turkish flora

Introduction

When broadly circumscribed, *Arnebia* Forsskål (1775: 62) is a genus of Boraginaceae tribe Lithospermeae including about 30 annual or perennial herbs distributed in SW and C Asia, Himalaya, NE Africa and SE Mediterranean. Diagnostic morphological traits are the fruiting calyx, often hardening and tightly enclosing the nutlets, the corolla without faucal appendages, the usually di- or tetrastigmatic flowers, with the style once or twice forked, and the ovoidal-subglobose nutlets, often ventrally keeled and with ornamented surface. Also very characteristic are the pollen grains, which bear two rows of pores, one about each end of the oblong grain, often constricted at the equator and asymmetrical (Johnston 1954; Huynh 1971; Weigend *et al.* in press).

According to Johnston (1954), two sections are comprised in the genus, one (sect. “*Euarnebia*”) consisting of the only annual species *A. tinctoria* Forsskål (1775: 63; = *A. tetrastigma* Forsskål 1775: 62, the type species of the genus described from Egypt), and one (sect. *Strobilia* (Don 1838: 327) Johnston 1954: 55) subdivided in three subgroups based on the life-cycle (annual/perennial) and the presence of a pubescent annulus at the base of the corolla tube.

Previously, some of the perennial species that lack an annulus, such as *A. densiflora* (Ledeb. ex Nordmann 1837: 312) Ledebour (1847–1849: 140) and *A. benthamii* (Wall. ex Don 1838: 333) Johnston (1954: 56), have been placed in the separate genus *Macrotomia* DC. ex Meissner (1837–1842: 281), though more recently these have been included in *Arnebia* (Edmondson 1978; Strid & Tan 1993; Zhu 1982; Zhu *et al.* 1995), and *Macrotomia* is no longer recognized (see also Riedl 1964, 1971).

On the other hand, a single species, *A. pulchra* (Willd. ex Römer & Schultes 1819: 756) Edmondson (1977: 33; based on *Lycopsis pulchra* Willd. ex Roem. & Schult.) was considered outside *Arnebia* by most authors and already placed in the separate monotypic genus *Aipyanthus* Steven (1851: 599), with the misapplied name *Aipyanthus echioides* (Linnaeus 1762: 199) Steven (1851: 600; formally based on *Nonea echioides* (L.) Römer & Schultes 1819: 71), then

in the (illegitimate) genus *Echioides* Ortega (1773: 7) by Johnston (as *E. longiflora* (Koch 1849: 640) Johnston 1954: 58), and, finally, again in the monotypic genus *Huynhia* Greuter (1981: 37) with the correct name of *H. pulchra* (Willd. ex Roem. & Schult.) Greuter (1981: 37; see also Riedl 1993).

Indeed, this attractive Irano-Turanian species differs from members of *Arnebia* and other related genera of Lithospermeae in outstanding features such as the stamens inserted at different heights, instead of forming a whorl, and the pollen grains with a peculiar structure as described in detail by Huynh (1971). Seibert (1978) also kept this species in a separate genus based on fruit characters, so that *Huynhia* is currently accepted as a good monotypic genus of Lithospermeae (Weigend *et al.* in press).

Unclear evidence concerning the phylogenetic relationships of *Arnebia*, *Macrotomia* and *Huynhia* emerged from recent investigations on Boraginaceae and tribe Lithospermeae. While results of Cecchi & Selvi (2009), Nazaire & Hufford (2012) and Weigend *et al.* (2009, 2013) suggested the monophyly of *Arnebia* including *Macrotomia* and the divergent position of *Huynhia*, those of Cohen (2014) indicated that the latter genus is sister to *Arnebia* also inclusive of *Macrotomia densiflora*. However, all of these studies included only a very few members (2–4) of *Arnebia* s.l., which prevented to obtain a more complete picture and deeper insights into the relationships within the group and the affinities of several rare or endemic species.

One of these taxa is *A. purpurea* Erik & Sümbül (1986: 151), described from southern Turkey and only known from a small area of the Taurus mountains (see also Davis *et al.* 1988; Ekim 2009). During a field trip in this area we could collect this remarkable endemic in two different localities which provided the opportunity to elucidate its affinities using molecular and morphological tools. To this purpose, we included all available species of *Arnebia*, *Huynhia* and several members of Lithospermeae in a DNA phylogeny based on nuclear ITS and plastid *trnL-trnF*, and examined taxonomically important characters and micro-characters with SEM, especially pollen.

The results of this study brought to the light new evidence on the systematic position of *A. purpurea* and the circumscription of genus *Huynhia*.

Material and Methods

Plant material—Native populations of species of *Arnebia* s.l. and *Huynhia* were studied and sampled by the authors during field trips in the eastern and south Mediterranean countries. *Huynhia* was collected in the highlands of eastern Anatolia in June 2000, while *A. purpurea* was found in June 2013 on the Taurus chain (S Turkey), in a first site not far from the type locality around Gazipaşa (Gökbel plateau; Fig. 1A–C), and in a second one ca. 35 km to the east (Karahasan pass between the villages of Ermenek and Taskent). Geographical details on these sites are given in Appendix 1, together with the full list of examined taxa and voucher information. Additional material of *Arnebia* species for morphological observations was obtained from herbarium collections mainly in FI, FI-W, B and G.

DNA extraction and amplification—Genomic DNA of the new *Arnebia* accessions was extracted from silica-gel dried samples of leaf tissue using a modified 2xCTAB protocol (Doyle & Doyle 1990). Amplification of the ITS region of nuclear DNA, including ITS1, 5.8S and ITS2, and of *trnL-trnF* IGS followed the procedure described in Cecchi *et al.* (2014).

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, USA).

Sequence alignment and phylogenetic analyses—Original sequences of the *Arnebia* accessions were treated as described in Cecchi *et al.* (2014). Three datasets were prepared for phylogenetic analyses, ITS, IGS and combined ITS-IGS, retrieving most of sequences from INSDC (accession numbers are given in Appendix 1). The ITS1-5.8S-ITS2 dataset included 46 ingroup taxa, of which 13 accessions (11 species) of *Arnebia* s.l. (including *Huynhia*) and the others representing the great majority of old-world Lithospermeae (17 genera). The *trnL-trnF* IGS dataset included 28 ingroup taxa, of which ten accessions (nine species) of *Arnebia* s.l. and a wide range of old-world Lithospermeae involved (13 genera). Different sample size of the two datasets was because IGS sequences were available for fewer taxa than for ITS, but this did not apparently cause inconsistencies between the resulting phylogenies also due to the low resolution power of IGS. Gaps were coded as separate characters according to Simmons & Ochoterena (2000) using FastGap v.1.0.8 (Borchsenius, 2009), and appended at the end of the datasets.

