

## Molecular Systematics of Boraginaceae Tribe Boragineae Based on ITS1 and *trnL* Sequences, with Special Reference to *Anchusa s.l.*

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Received: 28 April 2003 Returned for revision: 6 August 2003 Accepted: 31 March 2004

- **Background and aims** Boragineae is one of the main tribes of Boraginaceae, but delimitation and intergeneric classification of this group are unclear and have not yet been studied using DNA sequences. In particular, phylogenetic relationships in *Anchusa s.l.* still need to be elucidated in order to assess its taxonomic boundaries with respect to the controversial segregate genera *Hormuzakia*, *Gastrocotyle*, *Phyllocara* and *Cynoglottis*.
- **Methods** Phylogenetic relationships among 51 taxa of tribe Boragineae were investigated by comparative sequencing of the *trnL*<sub>UAA</sub> intron of the plastid genome and of the ITS1 region of the nuclear ribosomal DNA. Exemplar taxa from 16 genera of Boragineae and all subgenera of *Anchusa s.l.* were included, along with two selected outgroups from tribes *Lithospermeae* and *Cynoglosseae*.
- **Key results** Phylogenies generated by maximum parsimony and combined ITS1-*trnL* sequences support the monophyly of the tribe and a split into two clades, *Pentaglottis* and the remainder of Boragineae. The latter contains two large monophyletic groups. The first consists of three moderately to well-supported branches, *Borago-Symphytum*, *Pulmonaria-Nonea* and *Brunnera*. In the *Pulmonaria-Nonea* subclade, the rare endemic *Paraskevia cesatiana* is sister to *Pulmonaria*, and *Nonea* appears to be paraphyletic with respect to *Elizaldia*. The second main group corresponds to the well-supported clade of *Anchusa s.l.*, with the megaphyllic, polyploid herb *Trachystemon orientalis* as sister taxon, although with low support. *Anchusa s.l.* is highly paraphyletic to its segregate genera and falls into four subclades: (1) *Phyllocara*, *Hormuzakia*, *Anchusa* subgenus *Buglossum* and *A.* subgenus *Buglossoides*; (2) *Gastrocotyle*; (3) *A.* subgenus *Buglossellum* and *Cynoglottis*; and (4) *A.* subgenus *Anchusa*, *Lycopsis* and *Anchusella*. All species of *Anchusa* subg. *Anchusa*, including the South African *A. capensis*, are included in a single unresolved clade. *Anchusa* subgenus *Limbata* is also included here despite marked divergence in floral morphology. The low nucleotide variation of ITS1 suggests a recent partly adaptive radiation within this group.
- **Conclusions** Molecular data show that nine of the usually accepted genera of the Boragineae consisting of two or more species are monophyletic: *Anchusella*, *Borago*, *Brunnera*, *Cynoglottis*, *Gastrocotyle*, *Hormuzakia*, *Nonea*, *Pulmonaria* and *Symphytum*. In addition, the tribe includes the four monotypic genera *Paraskevia*, *Pentaglottis*, *Phyllocara* and *Trachystemon*. The morphologically well-characterized segregate genera in *Anchusa s.l.* are all confirmed by DNA sequences and should be definitively accepted. Most of the traditionally recognized subgenera of *Anchusa* are also supported as monophyletic groups by both nuclear and plastid sequence data. In order to bring taxonomy in line with phylogeny, the institution of new, independent generic entities for subgenera *Buglossum*, *Buglossellum* and *Buglossoides* and a narrower but more natural concept of *Anchusa* are advocated.

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**Key words:** *Anchusa*, *Boraginaceae*, *Boragineae*, ITS1, molecular systematics, phylogeny, taxonomy, *trnL*.

### INTRODUCTION

Boragineae Bercht. & J.Presl (=Anchuseae DC.) is one of the major tribes (approx. 170 species) of Boraginaceae Juss. s.s. (approx. 2000 species), a family of the euasterid I clade (Angiosperm Phylogeny Group II, 2003) recently been shown to be paraphyletic, if defined in the traditional broad sense (e.g. *sensu* Gürke, 1893). Hydrophyllaceae R.Br. ex Edwards are sister to a clade formed by Ehretiaceae Mart. ex Lindl. (including Lennoaceae Solms), Cordiaceae R.Br. ex Dumort. and Heliotropiaceae Schrad. (Böhle and Hilger, 1997; Ferguson, 1999; Gottschling *et al.*, 2001, Diane *et al.*, 2002) with all except Lennoaceae usually included as subfamilies in the Boraginaceae s.l. Relationships within and between the tribes of Boraginaceae s.s. are still not well understood, mainly due to

insufficient sampling of many groups in the phylogenetic analyses so far published (e.g. Långström and Chase, 2002).

Boragineae are morphologically well characterized by faucal corolla appendages (=fornices) and by strophiolate mericarpid attached basally on a planar gynobase. These mericarpid show a more or less thickened basal annulus surrounding a plug-like scar and usually have an elaiosome for ant dispersal. The tribe is native to the Old World only and has its major centre of diversity in the Mediterranean basin and adjacent Western Asia, with only two species of *Anchusa* subgenus *Anchusa* ranging into Eastern subtropical Africa and the Cape region.

Johnston (1924) and Riedl (1963) regarded Boragineae as a natural group possibly originated from Lithospermeae, with Eritrichieae/Cynoglosseae representing the 'neighbouring' lineage. Recent studies of Boraginaceae based on ribosomal ITS1 and plastid *atpB* sequences (Gottschling

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et al., 2001; Långström and Chase, 2002) have corroborated this view, although taxon sampling in both analyses was not sufficient to reliably demonstrate the monophyly of the tribe.

Boragineae have been subject to conflicting treatments with regard to their circumscription and number of genera recognized depending on the weight attributed by the different authors to fruit and floral characters. In the early systems based entirely on mericarpid morphology and attachment (De Candolle, 1846; Bentham, 1876; Gürke, 1893) nine genera were recognized, including the genus *Alkanna* Tausch. Johnston (1924) accepted 12 genera, including *Lithodora* Griseb., but excluding *Alkanna*, whereas Melchior (1964) adopted a 'lumping' approach and reduced the tribe to eight genera. Steps towards an apparently more natural treatment were made by Guşuleac (1923, 1928), the most important monographer of the tribe, who reduced Boragineae to those taxa with faucal appendages in the corolla, moving *Lithodora* and *Alkanna* to Lithospermeae. In his system, the tribe consisted of 11 genera, with *Elizaldia* Willk. reduced to synonymy in *Nonea* Medik. Guşuleac's treatment was largely followed by Riedl (1963), who recognized 13 genera with a narrowly defined *Anchusa* L. and *Elizaldia* separate from *Nonea*.

To date, however, there is still uncertainty concerning the number and delimitation of the genera of Boragineae, mainly because little is known about the phylogenetic relationships in the tribe. While some of these genera are distinctive (e.g. *Borago* L., *Symphytum* L., *Brunnera* Steven and *Pentaglottis* Tausch), others have been historically controversial because of a weaker morphological characterization and reticulate patterns of variation.

*Anchusa s.l.* is the genus that has been subjected to the most variable treatments. Understood in a broad sense by most early authors, it was shown by Guşuleac to include distinct lineages that he regarded as separate genera. Guşuleac's exhaustive morphological studies (Guşuleac, 1927, 1928, 1929) resulted in the well-supported separation at genus level of species originally described under *Anchusa*, such as *Phyllocara aucheri* (DC.) Guşul., *Hormuzakia aggregata* (Lehm.) Guşul., *Gastrocotyle hispida* (Forssk.) Bunge and *Pentaglottis sempervirens* (L.) L.H. Bailey. He further subdivided the remainder of *Anchusa* (*Anchusa s.s.*) into six subgenera *Cynoglottis*, *Lycopsis*, *Buglossum*, *Buglossellum*, *Buglossoides* and (*Eu*) *Anchusa*. This elaborate system, however, was not followed by most later authors of flora treatments, who continued to adopt a traditionally broad concept of *Anchusa* [e.g. Chater, 1972 (*Fl. Europaea*); Chamberlain, 1979 (*Fl. Turkey*); Greuter et al., 1984 (*Med-Checklist*)].

Recent studies based on micromorphology, palynology and karyology (Bigazzi and Selvi, 1998, 2000, 2001; Bigazzi et al., 1999) have provided evidence for a narrow concept of *Anchusa* and widely supported Guşuleac's generic treatment. Furthermore, evidence has been provided for also maintaining *Cynoglottis* (Guşul.) Vural & Kit Tan and *Anchusella* Bigazzi, Nardi & Selvi, the latter originally described as *Anchusa* subgenus *Rivinia* Greuter (Greuter, 1965), as separate genera in view of their apomorphic features in reproductive structures (Vural and Tan, 1983; Bigazzi et al., 1997).

A second phylogenetically poorly known and controversial lineage is represented by *Pulmonaria* and *Nonea*, two apparently related genera each with numerous species. To this lineage belongs the enigmatic species *Paraskevia cesatiana* (Fenzl. & Friedr.) W. Sauer & G. Sauer (Sauer and Sauer, 1980), originally described as *Anchusa cesatiana* (von Friedrichsthal, 1838), but later variously combined under *Pulmonaria* (Boissier, 1879) and *Nonea* (Boissier, 1849; Guşuleac, 1929; Greuter, 1981). *Nonea* also has close relationships with the North African genus *Elizaldia*, to which it is probably paraphyletic as suggested by a recent morphological analysis (Selvi et al., 2002).

