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

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. Längström and Chase, 2002).

Boragineae are morphologically well-characterized by faecal corolla appendages (=fornices) and by strophiolate mericarps attached basally on a planar gynobase. These mericarps 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/Cynoglossaceae representing the ‘neighbouring’ lineage. Recent studies of Boragineae based on ribosomal ITS1 and plastid atpB sequences (Gottschling

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

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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; Türke, 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 Guşulec (1923, 1928), the most important monographer of the tribe, who reduced Boragineae to those taxa with faecal appendages in the corolla, moving *Lithodora* and *Alkanna* to Lithospermeae. In his system, the tribe consisted of 11 genera, with *Elizalda* Willk. reduced to synonymy in *Nonea* Medik. Guşulec’s treatment was largely followed by Riedl (1963), who recognized 13 genera with a narrowly defined *Anchusa* L. and *Elizalda* 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 Guşulec to include distinct lineages that he regarded as separate genera. Guşulec’s exhaustive morphological studies (Guşulec, 1927, 1928, 1929) resulted in the well-supported separation at genus level of species originally described under *Anchusa cesatiana* (von Friedrichshal, 1838), but later variously combined under *Pulmonaria* (Boissier, 1879) and *Nonea* (Boissier, 1849; Guşulec, 1929; Greuter, 1981). *Nonea* also has close relationships with the North African genus *Elizalda*, 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* intron of the plastid genome is appropriate for investigations of relationships between species and genera. The usefulness of both markers in the systematics of Boragineales 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 amphiolium*, 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 Sequagel-6 (National Diagnostics, Atlanta, GA, USA). The bio-
tinylated PCR products were transferred onto a Biodyne A
### Table 1. List of taxa investigated with internal DNA number, origin and voucher specimens, and GenBank accession