An additional dataset consisting of concatenated ITS-IGS sequences plus coded gaps was also prepared for a combined analysis (25 ingroup taxa). Congruence between the two single-marker datasets and respective trees was inferred from the absence of conflicting well-supported clades in the resulting trees, according to Wiens (1998).

Four taxa of the tribes Echiochileae and Boragineae were selected as outgroup members, based on their relationships to Lithospermeae (Långström & Chase 2002; Weigend *et al.* 2013).

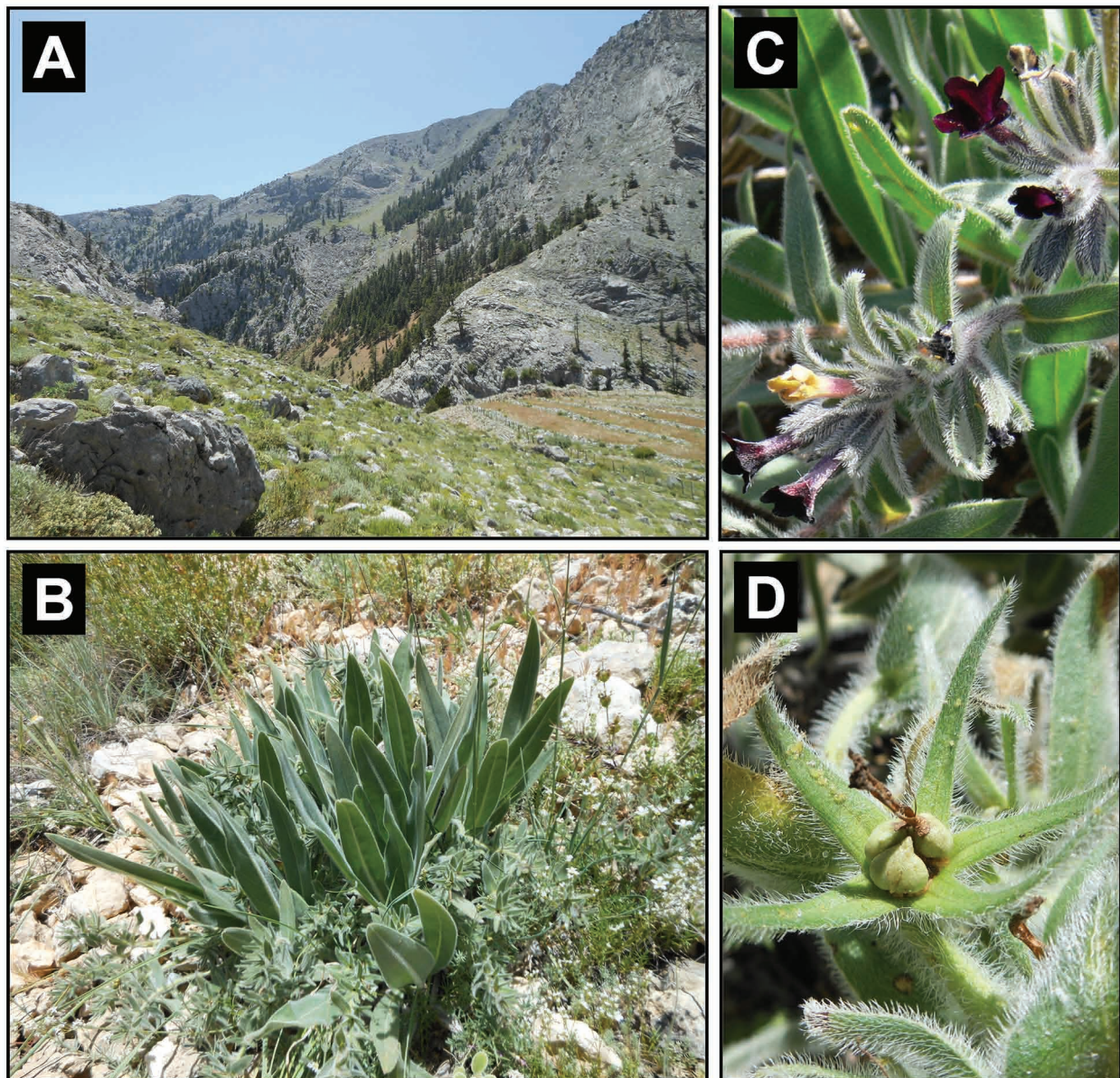


FIGURE 1. *Arnebia purpurea* Erik & Sümbül on Gökbel plateau (Turkey, Taurus mountains), June 2013. A) landscape and habitat of the species; B) whole plant; C) cymes with flowers; D) fruiting calyx with nutlets (photos: A,C,D by L. Cecchi; B by M. Nepi).

Phylogenetic analyses were performed using Maximum Parsimony and Bayesian methods. 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) 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 1.000 replicates, using TBR branch-swapping, 10 random taxon entries per replicate and MULTREES option on.

The data sets were also analysed using Bayesian inference of phylogeny with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Based on jModeltest (Posada 2008), the best fitting models of nucleotide substitution were GTR for ITS, 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 a 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 (Page 1996), with indication of Bayesian Posterior Probabilities (PP) values for the internal tree nodes.

Micromorphology (SEM)—Pollen grains from dry specimens were rehydrated in a solution of Aerosol-OT 20% and then observed with a FEI ESEM-QUANTA 200 working at 30 kV.

