Molecular phylogenetics and morphology support two new genera (*Memoremea* and *Nihon*) of Boraginaceae s.s.

ANA OTERO¹ ², PEDRO JIMÉNEZ-MEJÍAS¹, VIRGINIA V ALCÁRCEL² & PABLO V ARGAS¹

¹Dpto. de Biodiversidad y Conservación. Real Jardín Botánico (RJB-CSIC). Plaza de Murillo 2. 28014 Madrid. España (anaosterogomez@gmail.com).

Abstract

*Boraginaceae s.s.* (Cynoglosseae s.l.) comprises ca. 29 species of annual and perennial plants distributed in three main disjunct areas: Western Palearctic, Japan and SW of North America. This paper uses micromorphological and DNA data to reassess the monophyly and taxonomy of the genus. Morphological characters of 15 species of *Omphalodes* and four closely-related genera were analysed using SEM. A total of 82 ITS and 68 trnL–trnF sequences were newly sequenced, including 14 species of *Omphalodes* and three genera of the tribe Cynoglosseae. Phylogenetic analyses of 57 genera (186 species) of Boraginaceae indicated that *Omphalodes* as currently circumscribed is formed by three independent lineages, which were supported by morphological characters of the fruit. As a result, and in the interest of a more natural classification, two new genera are described to accommodate *Omphalodes scorpioides* (*Memoremea*) from Europe and the Japanese species (*Nihon*). *Memoremea* is distinguished from all the other species previously included in *Omphalodes* by the apical attachment scar and the hollow nutlet margin. *Nihon* is easily discriminated by the abrupt change of margin ornamentation towards the nutlet aperture. We also provided a taxonomic treatment that proposes the lowest number of nomenclature changes, although six new combinations are required.

Key words: Carpology, DNA sequences, Scanning Electron Microscopy, Systematics

Introduction

*Boraginaceae s.s.* (=Boraginaceae subfam. Boraginae; Al-Shehbaz, 1991; Gürke, 1893) comprises 112 genera and about 1600 species (Stevens 2001 onwards) of herbaceous plants and shrubs. Between four and 13 tribes have been traditionally recognized within the Boraginaceae s.s. based on morphological characters (De Candolle 1846, Baillon 1890, Gürke 1893, Johnston 1924, Popov 1953, Al-Shehbaz 1991, Riedl 1997). Nutlet macromorphology has traditionally been used to divide Boraginaceae into either 13 (Popov 1953) or six (Riedl 1997) tribes. However, molecular phylogenies of the family are largely congruent with synthetic treatments that reduce tribal division into four tribes: Lithospermeae, Boragineae, Echiochileae and Cynoglosseae s.l. (*Långström & Chase* 2002, *Långström & Oxelman* 2003, *Weigend* et al. 2010, *Nazaré & Hufford* 2012, *Weigend et al.* 2013, Cohen 2014). The three first tribes are each supported by autoapomorphic carpological characters: Echiochileae is characterized by a basal or submedial attachment scar and a flat to pyramidal gynobase (*Långström & Chase* 2002); Lithospermeae mostly present ovoid, keeled, slightly compressed and strongly incurved nutlets with a broad basal attachment scar and a flat gynobase (*Långström & Chase* 2002, *Weigend* et al. 2010; and Boragineae is basally attached with planar gynobase and basal annulus surrounding the scar (*Hilger* et al. 2004). The fourth tribe (Cynoglosseae s.l.), recognized based on molecular phylogenetics, includes a set of morphologically heterogeneous subtribes, showing the widest variety of nutlet morphology and ornamentation, including deeply dentate margins, glocidia, papillae or even surface completely smooth, and gynophore configuration from nearly flat to pyramidal. Some genera also have a more or less thickened wing. The most recent phylogenetic reconstructions (*Cohen* 2014; *Weigend et al.* 2013) recovered six well-supported major groups within Cynoglosseae s.l.: Trichodesmeae, Eritrichieae, Myosotideae, *Omphalodes* s.s., *Mertensia* clade, and Cynoglosseae s.s. This latter comprises *Cynoglossum* Linnaeus (1753: 134) and related genera (*e.g.* *Paracaryum* Boissier (1849: 128), *Rindera* Pallas (1771: 486), *Solenanthus* Ledebour (1829: 8) and *Trachelanthus* Kunze (1850: 665)), several East Asian genera (*e.g.* *Bothriospermum* Bunge (1831: 47), *Microula* Bentham (1876: 853)), and the taxa previously considered within the subtribe Cryptanthinae.
Omphalodes Miller (1754: 968) is a genus of the family Boraginaceae, known by the common English name of “navelwort” or “navel seeds” (Weigend et al. 2013), traditionally included in the tribe Cynoglosseae s.s. (De Candolle 1846, Gürke 1893, Johnston 1924, Riedl 1997, Valdés 2004; see revision in Nazaire & Hufford 2012). Omphalodes comprises ca. 29 herbaceous annual and perennial species (Table 1). They are distributed in three disjunct areas: the Western Palaearctic (Popov 1953, Tutin et al. 1972, Davis 1978, Nasir 1989, Fernández & Talavera 2012), Japan (Ka 1965, Yamazaki 1993), and SW North America (Nesom 2013). The largest number of species is found in the Mediterranean Region (11 spp.). The fruit of Omphalodes is a tetranutlet, with shortly cylindrical or ovoid, glabrous or hairy nutlets showing a great variation in the epidermis ornamentation (Fernández & Talavera 2012). The abaxial surface of the fruit is flattish, limited by a conspicuous winged margin that is entire, lobulated or dentate and inwardly curved or erect. The insertion of the fruit is elliptic, flat, without an appendage and attached to a more or less conical receptacle on its upper half, leaving an ovate to deltoid scar. The embryo is usually erect or exceptionally curved as in O. scorpioides Schrank (1812: 222) (Popov 1953).