<table>
<thead>
<tr>
<th>Taxa</th>
<th>DNA no.</th>
<th>Origin and voucher**</th>
<th>GenBank accession</th>
<th>trnL/ITS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anchusa aegyptiaca (L.) DC.</td>
<td>695</td>
<td>Cyprus: Hilger 00/1 (BSB)</td>
<td>AY383255/AY383294</td>
<td></td>
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<tr>
<td>Anchusa affinis R.Br.</td>
<td>1037</td>
<td>Saudi Arabia: Lavranos &amp; Collenette 18390 (FI)</td>
<td>AY383279/</td>
<td></td>
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<tr>
<td>Anchusa azurea Mill.</td>
<td>619</td>
<td>Cyprus: Hilger 00/18 (BSB)</td>
<td>AY383254/AY383293</td>
<td></td>
</tr>
<tr>
<td>Anchusa capellii Moris.</td>
<td>780</td>
<td>Italy, Sardinia: Bigazzi &amp; Selvi 99.002 (FI) BSB</td>
<td>AY383257/AY383297</td>
<td></td>
</tr>
<tr>
<td>Anchusa capensis Thunb.</td>
<td>990</td>
<td>South Africa: Orange Free State (FI)</td>
<td>AY383269/AY383311</td>
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<tr>
<td>Anchusa cespitosa Lam.</td>
<td>287</td>
<td>Greece, Crete: Hilger 98/11 (FI, BSB)</td>
<td>AY383268/AY383310</td>
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<tr>
<td>Anchusa crispa Viv. ssp. crispa</td>
<td>791</td>
<td>France, Corsica: Bigazzi &amp; Selvi 99.005 (FI)</td>
<td>AFS30603/A071853</td>
<td></td>
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<tr>
<td>Anchusa formosa Selvi, Bigazzi &amp; Bacchetta</td>
<td>781</td>
<td>Italy, Sardinia: Bigazzi &amp; Selvi 97.006 (FI)</td>
<td>AY383258/AY383309</td>
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<tr>
<td>Anchusa leptophylla Roem. &amp; Schult.</td>
<td>633</td>
<td>Turkey: Carle &amp; Künschner 4032 (BSB)</td>
<td>AFS30604/AY383298</td>
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<tr>
<td>Anchusa leucantha Selvi &amp; Bigazzi</td>
<td>862</td>
<td>Greece: Bigazzi &amp; Selvi 01.17 (FI, BSB)</td>
<td>AY383267/AY383309</td>
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</tr>
<tr>
<td>Anchusa limbata Boiss. &amp; Heldr.</td>
<td>1158</td>
<td>Turkey: Bigazzi &amp; Selvi 02.01 (FI, BSB)</td>
<td>AY383260/AY383301</td>
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<tr>
<td>Anchusa milleri Sprengel</td>
<td>623</td>
<td>Israel: Hilger 21/94. (FI, BSB)</td>
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<tr>
<td>Anchusa ochroleuca M.Bieb.</td>
<td>373</td>
<td>Bulgaria: Hilger 97/21 offspring (BSB)</td>
<td>AY383261/AY383302</td>
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<tr>
<td>Anchusa officinalis L.</td>
<td>672</td>
<td>Germany: Hilger 2000 (BSB)</td>
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<tr>
<td>Anchusa pusilla Guajul.</td>
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<tr>
<td>Anchusa sanftbractiata Bigazzi &amp; Selvi</td>
<td>811</td>
<td>Greece: Bigazzi &amp; Selvi 99.016 (FI, BSB)</td>
<td>AY383262/AY383303</td>
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<tr>
<td>Anchusa strieguisa Bank. &amp; Sol.</td>
<td>618</td>
<td>Cyprus: Hilger 00/16 (BSB)</td>
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<tr>
<td>Anchusa stylosa M. Bieb.</td>
<td>861</td>
<td>Turkey: Bigazzi &amp; Selvi 01.13 (FI, BSB)</td>
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<tr>
<td>Anchusa thessaloi Boiss. &amp; Spruner</td>
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<td>Turkey: Bigazzi &amp; Selvi 97.022 (FI, BSB)</td>
<td>AFS30599/AY383296</td>
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<tr>
<td>Anchusa undulata L. ssp. hybridata (Ten.) Bég.</td>
<td>616</td>
<td>Cyprus: Hilger 00/12 (BSB)</td>
<td>AY383259/AY383300</td>
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<tr>
<td>Anchusella cretica (Mill.) Bigazzi, Nardi &amp; Selvi</td>
<td>667</td>
<td>Italy: Bigazzi &amp; Selvi 00.33 (FI)</td>
<td>AYO0709/A045716</td>
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<tr>
<td>Anchusella variegata (L.) Bigazzi, Nardi &amp; Selvi</td>
<td>857</td>
<td>Greece: Bigazzi &amp; Selvi 01.10 (FI)</td>
<td>AY383265/AY383306</td>
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<tr>
<td>Borago officinalis L.</td>
<td>671</td>
<td>Germany (Berlin cult.): Hilger (BSB)</td>
<td>AY383245/AY383323</td>
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<tr>
<td>Borago pygmaea (DC.) Greuter</td>
<td>375</td>
<td>Germany (cult. H.Berlin-Dahlem): Hilger</td>
<td>AY383244/AY383298</td>
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<tr>
<td>Brunnera macrophylla (Adams) I.M.Johnst.</td>
<td>628</td>
<td>Germany (cult. H.Berlin-Dahlem): Hilger</td>
<td>AY383249/AY383298</td>
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<tr>
<td>Brunnera orientalis (Schenk) I.M.Johnst.</td>
<td>829</td>
<td>Turkey: Bigazzi &amp; Selvi 00.21 (FI)</td>
<td>AY383250/AY383298</td>
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<tr>
<td>Buglossoides arvensis (L.) I.M.Johnst.</td>
<td>829</td>
<td>Turkey: Bigazzi &amp; Selvi 00.21 (FI)</td>
<td>AY383250/AY383298</td>
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<tr>
<td>Cynoglossum aucheri (DC.) Gus (L.) L.H.Bailey</td>
<td>668</td>
<td>Italy: Bigazzi &amp; Selvi 97.015 (FI)</td>
<td>AY383254/AY383323</td>
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<tr>
<td>Cynoglossum arvense (L.) I.M.Johnst.</td>
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<td>Germany: Hilger 2000 (BSB)</td>
<td>AY383250/AY383298</td>
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<tr>
<td>Elizaldia calycina (Roem. &amp; Schult.) Maire ssp.</td>
<td>706</td>
<td>Morocco: Lewalle 10884 (RNG)</td>
<td>AY383264/AY383305</td>
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<td>Gastrocoryne hispida (Forsk.) Bunge</td>
<td>674</td>
<td>Jordan: Bajierle &amp; al. 17.3.86 (FI)</td>
<td>AYO0703/A045710</td>
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<td>Gastrocoryne macrodonica (Degen &amp; Dörf.)</td>
<td>682</td>
<td>Greece: Bigazzi &amp; Selvi 99.009 (FI, BSB)</td>
<td>AYO0706/A045713</td>
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<tr>
<td>Hormuzakia aggregata (Lehm.) Gusul.</td>
<td>693</td>
<td>Israel: Bigazzi &amp; Selvi 96.015 (FI)</td>
<td>AY383252/AY383291</td>
<td></td>
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<tr>
<td>Hormuzakia negevensis (Danin) Danin &amp; Hilger</td>
<td>664</td>
<td>Israel: Danin 24.97 (HUJ)</td>
<td>AYO0707/A045714</td>
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<tr>
<td>Lycopsis arvensis L.</td>
<td>624</td>
<td>Germany: Hilger &amp; Werres 27.5.99 (BSB)</td>
<td>AYO0707/A045714</td>
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<td>Lycopsis orientalis L.</td>
<td>831</td>
<td>Turkey: Bigazzi &amp; Selvi 00.10 (FI)</td>
<td>AY383277/A045319</td>
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<tr>
<td>Nonea lutea (Desr.) DC.</td>
<td>630</td>
<td>Germany (cult. H. Berlin-Dahlem): Hilger (BSB)</td>
<td>AY383274/A045316</td>
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<td>Nonea pulla DC.</td>
<td>661</td>
<td>Germany: Hensen 28.00 (BSB)</td>
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<tr>
<td>Nonea vesicaria (L.) Reichb.</td>
<td>1311</td>
<td>Morocco: Podlech 51525 (ITS1, M)</td>
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<td>Parakezia cespitosa (Fenzl &amp; Friedr.)</td>
<td>1252</td>
<td>Sicily: Bigazzi &amp; Selvi 97.038 (trnL, FI)</td>
<td>AY383263/</td>
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</tr>
<tr>
<td>Pencapitella serperevires (L.) L.H.Bailey</td>
<td>668</td>
<td>Italy (cult.): Bigazzi &amp; Selvi</td>
<td>AFS30598/AY383286</td>
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<tr>
<td>Phyllocastrum Trinule (DC.) Gujul.</td>
<td>670</td>
<td>Turkey: Bigazzi &amp; Selvi 97.041 (FI)</td>
<td>AY383251/AY383290</td>
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<td>Plumbago angustifolia L.</td>
<td>744</td>
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<tr>
<td>Plumbago mollis Wulf.</td>
<td>755</td>
<td>Germany (cult.): Hilger (BSB)</td>
<td>AY383273/AY383315</td>
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<td>Plumbago obscura Dumort.</td>
<td>681</td>
<td>Germany (cult.): Hilger (BSB)</td>
<td>AY383270/AY383312</td>
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<td>Plumbago picta Royu</td>
<td>761</td>
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<td>AY383272/AY383314</td>
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<td>Symphytum ctenicum (Willd.) Greuter &amp; Rech.fil.</td>
<td>284</td>
<td>Greece, Crete: Hilger (BSB)</td>
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<td>Symphytum tuberosum L.</td>
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<tr>
<td>Trachyspermum orientalis (L.) G.Don</td>
<td>666</td>
<td>Turkey: Bigazzi &amp; Selvi 00.06 (FI)</td>
<td>AY383248/AY383287</td>
<td></td>
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</tbody>
</table>