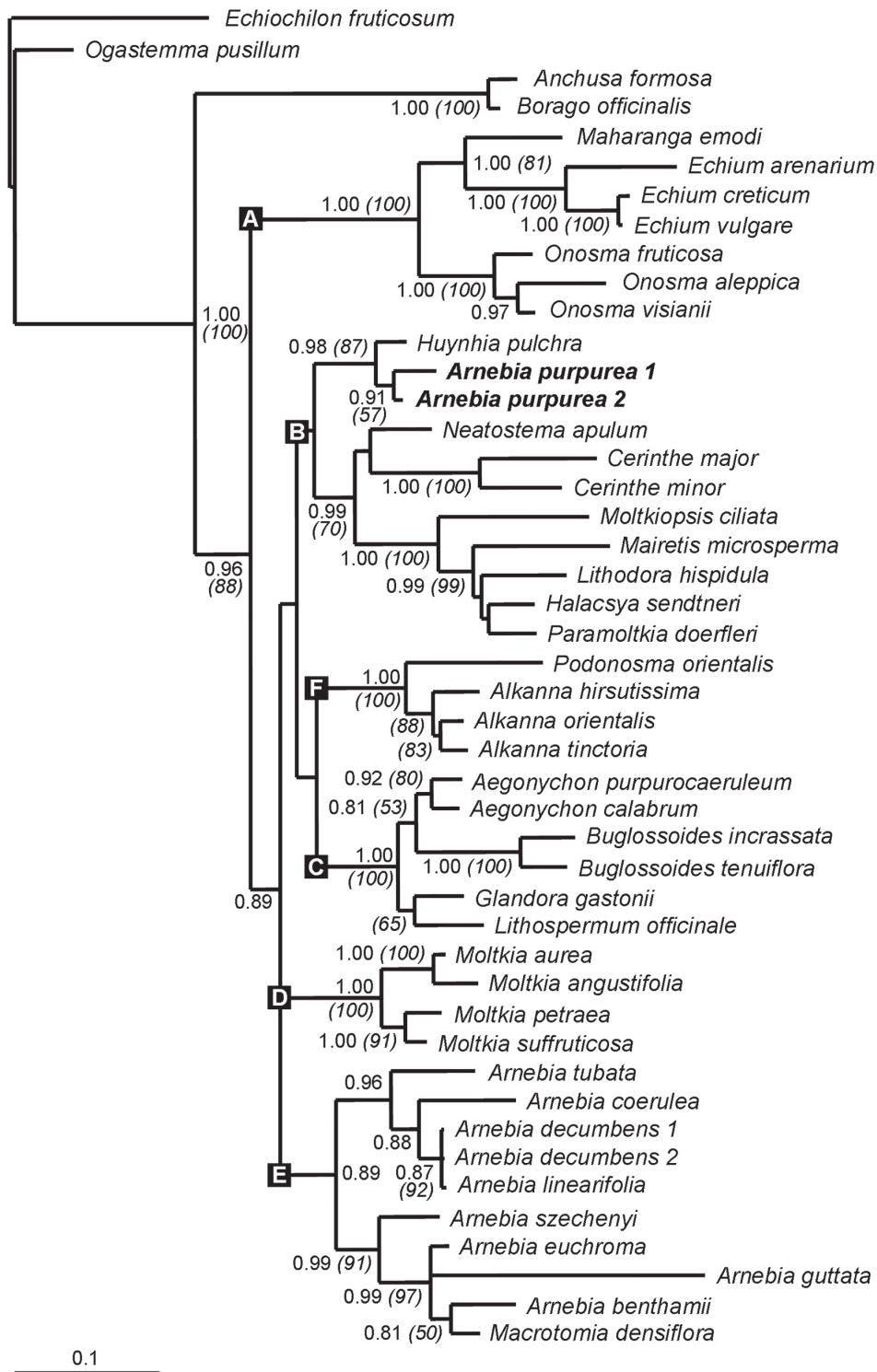


FIGURE 2. Bayesian 50% majority-rule consensus tree from the ITS dataset, with posterior probability values (PP) and bootstrap support (BS: in brackets and italics) shown near statistically supported nodes; the main clades of Lithospermeae are indicated with small squares and letters according to Cecchi & Selvi (2009).

Results

Nuclear ITS-5.8S dataset—The aligned matrix included a total of 805 positions, with coded gaps in pos. 683–805. In the Maximum Parsimony analysis, 267 characters were constant, 177 variable but non-informative, and 361 variable and parsimony informative. The heuristic search produced 54 most parsimonious trees with $L = 1662$, Consistency index (CI) = 0.51 and Retention index (RI) = 0.67. The strict consensus was topologically largely congruent with the 50 majority-rule consensus tree produced by the Bayesian analysis, which is shown in Fig. 2 with bootstrap (BS) and posterior probability values (PP).

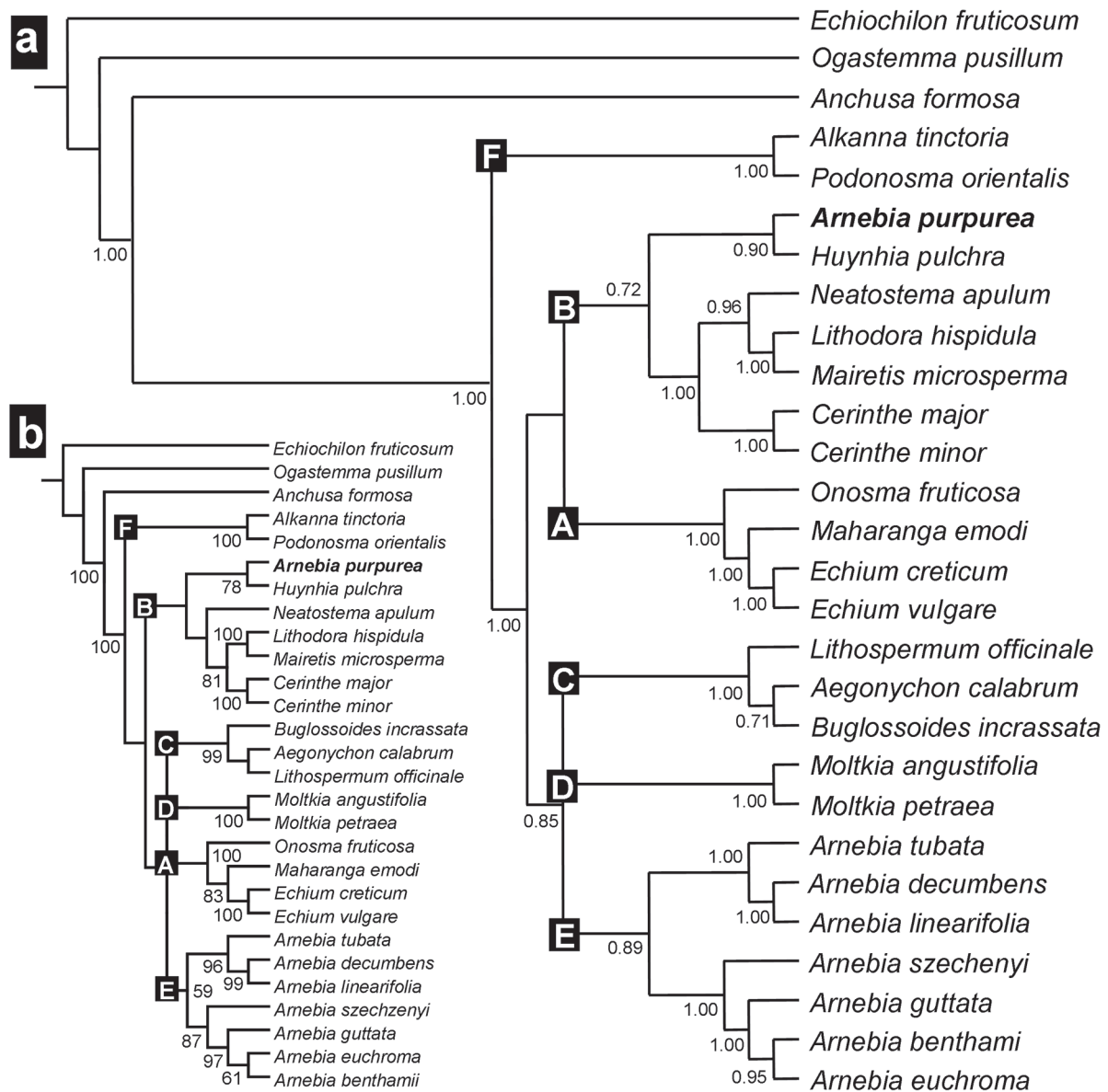


FIGURE 3. a) Bayesian 50% majority-rule consensus tree from the combined ITS-IGS dataset, with posterior probability values (PP) > 0.70; b) Strict consensus tree from Maximum Parsimony analysis with bootstrap support values to statistically supported nodes (> 50%); the main clades of Lithospermeae are indicated with small squares and letters according to Cecchi & Selvi (2009).