**TABLE 1**: List of the species of Omphalodes recognized in the most recent taxonomic treatments (Popov (1953), Nesom (2013), Ka (1965), Tutin et al. (1972), Yamazaki (1993), Strid and Tan (2005), Kadota (2009), Fernández and Talavera (2012) and Euro+Med (2006–present)). Superindices 1 and 2 indicate those species included in the DNA and SEM study respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution</th>
<th>Annual/Perennial</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Western Palearctic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O. brassicifolia Sweet.</td>
<td>CW and S Iberian Peninsula</td>
<td>Annual</td>
</tr>
<tr>
<td>O. cappadocica D.C.</td>
<td>Caucasus</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. caucasica Brand</td>
<td>Caucasus</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. commutata G.López</td>
<td>S Iberian Peninsula</td>
<td>Annual</td>
</tr>
<tr>
<td>O. kasinetzovi Kolak</td>
<td>Caucasus</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. luzinskyanae Wilk.</td>
<td>CW Portugal</td>
<td>Annual</td>
</tr>
<tr>
<td>O. linifolia Moench</td>
<td>Iberian Peninsula, W France to Crimea and W Caucasus</td>
<td>Annual</td>
</tr>
<tr>
<td>O. littoralis Lehmi.</td>
<td>NW Iberian Peninsula, SW France</td>
<td>Annual</td>
</tr>
<tr>
<td>O. lojkae Sommier &amp; Levier</td>
<td>Caucasus</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. luciliae Boiss.</td>
<td>Greece, N Iraq, W Iran</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. nitida Hoffmannans. &amp; Link</td>
<td>NW Iberian Peninsula</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. ripeyana P.H.Davis</td>
<td>Anatolia</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. rupestris Rupr ex Boiss.</td>
<td>Caucasus</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. runemarkii Strid &amp; Kit Tan</td>
<td>Greece</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. scorpioides Schrank.</td>
<td>C and NE Europe</td>
<td>Biennial</td>
</tr>
<tr>
<td>O. verna Moench.</td>
<td>E Mediterranean and C Europe</td>
<td>Perennial</td>
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<tr>
<td><strong>Japan</strong></td>
<td></td>
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<tr>
<td>O. akiensis Kadota</td>
<td>Honshu, Hiroshima</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. japonica Maxim.</td>
<td>Honshu</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. krameri Franch. &amp; Sav.</td>
<td>Hokkaido, N to C, Honshu</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. laevisperma Nakai</td>
<td>C Honshu</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. prolifera Ohwi</td>
<td>C and W Honshu</td>
<td>Perennial</td>
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<tr>
<td><strong>N. America</strong></td>
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<tr>
<td>O. aliena A.Gray ex Hemsl.</td>
<td>S Texas and Mexico (Nuevo León and C Coahuila)</td>
<td>Annual</td>
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<tr>
<td>O. alienoides Nesom</td>
<td>S Texas and Mexico (Coahuila)</td>
<td>Annual/Perennial</td>
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<tr>
<td>O. australis Nesom</td>
<td>Mexico (Puebla)</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. cardiophylla A.Gray ex Hemsl.</td>
<td>Mexico (Coahuila to Nuevo León and CW Tamaulipas)</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. carranzae Nesom</td>
<td>Mexico (NW Coahuila, Sierra del Carmen)</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. chiangii L.C.Higgins</td>
<td>Mexico (CN Coahuila)</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. erecta L.M. Johnston</td>
<td>Mexico (C Nuevo León to CW Tamaulipas)</td>
<td>Perennial</td>
</tr>
<tr>
<td>O. mexicana S.Watson</td>
<td>Mexico (C Nuevo León)</td>
<td>Perennial</td>
</tr>
</tbody>
</table>
Miller (1754) described *Omphalodes* following polynomial nomenclature. The first author using binomial nomenclature was Moench who described *O. linifolia* Moench (1794: 719) and *O. verna* Moench (1794: 420). *Omphalodes verna* was designated as the type species by Stafleu in Flora Neerlandica (van Ooststroom et al. 1961). De Candolle (1846) recognized four sections within the genus. Section *Eu-Omphalodes* De Candolle (1846:11) comprised the perennial European taxa *O. cappadocica* De Candolle (1846: 161), *O. luciliae* Boissier (1844: 41), *O. nitida* Hoffmannsegg & Link (1811: 194) and *O. verna*, and section *Maschalanthus* De Candolle (1846:11) included only *O. scorpioides*. With the exception of *O. scorpioides* which he retained in the monotypic section *Maschalantus*, Brand (1921) included taxa currently considered in other genera such as *Microla* and *Sinojohnstonia* Hu (1936: 201), together with all species of *Omphalodes* then known within section *Eu-Omphalodes*. The treatment of Popov (1953) proposed three sections based on habit and the shape and development of the nutlet gynophore. The section *Arctotertiariae* Popov (1953: 609) included perennials with small gynophores. This section was divided into two series: *Vernales* Popov (1953: 609) comprising Eurasian species from forest habitats (*O. verna*, *O. nitida*, *O. cappadocica*); and *Rupestræ* Popov (1953: 613) comprising species from rocky subalpine habitats in Asia Minor and the Caucasus (*O. kusnetzovii* Kolakovsky (1948: 62), *O. lojkae* Sommier & Levier (1892: 157) and *O. rupestris* Ruprecht ex Boissier (1879: 267)). The second section *Pseudoparacaryum* Popov (1953: 616) included annual species with large pyramidal gynophores (represented in the former USSR only by *O. linifolia*). The third section was the monotypic *Maschalanthus* that included only the biennial *O. scorpioides*, distributed in Eastern Europe and possessing a small gynophore.