In this paper, an overview of the phylogenetics of Boragineae, as inferred from DNA sequences from both plastid and nuclear non-coding regions, is provided. Special emphasis is applied to *Anchusa s.l.*, which has been the focus of our previous studies and for which a near-comprehensive sampling from its taxonomic and geographic range was possible. The combined use of two different markers with different evolutionary speed, the internal transcribed spacer region (ITS1) of nuclear ribosomal DNA and the more conserved *trnL*<sub>(UAA)</sub> intron of the plastid genome is appropriate for investigations of relationships between species and genera. The usefulness of both markers in the systematics of Boraginales has been shown in recent phylogenetic studies at different taxonomic levels (Böhle and Hilger, 1997; Gottschling and Hilger, 2001; Diane et al., 2002; Winkworth et al., 2002).

## MATERIALS AND METHODS

### *Plant material*

Silica gel preserved samples of leaf tissue from field collections or, in a few cases, from herbarium specimens were used for DNA extraction. The ingroup comprised 51 taxa (Table 1). *Buglossoides arvensis* and *Cynoglossum amplifolium*, of tribes Lithospermeae and Cynoglosseae, respectively, served as outgroups.

### *DNA extraction, marker amplification and sequencing*

The *trnL* primers (c and d) were those used by Taberlet et al. (1991), the ITS primers (P1 and P2) were those of Baldwin (1992). Genomic DNA was isolated using a modified 2 × CTAB extraction protocol [Doyle and Doyle, 1990; tissue ground in sea sand, 70 % (v/v) isopropanol substituted for the RNase step]. Approximately 40 mg of leaf tissue was used for each extraction. The DNA was amplified with Gibco BRL PCR kits. PCR products were cleaned with Qiagen QIAquick PCR purification columns, quantified with a 100 bp DNA ladder (MBI-Fermentas, St Leon-Rot, Germany), and cycle-sequenced with a GeneAmp PCR-System 2400 (Perkin Elmer, Boston, MA, USA). A Sequi-ThermExcel II sequencing kit (Epicentre Technologies, Madison, USA) was used with a stop-/loading-solution for terminating. Sequences were run on a GATC model 1500. Polyacrylamide gels were prepared using Sequa-Gel-6 (National Diagnostics, Atlanta, GA, USA). The biotinylated PCR products were transferred onto a Biotryne A

TABLE 1. List of taxa investigated with internal DNA number, origin and voucher specimens, and GenBank accession

Taxa	DNA no.	Origin and voucher**	GenBank accession <i>trnL</i> /ITS1
<i>Anchusa aegyptiaca</i> (L.) DC.	695	Cyprus: Hilger 00/1 (BSB)	AY383255/AY383294
* <i>Anchusa affinis</i> R.Br.	1037	Saudi Arabia: Lavranos & Collenette 18390 (FI)	AY383279/–
<i>Anchusa azurea</i> Mill.	619	Cyprus: Hilger 00/18 (BSB)	AY383254/AY383293
<i>Anchusa capellii</i> Moris	780	Italy, Sardinia: Bigazzi & Selvi 99.002 (FI) BSB	AY383257/AY383297
<i>Anchusa capensis</i> Thunb.	990	South Africa: Orange Free State (FI)	AY383269/AY383311
<i>Anchusa cespitosa</i> Lam.	287	Greece, Crete: Hilger 98/11 (FI, BSB)	AY383268/AY383310
<i>Anchusa crispa</i> Viv. ssp. <i>crispa</i>	791	France, Corsica: Bigazzi & Selvi 99.005 (FI)	AF530603/AY071853
<i>Anchusa formosa</i> Selvi, Bigazzi & Bacchetta	781	Italy, Sardinia: Bigazzi & Selvi 97.006 (FI)	AY383258/AY383299
<i>Anchusa leptophylla</i> Roem. & Schult.	633	Turkey: Carle & Kürschner 4032 (BSB)	AF530604/AY383298
<i>Anchusa leucantha</i> Selvi & Bigazzi	862	Greece: Bigazzi & Selvi 01.17 (FI, BSB)	AY383267/AY383309
<i>Anchusa limbata</i> Boiss. & Heldr.	1158	Turkey: Bigazzi & Selvi 02.01 (FI, BSB)	AY383260/AY383301
<i>Anchusa milleri</i> Sprengel	623	Israel: Hilger 21/94. (FI, BSB)	AY383256/AY383295
<i>Anchusa ochroleuca</i> M.Bieb.	373	Bulgaria: Hilger 97/21 offspring (BSB)	AY383261/AY383302
<i>Anchusa officinalis</i> L.	672	Germany: Hilger 2000 (BSB)	AY045703/AY045710
<i>Anchusa pusilla</i> Guşul.	727	Turkey: Bigazzi & Selvi 97.018 (FI)	AY383262/AY383303
<i>Anchusa samothracica</i> Bigazzi & Selvi	811	Greece: Bigazzi & Selvi 99.016 (FI, BSB)	AY383262/AY383303
<i>Anchusa strigosa</i> Banks. & Sol.	618	Cyprus: Hilger 00/16 (BSB)	AY383253/AY383292
<i>Anchusa stylosa</i> M. Bieb.	861	Greece: Bigazzi & Selvi 01.13 (FI, BSB)	AY383266/AY383308
<i>Anchusa thessala</i> Boiss. & Spruner	730	Turkey: Bigazzi & Selvi 97.022 (FI, BSB)	AF530599/AY383296
<i>Anchusa undulata</i> L. ssp. <i>hybrida</i> (Ten.) Bég.	616	Cyprus: Hilger 00/12 (BSB)	AY383259/AY383300
<i>Anchusella cretica</i> (Mill.) Bigazzi, Nardi & Selvi	667	Italy: Bigazzi & Selvi 00.33 (FI)	AY045709/AY045716
<i>Anchusella variegata</i> (L.) Bigazzi, Nardi & Selvi	857	Greece: Bigazzi & Selvi 01.10 (FI)	AY383265/AY383306
<i>Borago officinalis</i> L.	671	Germany (Berlin cult.): Hilger (BSB)	AY383245/AY383283
<i>Borago pygmaea</i> (DC.) Chater & Greuter	375	Germany (cult. H.Berlin-Dahlem): Hilger	AY383244/AY383282
<i>Brunnera macrophylla</i> (Adams) I.M.Johnst.	628	Germany (cult. H.Berlin-Dahlem): Hilger	AY383249/AY383288
<i>Brunnera orientalis</i> (Schenk) I.M.Johnst.	829	Turkey: Bigazzi & Selvi 00.28 (FI)	AY383250/AY383289
<i>Buglossoides arvensis</i> (L.) I.M.Johnst.	662	Greece, Crete: Kagiampaki 4/2000 (BSB)	AY383242/AY383280
<i>Cynoglossum amplifolium</i> A.DC.	645	Kenya: Fischer 5/2000 (BSB, FI)	AY383243/AY383281
<i>Cynoglottis barrelieri</i> (All.) Vural & Kit Tan	669	Italy: Bigazzi & Selvi 99.019 (FI)	AY045708/AY045715
<i>Cynoglottis cheikiana</i> Vural & Kit Tan ssp. <i>paphalagonica</i> (Bornm.) Vural & Kit Tan	830	Turkey: Bigazzi & Selvi 98.008 (FI, BSB)	AF530602/AY383307
<i>Elizaldia calycina</i> (Roem. & Schult.) Maire ssp. <i>multicolor</i> (Kunze) Chater	706	Morocco: Lewalle 10884 (RNG)	AY383264/AY383305
<i>Gastrocotyle hispida</i> (Forssk.) Bunge	674	Jordan: Baierle & al. 17.3.86 (BSB)	AY045705/AY045712
<i>Gastrocotyle macedonica</i> (Degen & Dörf.) Bigazzi, Hilger & Selvi	682	Greece: Bigazzi & Selvi 99.009 (FI, BSB)	AY045706/AY045713
<i>Hormuzakia aggregata</i> (Lehm.) Guşul.	693	Israel: Bigazzi & Selvi 96.015 (FI)	AY383252/AY383291
* <i>Hormuzakia negevensis</i> (Danin) Danin & Hilger	664	Israel: Danin 24.3.97 (HUJ)	AY383278/–
<i>Lycopsis arvensis</i> L.	624	Germany: Hilger & Werres 27.5.99 (BSB)	AY045707/AY045714
<i>Lycopsis orientalis</i> L.	831	Turkey: Bigazzi & Selvi 00.10 (FI)	AY383277/AY383319
<i>Nonea lutea</i> (Desr.) DC.	630	Germany (cult. H. Berlin-Dahlem): Hilger (BSB)	AY383274/AY383316
<i>Nonea pulla</i> DC.	661	Germany: Hensen 28.5.00 (BSB)	AY383275/AY383317
<i>Nonea vesicaria</i> (L.) Reichb.	1311	Morocco: Podlech 51525 (ITS1, M)	–/AY383304
“..”	1252	Sicily: Bigazzi & Selvi 97.038 ( <i>trnL</i> , FI)	AY383263/–
<i>Paraskevia cesatiana</i> (Fenzl & Friedr.) W. Sauer & G. Sauer	859	Greece: Bigazzi & Selvi 01.02 (FI, BSB)	AY383276/AY383318
<i>Pentaglottis sempervirens</i> (L.) L.H.Bailey	668	Italy (cult.): Bigazzi & Selvi	AF530598/AY383286
<i>Phyllocara aucheri</i> (DC.) Guşul.	670	Turkey: Bigazzi & Selvi 97.041 (FI)	AY383251/AY383290
<i>Pulmonaria angustifolia</i> L.	744	Italy: Frey 2000 (BSB)	AY383271/AY383313
<i>Pulmonaria mollis</i> Wulf.	755	Germany (cult.): Hilger (BSB)	AY383273/AY383315
<i>Pulmonaria obscura</i> Dumort.	681	Germany (cult.): Hilger (BSB)	AY383270/AY383312
<i>Pulmonaria picta</i> Rouy	761	Italy: Bigazzi & Selvi 91.002 (FI)	AY383272/AY383314
<i>Symphytum creticum</i> (Willd.) Greuter & Rech.fil.	284	Greece, Crete: Hilger (BSB)	AY383246/AY383284
<i>Symphytum tuberosum</i> L.	629	Germany (cult. H. Berlin-Dahlem): Hilger	AY383247/AY383285
<i>Trachystemon orientalis</i> (L.) G.Don	666	Turkey: Bigazzi & Selvi 00.06 (FI)	AY383248/AY383287