Arnebia was not retrieved as a monophyletic clade because of the position of *A. purpurea* as sister to *H. pulchra*. The two species clustered together in a monophyletic clade with good bootstrap (87% BS) and Bayesian (0.98 PP) support. They shared five SNPS and one 2-bp positions in the ITS1 region that were not present in anyone of the other species of *Arnebia*. This clade was suggested as sister (0.71 PP) to a well-supported assemblage of genera such as

Cerithe, *Neatostema*, *Mairetis* and others (clade B), but this relationship did not receive bootstrap support. All other members of *Arnebia* were retrieved in a clade with moderate support from Bayesian inference (0.89 PP), but not from bootstrap analysis. This consisted of two main sister branches: the first one (0.96 PP) included the annual members *A. linearifolia*, *A. decumbens*, *A. coerulea* and *A. tubata*, although the affinity of the latter species to this group did not receive bootstrap support; the second one was well supported (91% BS, 0.99 PP) and formed by the perennial species partly referred to genus *Macrotomia* in past times, such as *A. densiflora*, *A. benthamii* and *A. euchroma*.

The other well-supported groups were those of *Onosma*/*Maharanga*/*Echium* (clade A), *Moltkia* (clade D) *Alkanna*/*Podonosma* (clade F) and *Lithospermum*/*Glandora*/*Buglossoides*/*Aegonychon* (clade C).

Plastid *trnL-trnF* IGS dataset—The IGS alignment included a total of 995 positions, of which 72 coded gaps at the end of the matrix. Constant characters were 695, and 117 characters were parsimony informative. Heuristic search yielded 40 most parsimonious trees with L=426, CI=0.79 and RI=0.70. The 50-majority rule bootstrap tree and the consensus phylogram from Bayesian analysis were both strongly polytomized and therefore not shown here. Only the annual species of *Arnebia* formed a well supported clade (100% BS, 1.00 PP), and their sistership to *A. guttata* received very weak support (67% BS, 0.51 PP). *Arnebia purpurea* and *H. pulchra* shared a single SNP but were unresolved, as well as *A. szechenyi* Kanitz and the clade of *A. benthamii*/*A. euchroma* (57% BS, 0.99 PP).

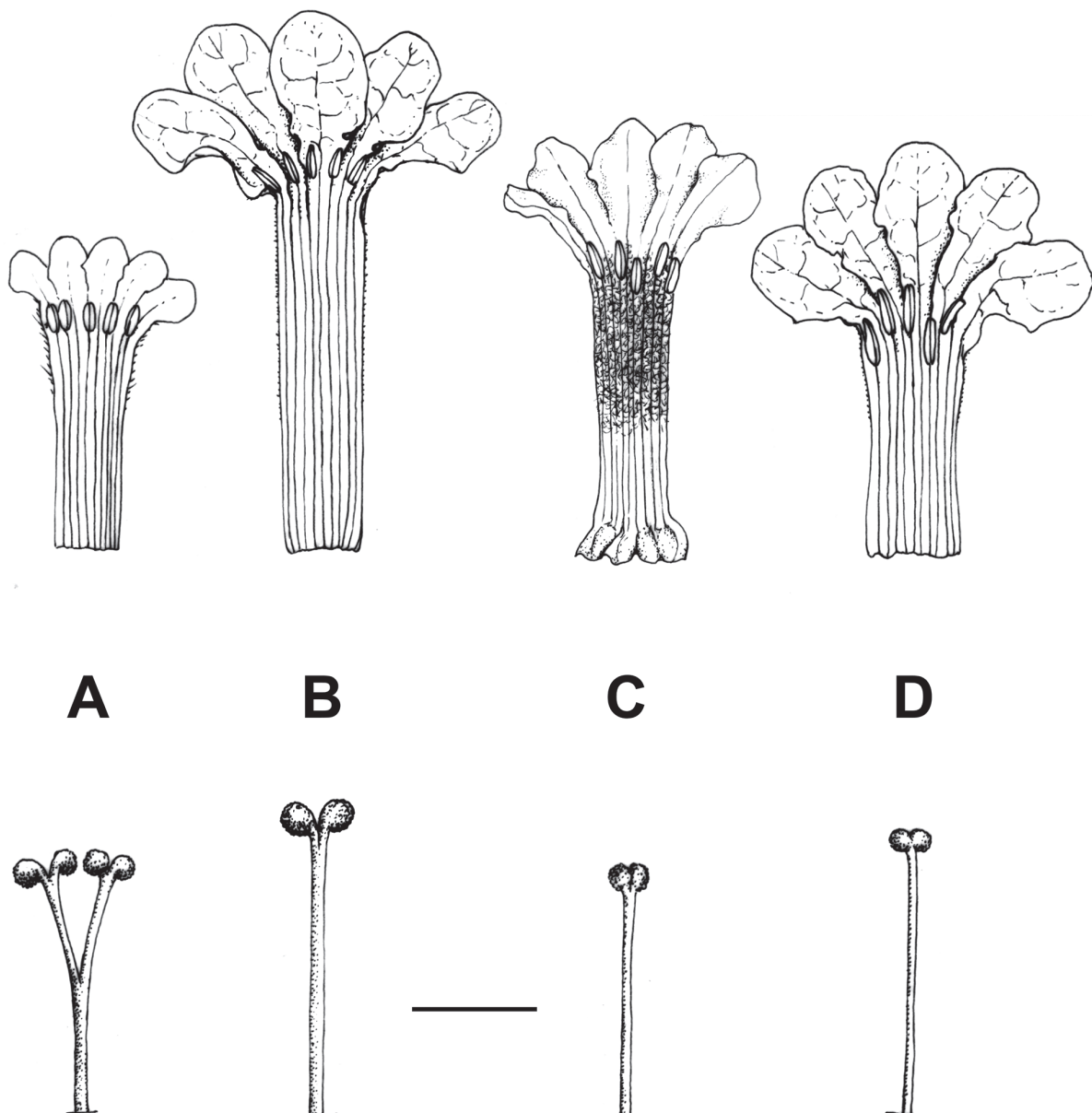


FIGURE 4. Open corolla, anther position, style and stigma in *Arnebia* and *Huynhia*. A) *A. linearifolia* (Cecchi, Coppi & Selvi HB 07.03); B) *A. densiflora* (Bigazzi & Selvi HB 02.45); C) *A. purpurea* (Cecchi, Selvi et al., HB 13.24); D) *H. pulchra* (Bigazzi & Selvi HB 00.09). Scale bar: B, D, corollas = 10 mm, styles = 2 mm; A, C, corollas = 20 mm, styles = 2 mm. Original drawings by L. Cecchi.