Recent phylogenetic studies of the Boraginaceae including nine species of *Omphalodes* (Weigend et al. 2013) have revealed its polyphyly, since *O. scorpioides* and the Japanese taxon *O. akiensis* Kadota (2009: 342) appear in independent lineages. The split of *Omphalodes* had already been noted based on morphology and palynology (Popov 1953, Pereira Coutinho et al. 2012). Additionally, the morphologically dissimilar *Myosotydium hortensia* (Decaisne) Bailon (1890: 333), a subantarctic megalherb from Chatham Island, was also placed within *Omphalodes s.s.* in phylogenetic studies (Heenan et al. 2011). All these studies imply the need for further investigation of the systematics of Cynoglosseae. Furthermore, it is notable that to date, little effort has been made to look into morphological characters that support monophyletic groups and boundaries within this tribe.

In the present study we performed a phylogenetic analysis of Cynoglosseae s.l. to evaluate the polyphyly of *Omphalodes* and provided a review of the taxonomy of the genus. In order to accurately analyze key taxonomic characters of *Omphalodes*, such as nutlet structure and ornamentation, we performed a scanning electron microscopy (SEM) study with representative sampling of fruits of Cynoglosseae s.l. based on clades, number of species, distribution areas and nutlet diversity. The main objectives were to: (1) identify monophyletic groups of *Omphalodes* and relatives, (2) find key morphological characters supporting those groups, and (3) propose taxonomic rearrangements needed for a more natural classification of the species.

Materials and methods

Phylogenetic study

**DNA and taxon sampling**

Two DNA regions were selected for the phylogenetic study, the nuclear ITS region and the plastid *trnL–trnF* region (including the *trnl* intron and the *trnL–trnF* spacer). Selection of both regions was based on previous studies (Weigend et al. 2010, Hasenstab-Lehman & Simpson 2012, Nazaire & Hufford 2012, Mozaaffar et al. 2013, Cohen 2014, Weigend et al. 2013). Forty genera (157 spp.) of the tribe Cynoglosseae s.l. were analysed (Appendix S1), which represents 71% of the total number of genera recognised. In total, we analyzed 67 (ITS) and 78 (*trnL–trnF*) sequences taken from previous studies and downloaded from the Genbank database, plus 82 (ITS) and 68 (*trnL–trnF*) samples sequenced specifically for this study (Appendix S1). As a result, taxon sampling was increased and 41 species and three genera that had not previously sequenced were included in our analysis (Appendix S1). Fourteen species of *Omphalodes* were sampled, which represents 50% of the total number of species recognised. Special effort was made to represent *Omphalodes* distributed in the western Palearctic where the main diversity centre occurs, resulting in the inclusion of 11 out of the 16 species in this region. Three more species of *Omphalodes* were analysed (one of eight from America; two of five from Japan). For outgroup samples, we included ITS sequences from 35 species of 23 genera and *trnL–trnF* sequences from 32 species of 22 genera representing the other Boraginaceae tribes and subfamilies. We also included three species of *Nicotiana* Linnaeus (1753: 180) (Solanaceae) from Genbank to root the tree, based on previous results (Nazaire & Hufford 2012) (Appendix S1).