\* Those taxa which were included only in the *trnL* analysis.

\*\* BSB = Herbarium, Institut für Biologie - Systematische Botanik und Pflanzengeographie, Freie Universität, Berlin; FI = Herbarium Universitatis Florentinae, Museo di Storia Naturale, Università di Firenze; RNG = Herbarium, Plant Science Laboratories, University of Reading; M = Herbarium, Botanische Staatssammlung, München; HUJ = Herbarium, Department of Evolution, Systematics and Ecology, Hebrew University, Jerusalem.

nylon membrane (Pall Filtron, Dreieich, Germany) and visualized by streptavidine/basic phosphatase.

#### Sequence alignment and phylogenetic analysis

Sequences were manually aligned with Align32 (Hepperle, 2001). Sequences are deposited in GenBank

(Table 1). Parsimony analysis was performed with PAUP 4.0b1 for PC (Swofford, 1998). A heuristic search analysis was run with ‘tree-bisection-reconnection’ (TBR) branch-swapping with accelerated transformation (ACCTRAN) optimization to infer branch lengths; MULTREES option on, ADDSEQ = random, ten randomized replicates. All

characters were weighted equally, and character state transitions were treated as unordered. Gaps were coded as separate characters according to the 'simple gap coding' method after Simmons and Ochoterena (2000). The bootstrap (BS) (Felsenstein, 1985) and jackknife (Farris *et al.*, 1996) were performed with 100 replicates (TBR branch-swapping, ten random taxon entries per replicate and MULTREES on); search = FASTSTEP and 10 000 replicates were used in *trnL* analysis because of computational time limitations.

## RESULTS

### Analysis of the *trnL* region

The aligned *trnL* data set (available from the authors upon request) is 469 bp in length with sequences varying from 419 base pairs (bp) (*Symphytum creticum*) to 460 bp (*Anchusa strigosa* and *A. azurea*). In the phylogenetic analysis, 42 sites were parsimony informative, 47 were uninformative and 380 were constant. Analysis of the *trnL* resolved the position of *Hormuzakia negevensis* and *Anchusa affinis*, two species of which ITS1 sequences were not available.

The heuristic search yielded 3380 most-parsimonious trees of tree length (L) = 134, consistency index (CI) = 0.813 and retention index (RI) = 0.903. The strict consensus tree is shown in Fig. 1. The monophyly of Boragineae is corroborated by 78 % BS and 71 % jackknife support. The ingroup falls into eight clades, the relationships of which remain unresolved: (1) *Pentaglottis*, (2) *Trachystemon*, (3) *Borago*, (4) *Symphytum*, (5) *Brunnera*, (6) *Phyllocara*, *Hormuzakia*, *Anchusa* subgenus *Buglossum* and subgenus *Buglossoides*, (7) *Gastrocotyle*, *Anchusella*, *Cynoglottis*, *Lycopsis*, *Anchusa* subgenus *Buglossellum*, subgenus *Anchusa* and subgenus *Limbata*, (8) *Nonea*, *Elizaldia*, *Pulmonaria* and *Paraskevia*. Most of these clades can already be recognized in a condensed alignment (outgroups, identical and non-informative positions removed) which shows the insertions/deletions (indels) (Fig. 2).

The monophyly of *Borago* (clade 3) is supported by 99 % BS and jackknife support; *B. pygmaea* and *B. officinalis* share at least six unique indels or substitutions. In clade 4 (99 % BS support), *S. creticum* forms a group with *S. tuberosum*. The relationship between *Borago* and *Symphytum* was not seen in the consensus tree, but it received BS/jackknife support of 59 % and 56 %, respectively. A shared deletion in position 286–288 characterizes the species of these two genera. Monophyly of *Brunnera* (clade 5) is supported by 94 % BS; a shared deletion at position 110–111 occurs in the two species of this genus (*B. macrophylla* and *B. orientalis*).

*Anchusa* in a broad sense is subdivided into two clades (6 and 7). One (clade 6) received BS 79 % and 76 % jackknife, and the other (clade 7) 70 % and 61 %, respectively. Both share an insertion at position 272–285 with the exception of *Phyllocara*, *Hormuzakia*, and *Anchusa* subgenus *Buglossoides*. In clade 6, the relationships between *Phyllocara*, *Anchusa* subgenus *Buglossum*, *Hormuzakia* plus *A. subgenus Buglossoides* remained unresolved. The clade with the two latter taxa received 62 % BS, as did

*A. subgenus Buglossoides* itself. Clade 7 was also supported by a shared deletion at position 85–88. The *Gastrocotyle* clade (77 % BS and 70 % jackknife) is sister to the remainder of clade 7, which is weakly supported (52 % BS). Within the latter, *Lycopsis arvensis* is sister to the terminal clade of *Anchusa* subgenus *Anchusa* (56 % BS) which also includes *Anchusella variegata*, *Anchusa* subgenus *Limbata* and the two African species *A. capensis* and *A. affinis*. All together, these taxa form a clade (82 % BS, 70 % jackknife) whose position is not resolved with respect to *Cynoglottis*, *Anchusa* subgenus *Buglossellum*, *Anchusella cretica* and *Lycopsis orientalis*. Relationships among the latter taxa also remain unresolved.

Clade 8, formed by *Pulmonaria*, *Nonea*, *Elizaldia* and *Paraskevia*, is weakly supported (57 % BS and 51 % jackknife), but a deletion at position 272–285 is shared by all the taxa of this clade. A close relationship is revealed between *Elizaldia calycina* and *Nonea vesicaria* (80 % BS and 66 % jackknife). This suggests paraphyly of *Nonea* relative to *Elizaldia*. The *trnL* sequences did not resolve the position of *Paraskevia* with respect to *Nonea pulla* and *Pulmonaria*. The monophyly of *Pulmonaria* is supported by 83 % BS and 75 % jackknife.

### Analysis of the ITS1 region

The topology of the trees based on ITS1 was almost identical to that resulting from the combined ITS1–*trnL* analysis (with lower resolution and support to the clades), and is therefore not presented or discussed separately. The position numbers in the next section refer to the positions in ITS1.

### Combined ITS1–*trnL* analysis

The combined ITS1–*trnL* data set (ITS1 positions 1–295, *trnL* 296–764, alignment available from the authors upon request) is 764 bp in length, with ITS1 sequences varying from 270 bp (*Borago pygmaea*, *Phyllocara aucheri* and *Anchusa thessala*) to 277 bp (*Pentaglottis sempervirens*). In the phylogenetic analysis, 183 sites were parsimony informative, 97 were uninformative and 484 were constant. As expected, ITS1 sequences are more variable than the *trnL* intron and thus gave a better resolution in part; however, the two markers gave substantially congruent trees, with the exception of the positions of *Anchusella cretica* and *Nonea pulla* (see below).

The heuristic search found six most-parsimonious trees, L = 740, CI = 0.605 and RI = 0.787, one of which is shown in Fig. 3. Boragineae are relatively weakly supported as a monophyletic group with 79 % BS and jackknife support. No shared indels for the whole ingroup were found in the ITS1, but some indels characterize the ingroup plus *Cynoglossum amplifolium* or the ingroup plus *Buglossoides arvensis*.