Combined ITS-IGS dataset—The aligned matrix consisted of 1783 characters, including coded gaps (pos. 1610~1783), of which 1018 were constant and 407 parsimony-informative. Maximum parsimony analysis retrieved only two trees with L=1716; CI=0.61 and RI=0.56, the consensus of which (Fig. 3b) was largely congruent with the 50% majority rule consensus tree from Bayesian analysis (Fig. 3a). *Arnebia pupurea* and *H. pulchra* clustered again in the same clade (78% BS; 0.90 PP), that was sister to clade B (inclusive of species of the genera *Neatostema*, *Lithodora*, *Mairetis*, *Cerintho*) but with low support (0.72 PP). In the MP and Bayesian trees, the rest of *Arnebia* was included in a single clade, though without strong support (59% BS, 0.89 PP). Both analyses confirmed the early split of this group in two well supported subclades, a first one with the annual species *A. linearifolia*, *A. tubata* and *A. decumbens* (96% BS, 1.00 PP) and a second one with the perennials *A. szechenyi*, *A. guttata*, *A. benthamii* and *A. euchroma* (87% BS, 1.00 PP).

Morphology—General and detailed characters of vegetative parts, flowers and fruits of *Huynhia* and *Arnebia* are already well known from various literature descriptions, while only two standard descriptions are available for *A. purpurea*. Herbarium material of this species is very scarce. Here, two characters are worth of mention because of their taxonomic significance, one concerning the androecial arrangement and one the stigma structure. In *Huynhia* and *A. purpurea* the epipetalous stamens are inserted through very short filaments at different heights within the corolla tube, e.g. three higher at the throat and two lower down in the tube (Fig. 4C,D). This character is less evident in *A. purpurea*, where the anthers of the two lower stamens reach ca. half of the anther length of the three higher stamens, rather than less than half in *Huynhia*. Based on our observations and current knowledge, the stamens in *Arnebia* (incl. *Macrotomia*) are whorled in the upper part of the corolla tube (Fig. 4A,B). Also, the style of most *Arnebia* s.l. species is shortly 2- or 4-forked, with each branch ending in a stigmatic portion (Fig. 4A), while it is entire and with capitate, shallowly bilobed stigma in *Huynhia* and *A. purpurea* (Fig. 4C,D). An intermediate condition is found in *A. densiflora*, where the cleft dividing the two lobes of the stigma reaches the style only shallowly (Fig. 4B). Heterostyly was not observed in *A. purpurea*, though it frequently occurs in *Arnebia* s.l.

Pollen characters provide other elements of taxonomic significance and these are summarized in Table 1. *Arnebia purpurea*, here investigated for the first time, turned out to be palynologically closer to *Huynhia* than to *Arnebia* (incl. *Macrotomia*). The key character shared by the two species is the presence of a single row of endoapertures along the equatorial belt, where the exine is thinner than towards the two polar regions. In both *Arnebia* and the allied, monotypic *Stenosolenium* Turczaninow (1840: 253; not included in molecular phylogeny), the germination pores are characteristically doubled, because each ectoaperture has two endoapertures located at the two opposite “emispheres” of the grain towards the polar regions. These are divided by the equatorial belt which is provided by a thickened exine. In addition, the grains of *Huynhia* and *A. purpurea* have a symmetrical, elliptical shape and narrowly fusiform ectoapertures without ornamented membrane (Fig. 5A,B), while *Arnebia* and *Stenosolenium* have strongly oblong, asymmetrical grains, often constricted at the equator and with rectangular-fusiform ectoapertures with ornamented membrane, usually separated by prominent costae (Fig. 5C–F). Pollen characters are therefore congruent with molecular data in supporting the affinity between *A. purpurea* and *Huynhia*.

The two species differ in the higher number of apertures which is 6(7) in the former (Fig. 5A) vs. 9 in the latter (Fig. 5B), while in other species of *Arnebia* and *Stenosolenium* there are usually (4)–5(6) apertures (Fig. 5C–F).

Discussion

The present study provides a small but, in our opinion, significant contribution to a better understanding of relationships and systematics of a still little-known group of old-world Lithospermeae. Previous investigations, in fact, included only very few species of *Arnebia* s.l. and could not satisfactorily address the problem of the monophyly of this genus (Thomas *et al.* 2008; Cecchi & Selvi 2009; Weigend *et al.* 2009, 2013; Nazaire & Hufford 2012; Cohen 2014). Taxon sampling of our investigation is still largely incomplete because of the difficulty in obtaining material of several Asian taxa.

TABLE 1. Main pollen characters of the taxa examined (in alphabetical order), with country of origin and voucher specimens

| | P (µm) | E (µm) | P/E | Shape (*) | No. apert. | Aperture length (µm) | Endoapert. position | Ectoapert. shape (**) | tectum | Pollen type | Origin | Voucher |
|---|--------|--------|------|-----------|------------|----------------------|---------------------|-----------------------|--------------|--------------------|-------------|---------------------------------------|
| <i>Arnebia decumbens</i> (Vent.) Coss. & Kralik | 32 | 21.8 | 1.46 | r/cc | 5/6 | 32 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Tunisia | Bigazzi & Selvi HB 04.34 (FIAF) |
| <i>Arnebia densiflora</i> Ledeb. | 40.9 | 28.4 | 1.44 | r/cc | 4 | 40.3 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Turkey | Bigazzi & Selvi HB 02.45 (FIAF) |
| <i>Arnebia griffithii</i> Boiss. | 24.8 | 14.2 | 1.74 | r/cc | 5/6 | 24.8 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Afghanistan | Griffith 5953 (FI) |
| <i>Arnebia hispidissima</i> DC. | 18.8 | 11.1 | 1.69 | r/cc | 6/7 | 18.6 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Arabia | Figari 1844, s.no. (FI) |
| <i>Arnebia perennis</i> A.DC. | 25.9 | 15.6 | 1.66 | r/cc | 5 | 25.9 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Nepal | Dainelli & Von Hofe 1930, s.no. (FI) |
| <i>Arnebia purpurea</i> Erik & Stümböl | 34 | 24 | 1.41 | ell | 6/7 | 20.1 | equatorial | f | (sub)psilate | Huynhia pulchra | Turkey | Cecchi, Selvi et al., HB 13.24 (FIAF) |
| <i>Arnebia tibetana</i> Kurz | 15.2 | 7.4 | 2.05 | r/cc | 5 | 15.2 | 2-polar | rf | (sub)psilate | Arnebia densiflora | Nepal | Dainelli & Von Hofe 1930, s.no. (FI) |
| <i>Arnebia transcaspica</i> Popov | 36.2 | 21.5 | 1.68 | r | 5-6 | 36.2 | 2-polar | rf | (sub)psilate | Arnebia densiflora | URSS | Belianina & Sojckova 1979, s.no. (FI) |
| <i>Huynhia pulchra</i> (Wild ex Roem. & Schult.) Greuter & Burdet | 30 | 22 | 1.36 | ell | 9 | 18.6 | equatorial | f | (sub)psilate | Huynhia pulchra | Turkey | Bigazzi & Selvi HB 00.09 (FIAF) |
| <i>Stenosolenium saxatile</i> (Pall.) Turez. | 24 | 12.8 | 1.87 | r/cc | 8 | 24 | 2-polar | rf | (sub)psilate | Arnebia densiflora | China | Giraldi 1897, s.no. (FI) |