Plant material for DNA extractions was obtained from herbarium specimens and field collections (Appendix S1).
All taxa were newly identified using the available taxonomic bibliography and verified with local floras: Europe (Tutin et al. 1972, Talavera et al. 2012), SW Asia (Riedl 1967, Davis 1978, Nasir 1989), former USSR (Popov 1953), China (Ge-ling et al. 1995), New Zealand (Allan 1961), Australia (Toelken 1986; Jeannes 1999), E Africa (Riedl & Edwards 2006; Thulin & Warfa 2006) and N America (Nesom 2013).

DNA extraction, amplification and sequencing

DNA was extracted from leaf tissue using DNeasy Plant Mini Kits (Qiagen, Valencia, California, USA) following the manufacturers protocol. PCR amplifications were performed in an Eppendorf Mastercycler Epgradient S (Hamburg, Germany). PCR conditions for ITS consisted of initial denaturation at 95 °C for 5 mins followed by 35 cycles of 95 °C for one minute, 48 °C for one minute, 72 °C for one minute, and a final elongation stage of 72 °C for ten minutes. PCR conditions for the trnL–trnF spacer are the same as those of the nuclear ones except for the annealing temperature (50 °C). The volume of genomic DNA was 1µl in both regions. Nested PCRs were needed to amplify old herbarium specimens. The amplifications of the trnL–trnF region were done with primers ‘c’ and ‘f’ (Taberlet et al. 1991). Two internal primers were designed using Geneious 5.4. (Drummond et al. 2011) for nested PCR of the trnL–trnF region: ‘trnL–trnF BOR F’ (5’ CCC GCA ATT AAT AAA AAT GGGC 3’) and ‘trnL–trnF BOR R’ (5’ ATA ATC AGG GGT CTA TGT 3’). The external primers ‘17SE’ and ‘26SE’ (Sun et al. 1994) were used for the amplification of the ITS region, with ‘ITS1’ and ‘ITS4’ (White et al. 1990) used for nested PCRs. PCR products were sequenced using the Macrogen Europe sequencing service (Amsterdam, The Netherlands).

Alignment and phylogenetic analyses

Two matrices were compiled. The ITS matrix included 186 accessions representing 163 spp and included 774 characters (hereafter called “nuclear matrix”). The trnL–trnF matrix comprised 180 accessions, representing 170 species and included 1218 characters (hereafter called “plastid matrix”). Two additional ITS and trnL–trnF reduced matrices were compiled including only the 146 samples for which sequence data was available for both regions.

Sequences were automatically aligned using Fast Fourier transform (MAFFT, Katoh et al. 2002) on the website platform EMBL-EBI (EMBL-EBI, 2013) and manually reviewed using Geneious 5.1.7. (Drummond et al. 2011).

Phylogenetic reconstructions were performed under Bayesian Inference (BI) using MrBayes v.3.2 (Ronquist & Huelsenbeck, 2003) in Biportal (Kumar et al. 2009). The nucleotide substitution model that best fitted each region (trnL–trnF and ITS1, 5.8S and ITS2) was inferred using JModelTest v. 0.1.1. (Posada 2008) The substitution model selected using the Akaike information criterion with correction (AICc) was SYM+G for ITS1 and ITS2, and GTR+G for the 5.8S region. This latter model was also selected for the plastid matrix. Bayesian inference was run for 50x10^6 generations, sampling every 1000 generations in four independent Markov chain Monte Carlo (MCMC). Four BI analyses were run, two with the complete nuclear and plastid matrices and two with the reduced ones.

The Approximate Unbiased test (AU; Shimodaira 2002) was used to explore discordance between the nuclear and plastid phylogenies and test for combinability. The AU test was performed in Treefinder (Jobb et al. 2004, Jobb 2007). The ITS and trnL–trnF majority-rule consensus trees obtained from the BI analyses were compared using 10^6 replicates. Competing hypotheses were rejected at a significance level of 0.05.