Nine major clades can be recognized (A–I). *Pentaglottis* is sister to the remainder of the tribe, which is then divided into two clades, A and B (though with BS and jackknife support <50 %). Clade A consists of three moderately to strongly supported branches: *Borago* plus *Symphytum* (C), *Brunnera* (D) and *Nonea/Elizaldia/Paraskevia/Pulmonaria*

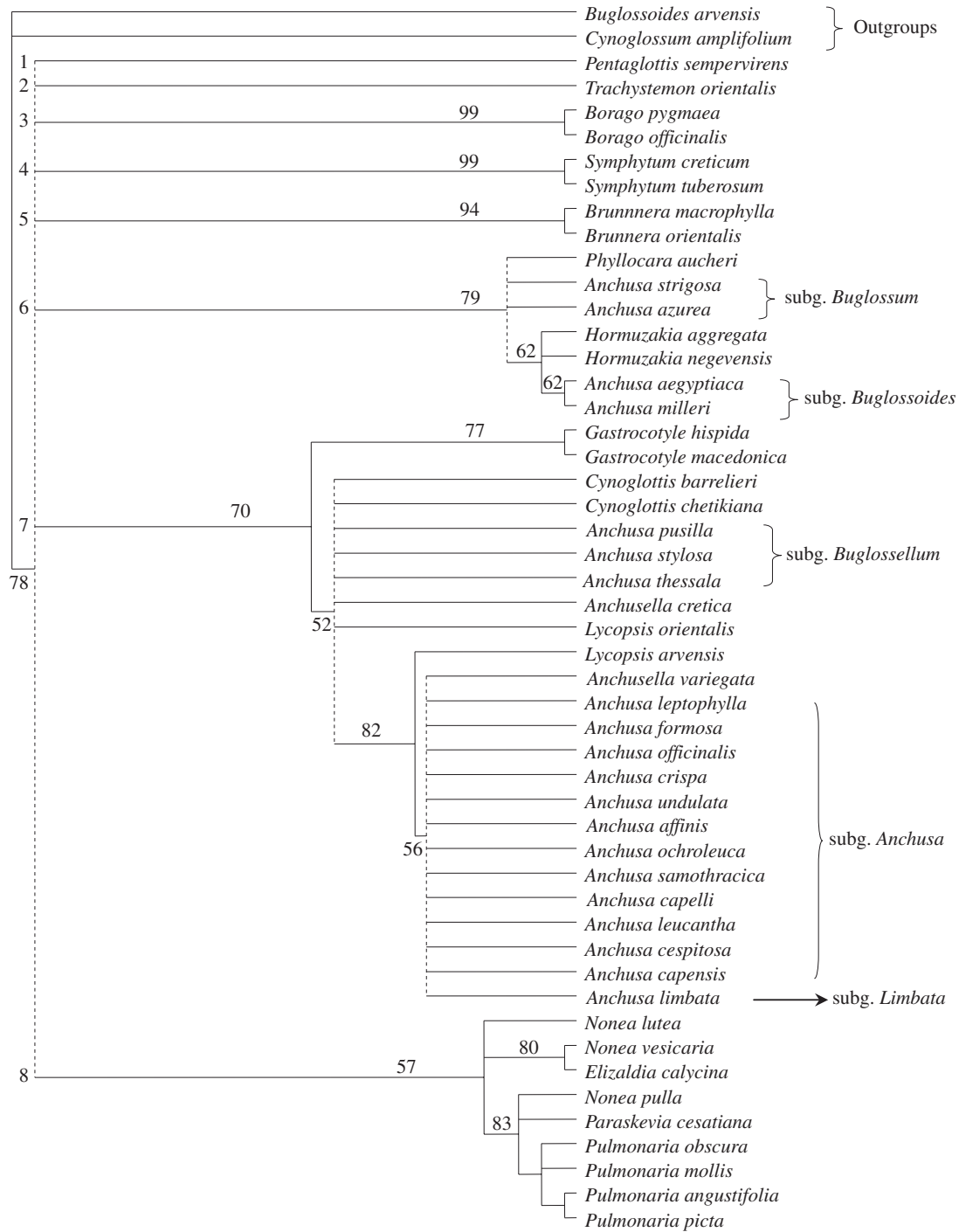


FIG. 1. Strict consensus of the 3380 most-parsimonious trees from *trnL* sequence data. Tree length (L) = 134, consistency index (CI) = 0.813, retention index (RI) = 0.903. The subgenera of *Anchusa s.l.* are indicated. The numbers above the branches are bootstrap percentages (percentages <50 % are not shown). Main polytomies are indicated by dotted lines and the main unresolved clades are numbered (1–8).

(E). *Brunnera* is sister to clades C and E but BS support for these two nodes is <50. Within clade C (73 % BS and 70 % jackknife), *Borago* and *Symphytum* are both supported by 100 % BS and jackknife. The monophyly of *Borago* is also supported by deletions at positions 83 and 120. A deletion at position 43 is restricted to clade E. Two points emerge in clade E. Firstly, *Elizaldia* is nested in *Nonea*, with which it

shares a 1 bp substitution at position 111, and the close relationship between *E. calycina* and *N. vesicaria* is confirmed by a shared insertion in ITS1 at position 101 (96 % BS and 94 % jackknife). In contrast to *trnL* alone, *N. pulla* is here part of the weakly supported *Nonea* clade. Secondly, *Paraskevia* is sister to *Pulmonaria* (84 % BS and jackknife). The monophyly of *Pulmonaria* is supported by ITS1



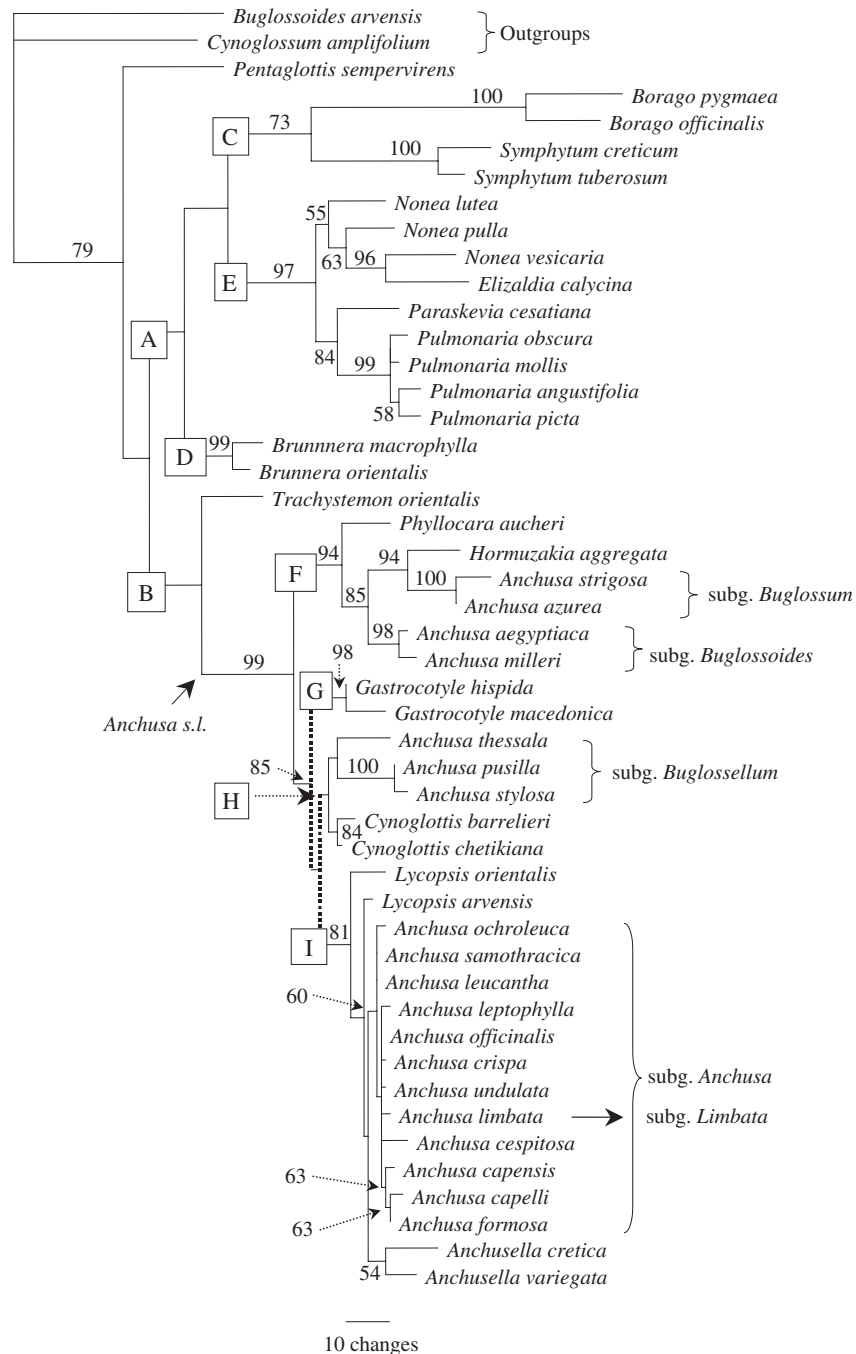


FIG. 3. One of the six most-parsimonious trees from ITS1-*trnL* sequences.  $L = 740$ ,  $CI = 0.605$ ,  $RI = 0.787$ . The subgenera of *Anchusa s.l.* are indicated. Letters on the branches indicate the main clades discussed in the text; numbers indicate bootstrap percentages (percentages < 50 % are not shown). Branch lengths are estimated under ACCTRAN. The branches collapsing in the strict consensus tree (not shown) are indicated by dotted lines.

insertions at positions 72–73 and 174, and by 99 % BS and jackknife.