* cc: constricted at equator; r: rectangular; ell: elliptical

** rf: rectangular-fusiform; f: fusiform

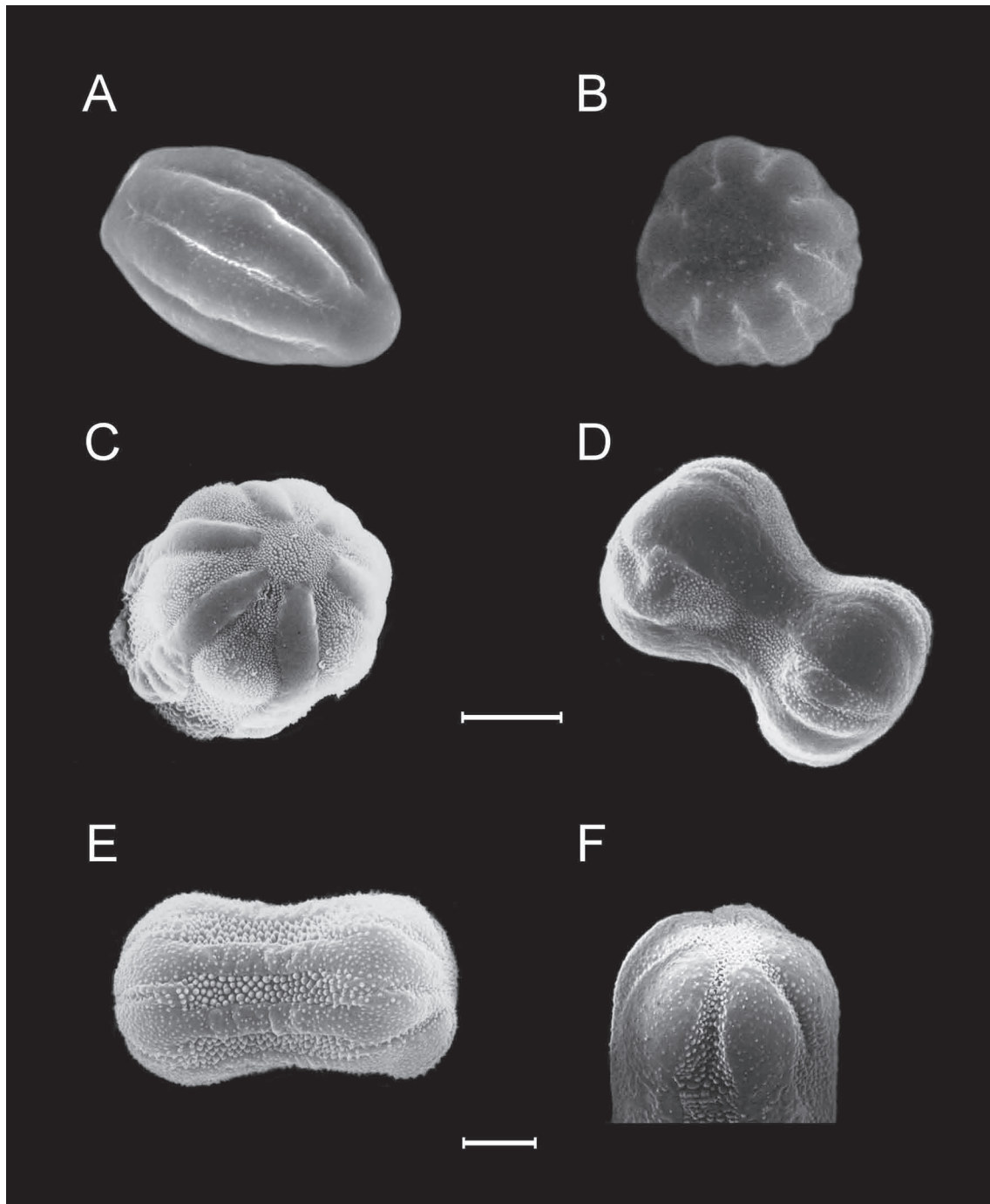


FIGURE 5. Pollen morphology in *Arnebia*, *Huynhia* and *Stenosolenium*. A) *A. purpurea* (Cecchi, Selvi *et al.*, HB 13.24); B) *H. pulchra* (Bigazzi & Selvi HB 00.09); C) *A. decumbens* (Bigazzi & Selvi HB 04.34); D) *A. densiflora* (Bigazzi & Selvi HB 02.45); E) *S. saxatile* (Giraldi 1897, s.no.); F) *A. perennis* (Dainelli & Von Hofe 1930, s.no.). Scale bars: A–D = 10 μ m; E–F = 5 μ m.

However, we show here that *Arnebia* is not monophyletic due to the position of *A. purpurea*, that resulted closely related and sister to *H. pulchra* in a well to moderately supported clade retrieved in the ITS and combined ITS-IGS analysis, respectively. The two latter species were in fact grouped in a different clade without direct relationship to *Arnebia*, though the backbone of the trees uniting the two lineages was not strongly supported. Lack of affinity between *Arnebia* and *Huynhia* emerged first in Cecchi & Selvi (2009) and this was supported in later analyses (Nazaire & Hufford 2012; Weigend *et al.* 2009, 2013) where, however, the position of the latter monotypic genus remained unclear. A different result was obtained in a recent broad-scale analysis of Boraginaceae using only plastid markers (Cohen 2014). Here, *Huynhia* was retrieved as sister to *Arnebia* (unfortunately represented by only the perennial species) plus *Macrotomia*, and *Arnebia* was assessed as non-monophyletic because *M. densiflora* (Ledeb. ex Nordm.) Macbride (1916: 56) was nested among its species.