Fruit morphology

A carpological study was carried out using Scanning Electron Microscopy (SEM) in order to evaluate morphological support for monophyletic groups in Cynoglosseae s.l. and Omphalodes. An exhaustive description of different fruit traits was performed for the 15 species of Omphalodes studied based on a total of 45 samples (Appendix S2). Although only one of the five Japanese Omphalodes was sampled, documented morphological variation for the species of Omphalodes in the archipelago is very limited (Ka 1965, Yamazaki 1993, Kadota 2009). Three mature nutlets were sampled per specimen, in order to obtain three views: abaxial and adaxial sides, and a cross section (to observe the inner side of the aperture). No prior treatment was done. The specimens were mounted directly onto metal stubs and metalized with gold-coating. Specimens were photographed with a Hitachi S3000N SEM. All photographs were revised and described focusing on six major characters: shape (mm), adaxial surface, scar, abaxial aperture (mm), margin and nutlet abaxial side epidermis (Table 2). In addition, representatives of the four genera recovered as the most closely related to Omphalodes s.s., O. scorpioides and the Japanese clade respectively (Asperugo Linnaeus (1753: 138), Bothriosperrum, Myosotidium, Thysanocarpus Hance (1862; 225); see Results and Figs 1–2) were also characterized in order to find shared traits. Unfortunately, no sample of Mertensia Roth (1797: 34) could be included because we found little material in good condition. We used the exhaustive descriptions of the fruits of this genus given by Popov (1953), as well as SEM photographs from Nazaire & Hufford (2012) that contributed to nutlet descriptions.

Additional observations were performed on 311 herbarium specimens at different states of ripening from eight
herbaria (A, B, M, MA, MBK, MSB, RSA, TEX) using a stereomicroscope in order to extend the SEM sampling. Key characters were evaluated based on taxonomic and phylogenetic results.
FIGURE 1 (continued)

TWO NEW GENERA OF BORAGINACEAE S.S.