* Those taxa which were included only in the trnL analysis.
** BSB = Herbarium, Institut für Biologie - Systematische Botanik und Pflanzengeographie, Freie Universität, Berlin; FI = Herbarium, University of Florence, 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-swatching 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) Phyllocca, Hormuzakia, Anchusa subgenus Buglossoides and subgenus Buglossooides, (7) Gastrocotyle, Anchusella, Cynoglossis, Lycopsis, Anchusa subgenus Buglossoellum, 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 Phyllocca, Hormuzakia, and Anchusa subgenus Buglossoides. In clade 6, the relationships between Phyllocca, Anchusa subgenus Buglossum, Hormuzakia plus A. subgenus Buglossooides remained unresolved. The clade with the two latter taxa received 62 % BS, as did A. subgenus Buglossooides 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 Cynoglossum, Anchusa subgenus Buglossoellum, 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, Phyllocca aucteri 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 Buglossooides 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
(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. 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).](image-url)
**Trachystemon orientalis**
**Borago pygmaea**
**Borago officinalis**
**Penaglottis sempervirens**
**Symphytum creticum**
**Symphytum tuberosum**
**Brummera macrophylla**
**Brummera orientalis**
**Phyllocarpa ancheri**
**Hormuzakia aggregata**
**Hormuzakia negevensis**
**Anchusa aegyptiaca**
**Anchusa milleri**
**Anchusa strigota**
**Anchusa azurea**
**Gastrocotyle hispida**
**Gastrocotyle macedonica**
**Anchusa thessala**
**Anchusa clyptus**
**Cynoglottis barrelieri**
**Cynoglottis chetikiana**
**Anchusa pusilla**
**Anchusa stylosa**
**Lycopsis orientalis**
**Lycopsis arvensis**
**Anchusa capelli**
**Anchusa leptophylla**
**Anchusa formosa**
**Anchusa officinalis**
**Anchusa crispa**
**Anchusa undulata**
**Anchusa limbata**
**Anchusa ochroleuca**
**Anchusa samothracica**
**Anchusa variegata**
**Anchusa leucantha**
**Anchusa cesiptosa**
**Anchusa capensis**
**Anchusa affinis**
**Nonea lutea**
**Nonea vesicaria**
**Elsalidia calycina**
**Nonea pulla**
**Paraskevia cesatiana**
**Pulmonaria obscura**
**Pulmonaria mollis**
**Pulmonaria angustifolia**
**Pulmonaria picta**