Including the latter species and the other members of *Macrotomia* in *Arnebia* or keeping these members of the “perennial clade” in a separate genus are both reasonable options in view of evidence presented here. Waiting for further investigations, we prefer to adopt a broad concept of *Arnebia* in line with Johnston’s (1954) treatment of Lithospermeae and the recent floristic literature (Edmondson 1978; Strid & Tan 1993; Zhu *et al.* 1995; Weigend *et al.* in press). In any case, our findings show that none of these two options actually allows to remove non-monophyly of *Arnebia* because of the position of *A. purpurea*.

Floral morphology provides other elements supporting the affinity of the latter species to *H. pulchra*, despite the weak external resemblance between the two species. Indeed, *A. purpurea* resembles more CW Asian species such as *A. euchroma* (Royle 1833–1840: 35) Johnston (1924: 49) which have probably led to suppose a relationship between them (Erik & Sümbül 1986). However, the asymmetrical stamen arrangement, already reported in the original description of the species (Erik & Sümbül 1986, see also Davis *et al.* 1988), is of the same type in the two species, though the difference in the height of the two lower stamens and the three higher ones in *A. purpurea* is not as marked as in *H. pulchra* (Fig. 4C,D). In addition, they share the entire style with capitate-bilobed stigma, while in *Arnebia* (incl. *Macrotomia*) the style is frequently 2- or 4-forked (Johnston 1954).

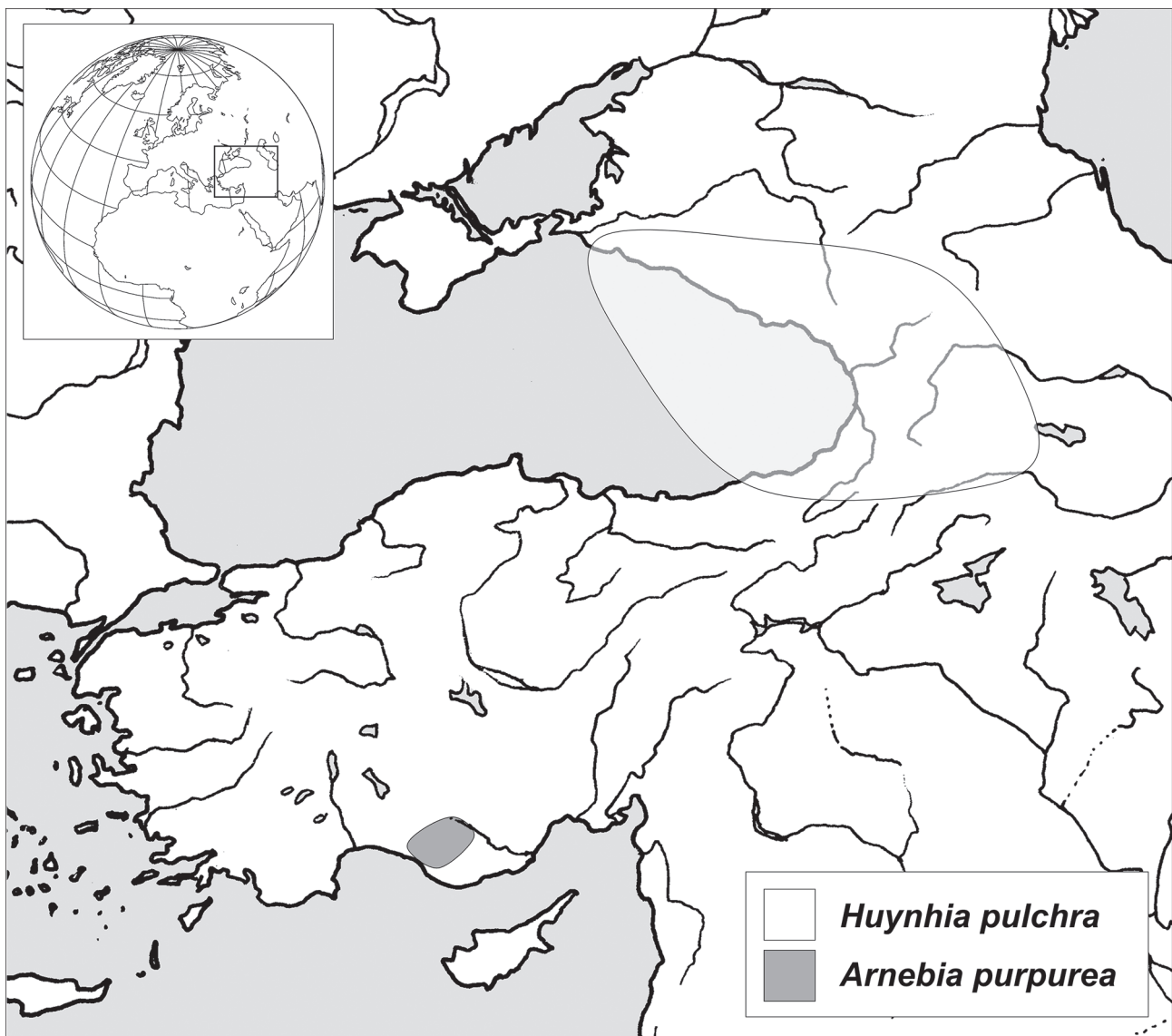


FIGURE 6. Disjunct distribution range of *Huynhia pulchra* and *Arnebia purpurea* in Turkey and the Caucasian area.

Palynomorphology corroborates the molecular findings even more strongly. Our observations confirm the peculiar structure of the grains in *Arnebia* (including *Macrotomia*), as already known from previous investigations (e.g., Johnston 1954; Huynh 1971; Qureshi *et al.* 1989; Perveen *et al.* 1995; Liu *et al.* 2010), and the sharply different traits of *H. pulchra* as described by Johnston (1954) and Huynh (1971), upon which the genus *Huynhia* was based (Greuter 1981). Based on this, Cohen’s (2014: 15) statement that “pollen with 8–12 subequatorial pores is a synapomorphy of the clade

composed of these three genera (*Huynhia*+*Macrotomia*+*Arnebia*)” cannot be supported. In fact, grains of *Arnebia* (incl. *Macrotomia*) can have up to seven ectoapertures, hence up to 14 pores close to the polar regions, while those of *H. pulchra* have nine apertures provided each with a single pore along the equatorial belt (Huynh 1971). Therefore, no clear palynological synapomorphy is shared between *Arnebia*/*Macrotomia* and *Huynhia*. Here, we show that *A. purpurea* is closer to *Huynhia* than to *Arnebia* especially in the presence of a single row of endoapertures along the equatorial belt, though it differs in the number of apertures, 6(7) vs. 9 as in *H. pulchra*. Hence, there is a palynological confirmation of the molecular data, corroborating the phylogenetic signal of pollen characters in Lithospermeae (Johnston 1954; Díez *et al.* 1986; Cecchi & Selvi 2009; Weigend *et al.* 2009; Liu *et al.* 2010; Cohen 2011).