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FIGURE 1. Bayesian majority rule consensus tree based on plastid (trnL-trnF) sequences. Numbers below nodes are Bayesian posterior probabilities, some indicated by solid arrows. Major clades are indicated. SEM photographs of species of Omphalodes are shown in their respective clades, some indicated by lined arrows. Scale bar represents the number of substitutions per site and is positioned at the end of the figure.
**TABLE 2.** Main carpological features of the different species of *Omphalodes* studied and the three sister taxa *Asperugo procumbens*, *Bothriospermum*, *Mertensia*, *Myosotidium* and *Thyrocarpus*. The measures provided correspond to the largest sides. Superindex 1 indicates the description of *Mertensia* is based on Popov (1953).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Shape (mm)</th>
<th>Adaxial surface</th>
<th>Scar</th>
<th>Abaxial aperture (mm)</th>
<th>Margin</th>
<th>Nutlet abaxial side epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>North American Taxa</strong></td>
<td></td>
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<tr>
<td><em>O. aliena</em></td>
<td>Ovoid, 2 × 2.5</td>
<td>Densely covered by short papillae and dense short papillose trichomes</td>
<td>Central, deltoid</td>
<td>2 × 1.7</td>
<td>Flat, wide; edge deeply dentate-lobate, both outer and inner sides with short rigid papillose trichomes, crowded in the tips of the lobes</td>
<td>Densely covered by short papillae and short sparse papillose trichomes, flattish</td>
</tr>
<tr>
<td><em>O. cardiophylla</em></td>
<td>Ovoid, 3.5 × 3</td>
<td>Densely covered by short papillae, and dense long smooth trichomes</td>
<td>Central, deltoid</td>
<td>3.5 × 3</td>
<td>Curved inward, wide; edge dentate-lobate; outer side densely covered by long smooth trichomes, inner side densely covered by short papillae</td>
<td>Very densely covered by short papillae and dense long smooth trichomes, with a scarcely prominent central rib</td>
</tr>
<tr>
<td><em>O. chiangii</em></td>
<td>Orbicular, 2.3 × 1.3</td>
<td>Densely covered by scale-like papillae</td>
<td>Central, deltoid</td>
<td>1.5 × 1.3</td>
<td>Strongly curved inward, wide, delimiting an air chamber, but not hollow; edge entire; outer side densely covered by scale-like papillae and long papillae at the top, inner side densely covered short papillae</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
<tr>
<td><strong>Annual European Taxa</strong></td>
<td></td>
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<tr>
<td><em>O. brassicifolia</em></td>
<td>Ovoid, 3.5 × 3</td>
<td>Densely covered by short papillae, and dense long smooth trichomes</td>
<td>Central, deltoid</td>
<td>3.5 × 3</td>
<td>Curved inward, wide; edge dentate-lobate; outer side with dense long smooth trichomes, inner side densely covered by short papillae</td>
<td>Very densely covered by short papillae and dense long smooth trichomes, with a scarcely prominent central rib</td>
</tr>
<tr>
<td><em>O. commutata</em></td>
<td>Subdeltoid, 2.1 × 2.1</td>
<td>Densely covered by short papillae and dense long smooth trichomes</td>
<td>Central, deltoid</td>
<td>1.3 × 1.3</td>
<td>Curved inward, narrow; edge entire; outer side densely covered by short papillae and sparse long smooth trichomes, inner side glabrous and smooth</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Taxon</th>
<th>Shape (mm)</th>
<th>Adaxial surface</th>
<th>Scar</th>
<th>Abaxial aperture (mm)</th>
<th>Margin</th>
<th>Nutlet abaxial side epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. kazinskyanae</em></td>
<td>Orbicular, 4.9 × 4.6</td>
<td>Densely covered by short papillae and sparse hooked trichomes</td>
<td>Central, deltoid</td>
<td>3 × 2.4</td>
<td>Curved inward, wide; edge slightly undulated; outer side densely covered by short papillae and sparse hooked trichomes, inner side smooth and glabrous</td>
<td>Densely covered by short papillae and sparse trichomes at the center, glabrous and wrinkled towards the edges, flattish</td>
</tr>
<tr>
<td><em>O. linifolia</em></td>
<td>Orbicular, 2.78 × 2.68</td>
<td>Densely covered by short papillae and disperse smooth trichomes, with dense long smooth trichomes around the scar</td>
<td>Central, deltoid</td>
<td>1.58 × 1.56</td>
<td>Curved inward, wide, hollow in its upper edge and delimiting a small air chamber; edge dentate, with ribs that ends on each tooth; outer side densely covered by short papillae, and long papillae towards the tips of the teeth, inner side glabrous, somewhat rough at the innermost border</td>
<td>Densely covered by short papillae, glabrous, with a scarcely prominent central rib</td>
</tr>
<tr>
<td><em>O. littoralis</em></td>
<td>Ovoid to deltoid, 2.8 × 2.7</td>
<td>Reticulate, with sparse long flat trichomes</td>
<td>Central, deltoid</td>
<td>1.9 × 2.2</td>
<td>Curved inward, wide; edge dentate; outer side densely covered by short papillae and sparse hooked trichomes; inner side smooth and glabrous</td>
<td>Densely covered by short papillae towards the edges, sparse long hooked trichomes at the center, flattish</td>
</tr>
<tr>
<td>Perennial Western Palearctic Taxa</td>
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<td></td>
</tr>
<tr>
<td><em>O. caucasica</em></td>
<td>Orbicular, 2.1 × 2.1</td>
<td>Densely covered by short papillae, and sparse long papillose trichomes</td>
<td>Central deltoid</td>
<td>1.9 × 1</td>
<td>Curved inward, wide; edge deeply dentate-lobate; outer side densely covered by long papillae and dense long trichomes crowded at the tips, inner side glabrous and smooth</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
<tr>
<td><em>O. cappadocica</em></td>
<td>Subdeltoid, 2.1 × 2.1</td>
<td>Densely covered by short papillae and dense long smooth trichomes</td>
<td>Central deltoid</td>
<td>1.3 × 1.3</td>
<td>Curved inward, narrow; edge entire; outer side densely covered by short papillae and sparse long smooth trichomes, inner side glabrous and smooth</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Taxon</th>
<th>Shape (mm)</th>
<th>Adaxial surface</th>
<th>Scar</th>
<th>Abaxial aperture (mm)</th>
<th>Margin</th>
<th>Nutlet abaxial side epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. luciliae</em></td>
<td>Ovoid, 2.9 × 1.6</td>
<td>Densely covered by short papillae and very sparse short papillose trichomes</td>
<td>Central, deltoid</td>
<td>2.7 × 1.5</td>
<td>Curved inward, narrow; edge entire; both outer and inner sides densely covered by short papillae</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
<tr>
<td><em>O. nitida</em></td>
<td>Ovoid, 2.8 × 2.3</td>
<td>Densely covered by short papillae and papillose spinulae that make transition to sparse papillose trichomes</td>
<td>Central, deltoid</td>
<td>2.7 × 1.9</td>
<td>Curved inward, wide; edge deeply dentate-lobate; outer side with sparse short papillose trichomes and spinules, crowded at the tips of the lobes, inner side glabrous and smooth</td>
<td>Densely covered by short papillae and sparse spinulae, glabrous, flattish.</td>
</tr>
<tr>
<td><em>O. verna</em></td>
<td>Orbicular, 2.1 × 1.7</td>
<td>Densely covered by short papillae and sparse long smooth trichomes</td>
<td>Central, deltoid</td>
<td>1.5 × 1.3</td>
<td>Curved inward, narrow; edge entire; outer side densely covered by short papillae and sparse long smooth trichomes, inner side reticulate and glabrous</td>
<td>Densely covered by short papillae, disperse long smooth trichomes, flattish</td>
</tr>
<tr>
<td>Japanese Taxon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. japonica</em></td>
<td>Orbicular, 2.4 × 2.1</td>
<td>Densely covered by short papilllose trichomes</td>
<td>Apical, deltoid</td>
<td>0.7 × 0.6</td>
<td>Strongly curved inward, hollow and delimiting an air chamber in its entire width, very wide; edge entire; outer side densely covered by short papillose trichomes and dispserse long spines in its external half, which is dark when ripe, that sharply changes towards the aperture to wrinkled and with sparsely covered by short papillae, whitish when ripe, inner side smooth and glabrous</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
<tr>
<td>Biennial European Taxon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. scorpioides</em></td>
<td>Orbicular, 2.8 × 2.5</td>
<td>Sparse short papillae and short, nearly smooth, trichomes</td>
<td>Apical, deltoid</td>
<td>2.5 × 1.5</td>
<td>Curved inward, wide, hollow and delimiting an air chamber in its entire width; edge entire; both outer and inner side with, sparse trichomes, rough in the innermost border</td>
<td>Sparsely covered by short papillae and long, nearly smooth, trichomes, flattish</td>
</tr>
</tbody>
</table>