**Fig. 2.** Condensed alignment scheme of *trnL* sequences (outgroups, identical and non-informative positions removed). Taxa are ordered according to the main insertions/deletions shared. Column numbers refer to nucleotide positions in the original alignment.
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. striigosa* and *A. azurea*, 100 %, both indices), and *Anchusa subgenus Buglossoides* (*A. aegyptiaca* and *A. milleri*, 98 %).
DISCUSSION

Infra-tribal 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 (Längström and Chase, 2002) that focused on higher taxonomic levels of Boragineae.

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 Längström 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 (= Caryolopha sempervirens). This is the only member of the tribe native to the Atlantic region of south-west Europe. It possesses autopomorphies in fruit morphology, such as eccentrically 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. *Brunniera*, *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 (89)–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 columnellae 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 Gušulec’s opinion (Gušulec, 1928) of a ‘Paleoborago’ ancestor shared by *Borago*, *Symphytum* and *Procopania*. The latter genus was instituted by the same author to accommodate *S. creticum* (*Procopania 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 exserted stamens. In more recent times, *Procopania* 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.

Brunniera (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 *Brunniera* is confirmed by strong BS and jackknife support in both ITS1 and *trnL* analyses, but its phylogenetic position remains unclear. Popov (1953) argued that *Brunniera* evolved through hybridization events between primary members of the Boragineae and Myosotideae and that it is a relict member of the Tertiary forest florals of the Euxine and western Siberian phytotokia (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 *Cynoglossis* (clade B) which was formerly supposed due to resemblance in flower and fruit morphology (Gušulec, 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 Peloponnesian. *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 exserted stamens, Gušuleac (1928) suggested a close relationship with Borago and Procopiation. 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 Phylloca, described to accomodate the annual Anatolian species Anchusa aucheri DC. (Gušuleac, 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; Gušuleac, 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 (Darin, 1995, 2000). A relationship between Hormuzakia and Anchusa subgenus Buglossoides was suggested by Gušuleac (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 Gušuleac (1928).

Clade H. In clade H the annual taxa of Anchusa subgenus Buglossellum and Cynoglossotis 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 Cynoglossotis. The monophyly of the latter genus is supported by the brachymorphic corollas (with short tube and rotate limb) and the small pollen grains like Brunera 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 Lithosermeae (e.g. Echiwm and Echiowhilion), Cynoglosseae (e.g. Caccinia) and Boragineae, and consequently he attached little taxonomic importance to this character in his treatment of the Old World Boragineidae. 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 autapomorphies such as the unbranched cymes, the coriulate 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 exserted scales (Chamberlain, 1977). Gušuleac (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. *Elizalda* 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 *Elizalda* 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 *Cynoglossit* (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, *Cynoglossit* 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*, *Cynoglossit*, *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), D. 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**