Within clade B (62 % BS and 63 % jackknife) *Trachystemon* is sister to the main lineage of *Anchusa s.l.*, the monophyly of which is strongly supported (99 % both indices and a shared deletion at position 50 of ITS1). ITS1 provides better resolution in this large group than *trnL*. A key point is that the genus *Anchusa*, even when

intended in a strict sense, is grossly paraphyletic. In fact, all of its subgenera (except A. subgenus *Limbata*) are sister groups of well-established genera rather than to *Anchusa* subgenus *Anchusa*. Within clade F (94 % BS and jackknife), the monotypic genus *Phyllocara* is sister to the three subclades of *Hormuzakia*, *Anchusa* subgenus *Buglossum* (*A. strigosa* and *A. azurea*, 100 %, both indices), and *Anchusa* subgenus *Buglossoides* (*A. aegyptiaca* and *A. milleri*, 98 %

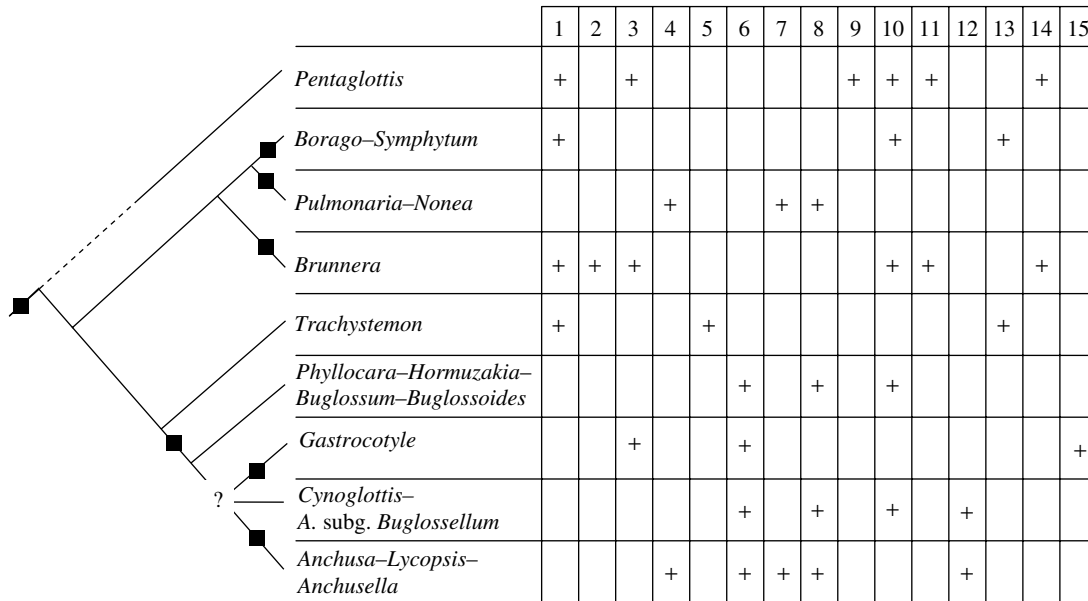


FIG. 4. Distribution of 15 morphological characters plotted onto the nine major clades of ITS1-*trnL* phylogeny of the Boragineae. A square on the clade indicates bootstrap support >85%. A cross indicates that all or the majority of the taxa of the clade possess the morphological character. Habit: (1) only perennial. Inflorescence and flower: (2) cymes always ebracteate; (3) corolla brachymorphic (with short tube and rotate to subrotate limb); (4) zygomorphy present in some taxa; (5) corolla scales in two series; (6) granular thickenings of cutin on the trichomes of the faucal scales; (7) stylar polymorphism present in some taxa; (8) stigma papillae with typical lageniform shape. Fruit: (9) mericarps excentrically stalked at the base; (10) mericarps erect or suberect in all or some of the taxa. Pollen: (11) grains small ( $P < 15 \mu\text{m}$ ); (12) grains with prolate shape; (13) mesocolpia with supratectum gemmae; (14) grains with (3)-4-(5) apertures; (15) apertures rhomboidal with conical processes on the colpus membrane.

both indices). Clade F is sister to the three clades G, H and I (85 % BS). Clade G (98 % both support indices) corresponds to *Gastrocotyle*, and is sister to clades H and I, though this relationship is not confirmed in the strict consensus tree (not shown). Clade H (51 % BS) comprises the two subclades of *Anchusa* subgenus *Buglossellum* (*A. thesala*, *A. stylosa* and *A. pusilla*) and *Cynoglottis* (84 % BS, 73 % jackknife). *Anchusa stylosa* and *A. pusilla* share an ITS1 deletion in position 132 and cluster together with 100 % BS and jackknife support. Clade I (81 % BS and 76 % jackknife) is formed by the two species of *Lycopsis* (not forming a clade), *Anchusella* plus *Anchusa* subgenus *Limbata* and all the taxa of *Anchusa* subgenus *Anchusa*. In contrast to *trnL* alone, *Anchusella* is monophyletic, though with low support, and sister to the rest of clade I, comprising all the taxa of subgenus *Anchusa* plus subgenus *Limbata* (60 % BS and 56 % jackknife). Species level relationships within this group remain largely unresolved. The Sardinian endemics *A. capellii* and *A. formosa* form a moderately supported (63 % BS) terminal clade sister to the South African *A. capensis*.

## DISCUSSION

### Infratribal relationships

Both nuclear and plastid DNA markers used in this analysis support the monophyly of Boragineae. Our results are largely congruent with the views of Johnston (1924), Guşuleac (1923, 1928) and Popov (1953: 207), who regarded the tribe as a ‘natural’ group of ‘ancient Mediterranean’

origin. No discrepancy occurs with respect to more recent studies based on ITS1 (Gottschling *et al.*, 2001) and plastid *atpB* (Långström and Chase, 2002) that focused on higher taxonomic levels of Boraginales.

To facilitate discussion, the backbone of the trees for Boragineae has been summarized and the distribution of 15 systematically relevant morphological characters plotted onto the nine major clades found in the ITS1-*trnL* analysis (Fig. 4).

The resolution of the deepest nodes of the phylogeny of the tribe remain poorly supported. In the *trnL* tree, eight main clades form an unresolved polytomy, whereas in the ITS1-*trnL* tree *Pentaglottis sempervirens* is sister to the remainder of the tribe with low support (BS and jackknife <50 %). In the Boragineae subtree from *atpB* sequences published by Långström and Chase (2002), *Pentaglottis* clusters with *Nonea* in a clade whose sister group is *Anchusa*, whereas *Borago* is sister to the remainder of the tribe.

*Pentaglottis sempervirens* ( $\equiv$  *Caryolopha sempervirens*). This is the only member of the tribe native to the Atlantic region of south-west Europe. It possesses autoapomorphies in fruit morphology, such as excentrically stalked mericarps, and in the form of the stigma, with a subconical receptive surface with densely crowded, granulose papillae (Guşuleac, 1928; Bigazzi and Selvi, 2000). Because of this and its unique karyotype of  $2n = 22$  small chromosomes, *Pentaglottis* had already been suggested to take an isolated position in Boragineae (Britton, 1951). Guşuleac (1928: 403) spoke of a ‘very ancient *Brunnera*–*Caryolopha* (*Pentaglottis*) type, which survives only in these two



genera'. The present results would support an isolated position for *Pentaglottis*, despite low BS support in the combined analysis.

The ITS1–*trnL* analysis indicates an early split of the tribe into two main lineages, the first (A) with three subclades (C, D and E), and the second (B) corresponding to *Anchusa s.l.* plus the monotypic genus *Trachystemon* (62 % BS). With the exception of *Nonea* and *Elizaldia*, the taxa of clade A are mainly perennial and mesophytic, and some of them are restricted to humid forest habitats of Pleistocene refugial areas (e.g. *Brunnera*, *Borago pygmaea*). On the contrary, xerophytism, less marked habitat specificity and annual growth are widespread in the taxa of clade B (except *Trachystemon*).

**Borago–Symphytum (C).** The relationship between these two genera is supported by 73 % BS assigned to clade C and by a 2-bp deletion in the *trnL* sequence. Morphologically, *Borago* and *Symphytum* share significant features, such as the (8)9–10(11)-aperturate pollen grains with a gemmate tectum, and the stigmatic receptive surface with skittle-like papillae, lacking the elaborated apical disk of most other Boragineae (Bigazzi and Selvi, 1998, 2000). Ecologically, predominance of mesophytism is another distinguishing aspect of this clade and supports a common ancestry of the two genera. On the other hand, numerous characters separate them in inflorescence, flower and fruit morphology. Among these, in *Symphytum* the cymes are ebracteate and the corolla is almost tubular with elongated, triangular scales, while in *Borago* cymes are bracteate and the corolla is rotate to campanulate with short scales. The latter is further characterized by pollen with branched columellae and thick exine, and there are two autapomorphic 1 bp deletions in the ITS1 sequence. The two genera are allopatric (except the widespread weed *Borago officinalis*), *Symphytum* being mainly south-east European–western Asiatic and *Borago* south-west Mediterranean. The support for the relationship between these two genera, though moderate, is in line with Guşuleac's opinion (Guşuleac, 1928) of a 'Paleoborago' ancestor shared by *Borago*, *Symphytum* and *Procopiana*. The latter genus was instituted by the same author to accommodate *S. creticum* (*Procopiana cretica*), a south Aegean species with floral morphology intermediate between *Borago* and *Symphytum* due to the corolla lobes being longer than the tube and the exerted stamens. In more recent times, *Procopiana* was accepted by some authors (Riedl, 1963; Pawlowski, 1971; Chater, 1972; Stearn, 1986) but not by others (Runemark, 1967; Wickens, 1969) who included it in *Symphytum*. The data presented here showed that *S. creticum* is nearly identical to *S. tuberosum* in both ITS1 and *trnL* sequences.