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Table 2. (Continued)

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Shape (mm)</th>
<th>Adaxial surface</th>
<th>Scar</th>
<th>Abaxial aperture (mm)</th>
<th>Margin</th>
<th>Nutlet abaxial side epidermis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other taxa associated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asperugo</td>
<td>3.5 × 2</td>
<td>Dense short papillae, compound papillae</td>
<td>Subapical, rounded</td>
<td>Flat narrow keel, densely covered by short papillae</td>
<td>Densely covered by short papillae, compound papillae</td>
<td></td>
</tr>
<tr>
<td>Bothriospermum</td>
<td>Ovoid to reniform, 2 × 1</td>
<td>Dense short and long papillae, compound papillae</td>
<td>Apical, linear</td>
<td>0.5 × &lt;0.4</td>
<td>Curved inward, narrow, edge entire, both outer and inner side densely covered by short papillae. Presence of an inner free layer wrinkled with disperse short smooth trichomes</td>
<td>Densely covered by short papillae, glabrous, flattish</td>
</tr>
<tr>
<td>Mertensia</td>
<td>Tetrahedral, 3 to 5 in length</td>
<td>Rugose or grumose, glabrous, keeled.</td>
<td>Basal, rounded</td>
<td>Absent</td>
<td>Rarely narrow wing with prickles</td>
<td>Rugose or grumose, glabrous</td>
</tr>
<tr>
<td>Myosotidium</td>
<td>Deltoid, 10 × 14</td>
<td>Slightly wrinkled</td>
<td>Central deltoid</td>
<td>9 × 10</td>
<td>Flat or curved outward, irregular in width; edge entire, irregular; both outer and inner sides smooth, slightly wrinkled, and glabrous</td>
<td>Slightly wrinkled, glabrous, flattish</td>
</tr>
<tr>
<td>Thyrocarpus</td>
<td>Ovoid to reniform, 2 × 1.4</td>
<td>Dense short and long papillae, compound papillae</td>
<td>Apical, linear</td>
<td>1.75 × 0.5</td>
<td>Curved inward, wide, edge dentate, both outer and inner side densely covered by short papillae, longer in tips. Presence of an innermost free layer wrinkled with sparse short smooth trichomes</td>
<td>Apparently missed</td>
</tr>
</tbody>
</table>

Results

Phylogenetic reconstructions

Our plastid (Fig. 1) and nuclear (Fig. 2) phylogenetic reconstructions are mainly in agreement with previous phylogenies of Boraginaceae (Långström & Chase 2002, Långström & Oxelman 2003, Weigend et al. 2010, Nazaire & Hufford 2012, Weigend et al. 2013, Cohen 2014). Results from the AU test reveal that the ITS topology is not rejected by the plastid dataset (difference in – LnL = 32.171, p = 0.9), whereas the plastid topology is rejected by the ITS dataset (difference in – LnL = 748.790, p < 0.001). Four topological incongruences were detected between the plastid and nuclear trees. One of these incongruences affects the internal resolution of our study group (O. verna, O. nitida, see below; Figs. 1–2). The remaining three incongruences will not be further discussed since they do not affect our study group (see Symphytum Linnaeus (1753: 136), Borago Linnaeus (1753: 136) and Harpagonella A. Gray (1876: 88) in Figs. 1–2). Because of these results, we did not perform a combined analysis. Accordingly, the phylogenetic results based on the nuclear and plastid datasets are shown independently.