At the present state of knowledge, we believe therefore that *A. purpurea* should be placed in *Huynhia*, to make *Arnebia* (incl. *Macrotomia*) monophyletic. Hence, *Huynhia* is no longer a monotypic genus of Lithospermeae and its distribution range becomes considerably wider to the west and the south of Turkey. In fact, *A. purpurea* is endemic to a narrow area of the central section of the Taurus chain (Ekim 2009), implying a considerable disjunction with respect to *H. pulchra*, which is distributed in NE Anatolia and the Caucasian area (Fig. 6).

It is finally worth to add that our phylogenetic results are primarily based on ITS due to the low resolution power of IGS, and this may not reveal other processes that can be responsible for the homogenization of ITS after natural hybridization events, such as concerted evolution (Fuertes Aguilar *et al.* 1999). Since the *Arnebia*/*Macrotomia* group is not strongly supported, nor is the backbone of the combined ITS-IGS tree uniting it to *Huynhia*, the possibility that further analyses with more or different molecular markers reveal different topologies and retrieve these two lineages as sister clades, or nested into each other, cannot be ruled out. Such scenario would support a wider circumscription of *Arnebia*, including *Huynhia*, as in several former treatments. While at present morphology and molecules clearly indicate that *A. purpurea* is sister to the latter in a separate clade, our phylogenies may not be conclusive concerning the intergeneric relationships in the group. Further studies using additional markers and a wider taxon sampling would be useful to address this issue.

Taxonomy

The placement of *A. purpurea* in the genus *Huynhia* is here proposed:

Huynhia purpurea (Erik & Sümbül) L.Cecchi & Selvi, *comb. nov.*

≡ *Arnebia purpurea* Erik & Sümbül in Notes Roy. Bot. Gard. Edinburgh 44: 151. 1986 (basionym).

Type: TURKEY. C4 Antalya, Gazipaşa: Sugözü köyü, Akçal tepesi, 1900–2000 m, 17 May 1983, *H. Sümbül* 3028 (holotype HUB, isotype E).

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Appendix 1.

List of taxa included in the phylogenetic analysis, with INSDC accession numbers (ITS/IGS; ITS when only one number). Detail collection localities and herbarium vouchers are given for only the taxa originally sequenced here.

INGROUP: *Arnebia benthamii* (Wall. ex G. Don) I.M. Johnst., KF287949/KF288032. *Arnebia coerulea* Schipcz., EU044856. *Arnebia decumbens* (Vent.) Coss. et Kralik: **1**) Syria (*Cecchi, Coppi & Selvi* HB 07.05, FIAF), KJ394991/HG939444; **2**) Tunisia (*Bigazzi & Selvi* HB 04.34, FIAF), EU919579. *Macrotomia densiflora* (Ledeb.) J.F. Macbr., EU919591. *Arnebia euchroma* I.M. Johnst., EF199848/EF199852. *Arnebia guttata* Bunge, EF199862/JX976910. *Arnebia tubata* (Bertol.) Sam.: Syria, 4 km west of Palmyra, subdesertic steppe, 400 m, 34°31.468' N, 38°13.351' E, *Cecchi, Coppi & Selvi* (HB 07.10, FIAF), LN626706/LN626708. *Arnebia linearifolia* DC., EU919580/HG939445. *Arnebia purpurea* S. Erik & Sömbül: **1**) Turkey, Alanya, rocky pastures 1 km NW of the Gökbül village (Gökbül plateau), 1665 m, 36°42.07'N–32°19.00'E, *Cecchi, Selvi et al.* (HB 13.24, FIAF), LN626704/LN626707; **2**) Turkey, Konya, rocky limestone slopes at pass Karahasan between Sariveliler and Taskent, 1800 m, 96°44.91 N–32°38.09' E, *Cecchi, Selvi et al.* (HB 13.38, FIAF), LN626705. *Arnebia szechenyi* Kanitz, EF199863/EF199855. *Alkanna hirsutissima* A.DC., EU919572. *Alkanna orientalis* Boiss., EU919575. *Alkanna tinctoria* (L.) Tausch, FJ763250/FJ763304. *Buglossoides incrassata* (Guss.) I.M. Johnst., FJ763191/FJ763255. *Aegonychon purpureocaeruleum* (L.) Holub, FJ789859. *Aegonychon calabrum* (Ten.) Holub, KJ394986/FJ763305. *Buglossoides tenuiflora* (L.f.) I.M. Johnst., KJ394967. *Cerinth major* L., FJ763244/FJ763298; *Cerinth minor* subsp. *minor* L., FJ763223/FJ763281. *Echium arenarium* Guss., EU919584. *Echium creticum* L., FJ763249/FJ763303. *Echium vulgare* L., FJ763247/FJ763301. *Halacsya sendtneri* Dörf., EU919588. *Huynhia pulchra* (Willd. ex Roem. & Schult.) Greuter & Burdet, FJ763219/FJ763278. *Glandora gastonii* (Benth.) L. *Cecchi et Selvi*, KJ394988. *Lithodora hispidula* (Sm.) Griseb., FJ789865/KF288068. *Lithospermum officinale* L., FJ763189/FJ763254. *Moltkia angustifolia* DC., FJ763252/FJ763306. *Moltkia aurea* Boiss., EU919594. *Moltkiopsis ciliata* (Forssk.) I. M. Johnst., EU919598. *Mairetis microsperma* (Boiss.) I.M. Johnst., EU919592/FJ763257. *Maharanga emodi* DC., FJ763207/FJ763269. *Moltkia petraea* (Tratt.) Griseb., FJ763194/FJ763258. *Moltkia suffruticosa* (L.) Brand subsp. *suffruticosa*, EU919597. *Neatostema apulum* (L.) I.M. Johnst., FJ763198/FJ763262. *Onosma aleppica* Boiss., EU919600. *Onosma visianii* Clem., EU919603. *Onosma fruticosa* Sibth. et Sm., FJ763196/FJ763260; *Paramoltkia doerfleri* (Wettst.) Greuter et Burdet, EU919606. *Podonosma orientalis* (L.) Feinbrun, FJ763253/FJ763307. OUTGROUPS: *Anchusa formosa* Selvi, Bigazzi & Bacch., GQ285226/GQ285251; *Borago officinalis* L., FJ763248. *Echiochilon fruticosum* Desf., AJ555908/FJ763310; *Ogastemma pusillum* (Coss. & Durieu ex Bonnet & Barratte) Brummitt, FJ763201/FJ763265.