**Brunnera (D).** This is a well-defined genus with three rhizomatous species in humid forests of Asia Minor, Caucasus and western Siberia. Morphological autapomorphies are the ebracteate inflorescences, small pollen grains with spinulose equatorial band and stigmas with irregularly cuspidate papillae. Karyologically, *B. macrophylla* and *B. orientalis* possess complements of  $2n = 12$  small chromosomes (the lowest number in the tribe known to date) with low heterochromatin content (approx. 4 %; Britton, 1951;

Bigazzi and Selvi, 2001). The monophyly of *Brunnera* is confirmed by strong BS and jackknife support in both ITS1 and *trnL* analyses, but its phylogenetic position remains unclear. Popov (1953) argued that *Brunnera* evolved through hybridization events between primary members of the Boragineae and Myosotideae and that it is a relict member of the Tertiary forest floras of the Euxine and western Siberian phytochoria (see also Edmondson, 1978). There is no evidence for this hybridization hypothesis, but the molecular data given here suggest it is a sistergroup to clades C and E though with BS <50 %. However, its position within clade A does not support the relationship with the genus *Cynoglottis* (clade B) which was formerly supposed due to resemblance in flower and fruit morphology (Guşuleac, 1928; Vural and Tan, 1983).

**Pulmonaria–Nonea (E).** Monophyly of this clade is weakly supported in the *trnL* analyses but confirmed by ITS1 sequences. The widely accepted assumption (e.g. Johnston, 1924) of a close relationship between *Pulmonaria* and *Nonea* is corroborated by molecular data. In the combined analysis, they are sister groups when treated in a wide sense. Morphologically, there are no characters exclusive to this large group. It is proposed to keep these two genera separate in line with traditional taxonomy, in contrast to Johnston (1924) and Greuter (1981). Monophyly of *Pulmonaria* is supported by two ITS1 insertions and 99 % BS and jackknife support. *Nonea* is morphologically and karyologically heterogeneous (Selvi and Bigazzi, 2002) and a wider species sampling of this genus is required for a better understanding of its infrageneric relationships.

Two other important points emerge in clade E. Firstly, both ITS1 and *trnL* sequences demonstrate that *Nonea* and *Elizaldia*, which differ only by the exerted stamens in *Elizaldia*, together form a monophyletic group. *Elizaldia calycina* is nested in *Nonea* and forms a terminal clade with *N. vesicaria*. This matches morphological evidence (see Selvi *et al.*, 2002), geographical patterns and chromosome data. In fact, *E. calycina* and *N. vesicaria* are sympatric over most of the Mediterranean belt of North Africa and are the only taxa in the group with  $2n = 2x = 30$ , a complement possibly originated via amphidiploidy from annual ancestors with  $x = 7$  and  $x = 8$  (Grau, 1971; Luque, 1995). Secondly, the ITS1 sequences indicate that the tetraploid species *Paraskevia cesatiana*, known only from three isolated localities in the mountains of the Greek Peloponnese (Sauer and Sauer, 1980), is sister to *Pulmonaria*, with which it forms a well-supported clade in the combined tree. Our results are substantially in line with Sauer's opinion (Sauer, 1987: 273) that *Paraskevia* may share an Early Tertiary ancestor with *Pulmonaria*, and the conservation of plesiomorphic characters (Selvi *et al.*, 2002) may be linked to its long geographic isolation in the Peloponnese. *Paraskevia* differs substantially from *Pulmonaria* in its non-rhizomatous root system, the absence of heterostyly and the prefloral development of foliage leaves.

**Trachystemon orientalis.** This is a large-leaved, rhizomatous herb with a hexaploid chromosome complement ( $2n = 6x = 54$ , pers. obs.). It occurs in humid forests along the southern Black Sea. The *trnL* phylogeny does not resolve its

relationships, whereas ITS1 sequences indicate a sister group relationship to *Anchusa s.l.* (clade B), but with weak support (62 % BS). Morphologically, there is no evidence for such a relationship. *Trachystemon orientalis* is characterized by striking autapomorphies, such as the corolla with two series of scales and contorted lobes, the pubescent filaments and the cystolithic trichomes of the adaxial leaf surface (Selvi and Bigazzi, 2001). Based on the corolla with short tube, long lobes and exerted stamens, Guşuleac (1928) suggested a close relationship with *Borago* and *Procopiana*. This assumption receives support from pollen (multiaperturate grains with gemmate tectum) and stigma characters (receptive surface with simple papillae lacking apical disk) which are common to these two genera (Bigazzi and Selvi, 1998, 2000). Thus, the discrepancy between molecular and morphological data suggests that additional analyses are needed to ascertain the affinities of *Trachystemon*.

*Anchusa s.l. (F–I)*. Both *trnL* and ITS1 show considerable phylogenetic divergence in *Anchusa s.l.*, whose monophyly is supported by 99 % BS in the combined analysis. Four main lineages emerge in this group, with clade F (94 % BS) as sister to a monophyletic group (85 % BS) consisting of the clades G, H and I.

*Clade F*. Clade F highlights relationships which were not previously suspected. It is a morphologically heterogeneous group mainly composed of south-east Mediterranean species with  $x = 8$  as base chromosome number. The monotypic genus *Phyllocara*, described to accommodate the annual Anatolian species *Anchusa aucheri* DC. (Guşuleac, 1928), is sister to the rest of this group. Morphologically it is an isolated species due to unique traits in its inflorescence, flower and pollen morphology (for full description, see Bigazzi *et al.*, 1999). The other two subclades correspond to *Anchusa* subgenus *Buglossoides* and to *Hormuzakia* plus *Anchusa* subgenus *Buglossum*. No common morphological characters distinguish these taxa from the rest of *Anchusa*, but they share a distinctive 6-bp insertion in the *trnL* sequences. *Hormuzakia aggregata*, a psammophytic species of arid habitats, also has autapomorphies (the congested-aggregate inflorescence and the helmet-shaped mericarps; Guşuleac, 1928; Bigazzi *et al.*, 1999). The *trnL* sequences show that the position of *Hormuzakia negevensis*, known only from a narrow area in the Negev desert, falls with *H. aggregata* and *Anchusa* subgenus *Buglossoides*; the two species of the latter form an independent clade with moderate BS support. The close affinity between *H. aggregata* and *H. negevensis* is supported by the helmet-shaped nutlets unique to these taxa (Danin, 1995, 2000). A relationship between *Hormuzakia* and *Anchusa* subgenus *Buglossoides* was suggested by Guşuleac (1928), who believed in a common ancestry from a Tertiary ‘*Buglossoides*’ type.

*Clade G*. Clade G corresponds to *Gastrocotyle*, a strongly supported genus with two disjunct, annual species (*G. hispida* and *G. macedonica*) characterized by striking synapomorphies in inflorescence, pollen and stigma morphology (Selvi and Bigazzi, 2000; Bigazzi *et al.*, 2002). The sister group of *Gastrocotyle* remains unclear, but there is no

molecular evidence for a close relationship with *Hormuzakia* as argued by Guşuleac (1928).

*Clade H*. In clade H the annual taxa of *Anchusa* subgenus *Buglossellum* and *Cynoglottis* are sister groups, although with low BS support. The low support received by *A. subgenus Buglossellum* is due to the sequence divergence of *A. thessala*. This is the only species of *Anchusa* with base chromosome number  $x = 6$  (Markova and Goranova, 1995) and erect mericarps like *Cynoglottis*. The monophyly of the latter genus is supported by the brachymorphic corollas (with short tube and rotate limb) and the small pollen grains like *Brunnera* and *Pentaglottis* (Vural and Tan, 1983), and by  $x = 9$ , a base chromosome number which is not found in *Anchusa* (Bigazzi and Selvi, 2001).

*Clade I*. In clade I *Lycopsis* and *Anchusella*, with zygomorphic flowers and annual habit, are sister to *Anchusa* subgenus *Limbata* and *A. subgenus Anchusa*, with consistently actinomorphic flowers and biennial/perennial habit. All these taxa have the base chromosome number  $x = 8$ .

Our phylogenetic reconstruction suggests that floral zygomorphy has appeared repeatedly in Boragineae, maybe as an insect-pollination specialization syndrome. This condition occurs, in slightly different forms, in the distant clades of *Anchusa* (I) and, partly, *Nonea* (E) (Selvi *et al.*, 2002). Johnston (1924) was already aware that tendency to zygomorphy occurs several times in Lithospermeae (e.g. *Echium* and *Echiochilon*), Cynoglosseae (e.g. *Caccinia*) and Boragineae, and consequently he attached little taxonomic importance to this character in his treatment of the Old World Boraginoideae. *Lycopsis* is characterized by corollas with a sigmoid tube and almost planar limb but it does not receive strong support (BS < 50 %). *Anchusella* has a straight tube and strongly oblique limb, but it is not monophyletic in the *trnL* analysis and is weakly supported even in the combined analysis. Such a weak support may be due to the deletion shown by *A. cretica* from position 166–173, which is probably not homologous with that in the *Symphytum* clade (Fig. 2). However, monophyly of *Anchusella* is corroborated by other outstanding morphological autoapomorphies such as the unbranched cymes, the corniculate stigma with embricate papillae, the pollen with spinulose aperture margins and, above all, the androecium with only two fertile stamens (Greuter, 1965; Bigazzi *et al.*, 1997).

Neither ITS1 nor *trnL* support the subgenus rank for the endemic *Anchusa limbata*. This species, known only from a single locality in south-west Anatolia (Bigazzi *et al.*, 2003), was separated as the monotypic subgenus *Limbata* Chamberlain & R Mill in view of its unique corolla with much reduced lobes and exerted scales (Chamberlain, 1977). Guşuleac (1928) tentatively referred it to genus *Hormuzakia*, but the present analysis shows that *A. limbata* is instead closely related to members of *Anchusa* subgenus *Anchusa*. The two subgenera together form a moderately supported clade, in which lack of good resolution of species-level relationships may indicate a recent, rapid and partly adaptive radiation in (semi)arid habitats of the Mediterranean and continental Europe. This is in line with the considerable morphological affinity, the usually perennial (rarely biennial) habit, the base chromosome number  $x = 8$  and the

low incidence of polyploidy. Some species groups in this clade show stylar polymorphism, i.e. the infraspecific occurrence of long-styled and short-styled populations associated with the control of self-incompatibility. Like floral zygomorphy, stylar polymorphism appears to be an advanced character and also occurs in *Pulmonaria* (clade E), thus providing another example of parallel evolution in the tribe. However, in *A. officinalis*, *A. leucantha* and *A. undulata* ssp. *hybrida* heterostyly is imperfect because style length is not clearly associated with the position of anthers in the corolla tube (Phillip and Schou, 1981; Selvi, 1998; Selvi and Bigazzi, 2003). Stylar polymorphism has not been documented for the species of *A.* subgenus *Anchusa* that form a weakly supported terminal clade, the Sardinian endemics *A. capellii* and *A. formosa*, and the South African *A. capensis*. Early divergence and common ancestry of the Sardinian endemics were hypothesized on the basis of morphological and karyological features (Selvi and Bigazzi, 1998), although the position of *A. crispa*, a third Corso-Sardinian endemic, is unresolved in our phylogeny. Another marker will be used to examine the monophyly of this group. Another point in need of further investigation is the South African–Mediterranean disjunction of *A. affinis* and *A. capensis*, both members of *Anchusa* subgenus *Anchusa*. At the moment, no explanation can be advanced for the relationship between *A. capensis* and the Sardinian endemics suggested by ITS1 sequences, and the position of *A. affinis* from Eritrea and Saudi Arabia remained unresolved in the *trnL* analysis.

#### Taxonomic consequences

Taxonomically, the main aspects emerging from the present study are:

- (1) *Elizaldia* and *Nonea* form a monophyletic group and the relationship between *N. vesicaria* and *E. calycina* is strongly supported. This confirms the results of a morphological analysis published recently (Selvi *et al.*, 2002). Further studies on this group are in progress, but at this moment there is no evidence for maintaining *Elizaldia* separate from *Nonea*.
- (2) From both morphological and molecular data, there is sufficient evidence for keeping *Paraskevia* separate from its sister taxon *Pulmonaria* at generic level.
- (3) *Anchusa s.l.* is a strongly supported monophyletic group, but treating it as a single genus would mean neglecting remarkable morphological and molecular divergence. Both lines of evidence allow us to accept *Anchusa* only in a narrow sense, keeping *Phyllocara*, *Hormuzakia*, *Gastrocotyle* and *Cynoglottis* (all originally described as species of *Anchusa*) as separate genera. *Lycopsis* and *Anchusella* are more closely related to *Anchusa* subgenus *Anchusa* but morphological aspects also support for both the genus rank. *Anchusa s.s.* in Guşuleac's concept is paraphyletic due to the position of the subgenera *Buglossum*, *Buglossoides* and *Buglossellum*.  
Therefore, our data indicate that a taxonomic splitting of *Anchusa* is needed in order to recognize the monophyletic groups. Nevertheless, the circumscription of

the new genera and the identification of their diagnostic characters is not straightforward and further phylogenetic analyses including morphological data are required. For example, the straight, erect mericarpid of *Anchusa* subgenus *Buglossum* is one of the characters upon which Guşuleac (1927, 1929) based this taxon, but the present analysis suggests that this type of mericarpid may have originated repeatedly as it is present in other distantly related taxa of Boragineae (in some species of *Nonea*, *Anchusa thessala*, *Cynoglottis* and, slightly modified, in *Brunnera*).

- (4) Based on the combined ITS1–*trnL* analysis, nine of the usually accepted genera of the Boragineae consisting of two or more species are monophyletic: *Anchusella*, *Borago*, *Brunnera*, *Cynoglottis*, *Gastrocotyle*, *Hormuzakia*, *Nonea*, *Pulmonaria* and *Symphytum*. In addition, the tribe includes the four monotypic genera *Paraskevia*, *Pentaglottis*, *Phyllocara* and *Trachystemon*. Our data do not support the monophyly of *Lycopsis*. The relationships and taxonomic status of *Symphytum creticum* could be better resolved through a wider taxon sampling of *Symphytum*. Finally, further studies will aim at providing morphological evidence for a more natural subdivision of *Anchusa* to bring taxonomy in line with phylogeny.

#### ACKNOWLEDGEMENTS

We thank the herbaria curators of BSB and M for providing us with leaf material and A. Biesek and C. Müller (Berlin) for technical assistance. E. Nardi (Firenze), M. Weigend (Berlin), R. Olmstead (Seattle), Dr M. Fay (Kew) and an anonymous reviewer provided very useful comments and discussion on an early version of the manuscript. This work has been partly funded by M.I.U.R. 40 % 2003 and University of Firenze.

#### LITERATURE CITED

- Angiosperm Phylogeny Group II. 2003.** An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants. *Botanical Journal of the Linnean Society* **141**: 399–436.
- Baldwin BG. 1992.** Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* **1**: 3–16.
- Bentham G. 1876.** *Boragineae*. In: Bentham G, Hooker JD, eds. *Genera plantarum* 2. London: Reeve & Co., 832–865.
- Bigazzi M, Selvi F. 1998.** Pollen morphology in the Boragineae Bercht & J Presl (Boraginaceae) in relation to the taxonomy of the tribe. *Plant Systematics and Evolution* **213**: 121–151.
- Bigazzi M, Selvi F. 2000.** Stigma form and surface in the tribe Boragineae (Boraginaceae): micromorphological diversity, relationships with pollen and systematic relevance. *Canadian Journal of Botany* **78**: 388–408.
- Bigazzi M, Selvi F. 2001.** Karyotype morphology and cytogeography in *Brunnera* and *Cynoglottis* (Boraginaceae). *Botanical Journal of the Linnean Society* **136**: 365–378.
- Bigazzi M, Duman H, Selvi F. 2003.** *Anchusa limbata* (Boraginaceae): contribution to the knowledge of an enigmatic species from SW Turkey. *Candollea* **58**: 339–349.
- Bigazzi M, Hilger HH, Selvi F. 2002.** Evidence from nuclear and chloroplast DNA for the placement of *Anchusa macedonica* in the genus *Gastrocotyle* (Boraginaceae). *Webbia* **57**: 173–180.

- Bigazzi M, Nardi E, Selvi F. 1997.** *Anchusella*, a new genus of *Boraginaceae* from the Central-Eastern Mediterranean. *Plant Systematics and Evolution* **205**: 241–264.
- Bigazzi M, Selvi F, Fiorini G. 1999.** A reappraisal of the generic status of *Gastrocotyle*, *Hormuzakia* and *Phyllocara* in the light of micromorphological and karyological evidence. *Edinburgh Journal of Botany* **56**: 229–251.
- Böhle U-R, Hilger HH. 1997.** Chloroplast DNA systematics of 'Boraginaceae' and related families: a goodbye to the old and familiar concept of five subfamilies. *Scripta Botanica Belgica* **15**: 30.
- Boissier E. 1849.** *Diagnoses Plantarum orientalium novarum* 1, no. 11. Paris: Ducloux, 95–96.
- Boissier E. 1879.** *Flora Orientalis* 4. Geneva: Georg, 162–170.
- Britton D. 1951.** Cytogenetic studies on the Boraginaceae. *Brittonia* **7**: 233–266.
- Chamberlain DF. 1977.** *Anchusa*. In: Davis PH, ed. Materials for a flora of Turkey XXXIV: Boraginaceae, Gentianaceae, Solanaceae. *Notes Royal Botanic Garden Edinburgh* **35**: 298–299.
- Chamberlain DF. 1979.** *Anchusa* L. In: Davis PH, ed. *Flora of Turkey and the East Aegean Islands* 6. Edinburgh: Edinburgh University Press, 388–402.
- Chater AO. 1972.** *Anchusa* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, eds. *Flora Europaea* 3. Cambridge: Cambridge University Press, 106–109.
- Danin A. 1995.** A new *Anchusa* from Israel. *Edinburgh Journal of Botany* **52**: 333–336.
- Danin A. 2000.** The nomenclature news of Flora Palaestina. *Flora Mediterranea* **10**: 101–172.
- De Candolle ALPP. 1846.** *Tribus Borrageae*. In: De Candolle ALPP, ed. *Prodromus systematis naturalis regni vegetabilis* 10. Paris: Masson; Leipzig: Michelsen, 1–56.
- Diane N, Förther H, Hilger HH. 2002.** A systematic analysis of *Heliotropium*, *Tournefortia* and allied taxa of the Heliotropiaceae (Boraginales) based on ITS1 sequences and morphological data. *American Journal of Botany* **89**: 287–295.
- Doyle JJ, Doyle JL. 1990.** Isolation of plant DNA from fresh tissue. *Focus* **12**: 13–15.
- Edmondson JR. 1978.** *Brunnera* Steven. In: Davis PH, ed. *Flora of Turkey and the East Aegean Islands* 6. Edinburgh: Edinburgh University Press, 387–388.
- Farris JS, Albert VA, Källersjö M, Lipscomb D, Kluge AG. 1996.** Parsimony jackknifing outperforms neighbor-joining. *Cladistics* **12**: 99–124.
- Felsenstein J. 1985.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.
- Ferguson DM. 1999.** Phylogenetic analysis and relationships in Hydrophyllaceae based on *ndhF* sequence data. *Systematic Botany* **23**: 253–268.
- Gottschling M, Hilger HH. 2001.** Phylogenetic analysis and character evolution of *Ehretia* and *Bourreria* (Ehretiaceae, Boraginales) and their allies based on ITS1 sequences. *Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie* **123**: 249–268.
- Gottschling M, Hilger HH, Wolf M, Diane N. 2001.** Secondary structure of the ITS1 transcript and its application in a reconstruction of the phylogeny of Boraginales. *Plant Biology* **3**: 629–636.
- Grau J. 1971.** Cytologische Untersuchungen an Boraginaceae. II. *Mitteilungen der Botanischen Staatssammlung München* **9**: 177–194.
- Greuter W. 1965.** Beiträge zur Flora der Südägäis. *Candollea* **20**: 167–218.
- Greuter W. (ed.) 1981.** Med-Checklist Notulæ, 3. *Willdenowia* **11**: 23–37.
- Greuter W, Burdet, HM, Long G. 1984.** Med-Checklist 1: *Pteridophyta*, 2nd edn. *Gymnospermae, Dicotyledones (Acanthaceae-Cneoraceae)*. Geneva: Conservatoire et Jardin Botaniques, 69–72.
- Gürke M. 1893.** *Boraginaceae*. In Engler A, Prantl K, eds. *Die natürlichen Pflanzenfamilien* IV (3a). Leipzig: Engelmann, 71–131.
- Guşuleac M. 1923.** Beiträge zur Systematik der Anchuseae. *Publicaţiunile Societăţii Naturaliştilor din Bucureşti* **6**: 79–92.
- Guşuleac M. 1927.** Die europäischen Arten der Gattung *Anchusa* Linné. *Buletinul Facultăţii Ştiinţe Cernăuţi* **1**: 73–123.
- Guşuleac M. 1928.** Die monotypischen und artenarmen Gattungen der Anchuseae (*Caryolopha*, *Brunnera*, *Hormuzakia*, *Gastrocotyle*, *Phyllocara*, *Trachystemon*, *Procopiania* und *Borago*). *Buletinul Facultăţii Ştiinţe Cernăuţi* **2**: 394–461.
- Guşuleac M. 1929.** Species *Anchuseae* generis Linn. hucusque cognitae. *Feddes Repertorium* **26**: 286–322.
- Hepperle D. 2001.** *Multicolor Sequence Alignment Editor*. Neuglobsow, Germany: Institute of Freshwater Ecology and Inland Fisheries.
- Johnston IM. 1924.** Studies in the *Boraginaceae*. III. 1. The Old World genera of the *Boraginoideae*. *Contributions of the Gray Herbarium of Harvard University* **73**: 42–78.
- Långström E, Chase MW. 2002.** Tribes of Boraginoideae (Boraginaceae) and placement of *Antiphytum*, *Echiochilon*, *Ogastemma* and *Sericostoma*: a phylogenetic analysis based on *atpB* plastid DNA sequence data. *Plant Systematics and Evolution* **234**: 137–153.
- Luque T. 1995.** Karyology of *Nonea* Medicus (Boraginaceae) in Spain; relationships between genera of Boragineae Barbier & Mathez (Anchuseae DC.). *Botanical Journal of the Linnean Society* **117**: 321–331.
- Markova M, Goranova V. 1995.** Mediterranean chromosome number reports no. 435–473. *Flora Mediterranea* **5**: 289–317.
- Melchior H. 1964.** Boraginaceae. In: Melchior H, ed. *A. Englers Syllabus der Pflanzenfamilien* edn. 12, 2. Berlin: Borntraeger, 431–434.
- Pawlowski B. 1971.** De genere *Procopiania* Guşuleac – Rodzaj *Procopiania* Guşuleac. *Fragmenta Floristica Geobotanica* **17**: 39–58.
- Phillip M, Schou O. 1981.** An unusual heteromorphic incompatibility system. Distyly, self-incompatibility, pollen load and fecundity in *Anchusa officinalis* (Boraginaceae). *New Phytologist* **89**: 693–703.
- Popov MG. 1953.** Anchuseae DC. In: Komarov VL, ed. *Flora SSSR, vol. 19*. Moskva-Leningrad: Akademii Nauk SSSR, 207–263.
- Riedl H. 1963.** *Anchusa* subgen. *Chamanchusa* subgen. nov. und das System der *Boraginoideae-Anchuseae*. *Österreichische Botanische Zeitschrift* **110**: 543–546.
- Runemark H. 1967.** Studies in the Aegean flora. XI. *Procopiania* (Boraginaceae) included into *Symphytum*. *Botaniska Notiser* **120**: 84–94.
- Sauer W. 1987.** The *Pulmonaria dacica* group: its affinities with central and south-east European allies and with the genus *Paraskevia* (Boraginaceae). *Plant Systematics and Evolution* **155**: 257–276.
- Sauer W, Sauer G. 1980.** *Paraskevia* gen. nov. mit *P. cesatiana* comb. nov. (Boraginaceae), eine endemische Gattung Griechenlands. *Phyton* **20**: 285–306.
- Selvi F. 1998.** Floral biometrics in the *Anchusa undulata* L. group (Boraginaceae) from the central-eastern Mediterranean. *Botanical Journal of the Linnean Society* **128**: 251–270.
- Selvi F, Bigazzi M. 1998.** *Anchusa* L. and allied genera (Boraginaceae) in Italy. *Plant Biosystems* **132**: 113–142.
- Selvi F, Bigazzi M. 2000.** Removal of *Anchusa macedonica* (Boraginaceae) from *Anchusa*. Evidence from phenetics and karyotype analysis. *Taxon* **49**: 765–778.
- Selvi F, Bigazzi M. 2001.** Leaf surface and anatomy in Boraginaceae tribe Boragineae with respect to ecology and taxonomy. *Flora* **196**: 269–285.
- Selvi F, Bigazzi M. 2002.** Chromosome studies in Turkish species of *Nonea* (Boraginaceae): the role of polyploidy and descending dispolyploidy in the evolution of the genus. *Edinburgh Journal of Botany* **59**: 405–420.
- Selvi F, Bigazzi M. 2003.** Revision of genus *Anchusa* (Boraginaceae-Boragineae) in Greece. *Botanical Journal of the Linnean Society* **142**: 431–454.
- Selvi F, Papini A, Bigazzi M. 2002.** Systematics of *Nonea* (Boraginaceae-Boragineae): new insights from phenetic and cladistic analyses. *Taxon* **51**: 719–730.
- Simmons MP, Ochoterena H. 2000.** Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49**: 369–381.
- Stearn WT. 1986.** The Greek species of *Symphytum* (Boraginaceae). *Annales Musei Goulandris* **7**: 175–220.
- Swofford DL. 1998.** *PAUP\**. *Phylogenetic Analysis Using Parsimony (and other methods)* vers. 4.0. Sunderland, MA: Sinauer.
- Taberlet P, Gielly L, Pautou G, Bouvet J. 1991.** Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.
- von Friedrichsthal E. 1838.** *Reise in den südlichen Theilen von Neu-Griechenland. Beiträge zur Charakteristik dieses Landes*. Leipzig: Engelmann.
- Vural M, Tan K. 1983.** New taxa and records from Turkey. *Notes from the Royal Botanic Garden Edinburgh* **41**: 65–75.
- Wickens GE. 1969.** A revision of *Symphytum* in Turkey and adjacent areas. *Notes from the Royal Botanic Garden Edinburgh* **29**: 157–180.
- Winkworth RC, Grau J, Robertson AW, Lockhart PJ. 2002.** The origins and evolution of the genus *Myosotis* L. (Boraginaceae). *Molecular Phylogenetics and Evolution* **24**: 180–193.