The polyphyly of Omphalodes (Omphalodes s.l.) is supported by our phylogenetic analyses since three well-supported clades are consistently recovered in the nuclear and plastid trees (Figs. 1–2): (1) the Omphalodes s.s. clade, (2) O. scorpioides, and (3) the Japanese Omphalodes clade.
FIGURE 2 (continued)

Trichodesma calcaratum
Trichodesma calycosum
Trichodesma incanum
Trichodesma aucheri

Omphalodes brassicifolia
Omphalodes linifolia
Omphalodes commutata
Omphalodes kazinskianae
Omphalodes littoralis subsp. gallaecia

Myosotidium hortensia
Omphalodes aliena

Omphalodes luciae
Omphalodes luciae
Omphalodes cappadocica
Omphalodes caucastica
Omphalodes nitida
Omphalodes verna
Omphalodes verna

Omphalodes scorpioides (Menorema scorpioides) O. scorpioides clade

Asperugo procumbens
Mertensia alpina
Mertensia oblongifolia subsp. nevadensis
Mertensia davurica
Mertensia sibirica
Mertensia virginica
Mertensia maritima
Mertensia maritima
Ogastemma pusillum
Echiochilum fruticosum
Echiochilum lithospermoides
Echiochilum persicum

Cynoglossae s.l.
Echiochilae
Mertensio-clade
FIGURE 2 (continued)

Symphytum bornmuelleri
  - Symphytum bulbosum
    - Brunnera sibirica
      - Brunnera orientalis
    - Pentaglotis sempervirens
      - Achusa italicca
        - Cynoglotris chetikiana subsp. paphagonica
      - Borago officinalis
        - Achusa formosa
        - Achusa crispa
    - Echinum vulgare
      - Echinum creticum
        - Alkanna sieberi
          - Alkanna tinctoria
        - Arnebia decumbens
    - Neotestma apium
      - Lithodora fruticosa
        - Cerinthe minor subsp. auriculata
          - Cerinthe major
        - Buglossoides calabra
          - Glandora diffusa
            - Buglossoides gastomi
        - Lithosperrum cinereum
        - Lithosperrum officinale
          - Onosmodium occidentale
          - Onosmodium virginianum
    - Plagiobothrys hispidus
      - Harpagonella palmieri
    - Pectocarya peninsularis
      - Pectocarya penicillata
    - Eremocarya microtricha
        - Cryptantha flavoculata
          - Oreocarya humilis
            - Oreocarya weberi
FIGURE 2 (continued)

- Actinocarya tibetica
  - Microula floribunda
  - Microula floribunda
  - Microula floribunda
  - Microula floribunda
  - Microula pseudotrichocarpa
    - Microula diffusa
    - Microula turbinata
    - Microula forrestii
    - Microula ovalifolia
    - Microula miliensis
    - Microula oblongifolia
    - Microula sikkimensis
    - Microula myosotidea
    - Microula ovalifolia
    - Microula diffusa
    - Microula stenophylla
    - Microula stenophylla
    - Microula sikkimensis
    - Microula younghusbandii
    - Microula pseudotrichocarpa
      - Microula tibetica var. tibetica
    - Microula tibetica
    - Microula pseudotrichocarpa
    - Microula pseudotrichocarpa
    - Cryptantha granulosa
      - Cryptantha maritima
        - Plagiobothrys jonesii
          - Cryptantha minima
            - Cryptantha nevadensis
            - Cryptantha foliosa
            - Cryptantha affinis
            - Cryptantha diffusa
              - Amsinckia calycina
                - Amsinckia eastwoodiae
                - Amsinckia intermedius
                  - Amsinckia spectabilis
                    - Johnstonella argustifolia
                      - Johnstonella parviflora
                        - Johnstonella racemosa

**FIGURE 2 (continued)**

Bayesian majority rule consensus tree based on nuclear (ITS) sequences. Numbers below nodes are Bayesian posterior probabilities, some indicated by solid arrows. Major clades are indicated. SEM photographs of species of *Omphalodes* are shown in their respective clades, some indicated by lined arrows. Scale bar represents the number of substitutions per site and is positioned at the end of the figure.
The plastid reconstruction reveals that *Omphalodes* accessions are placed in three independent well-supported clades (1 BPP each, Fig. 1). In particular, the Japanese clade is placed within Cynoglosseae s.s. and the remaining two clades (*Omphalodes* s.s. and *O. scorpioides*) appear in a basal polytomy of Cynoglosseae s.l. together with the Eritrichiae clade and the *Mertensia* clade (*Mertensia* and *Asperugo*). The *Omphalodes* s.s. clade includes all eleven of the Mediterranean and western Asia annual and perennial species studied plus *Myosotidium* (1 BPP, Fig. 1). Within this clade, the Mediterranean perennial species *O. verna* and *O. nitida* form a monophyletic group in a basal polytomy, together a subclade including the remaining species of *Omphalodes* s.s. It contains the five annual species from the Western Mediterranean clustering in a well-supported group (1 BPP, Fig. 1), while the Mediterranean and West Asian perennial species, *O. aliena* A.Gray ex Hemsl (1882: 377) and *Myosotidium* are unresolved. The second main clade of *Omphalodes* s.l. includes the two samples of the biennial species *O. scorpioides* (1 BPP, Fig. 1). Finally, the two Japanese species of *Omphalodes* form a clade (1 BPP, Fig. 1), and they group together with two Asian species (*Thyrocarpus sampsonii* Hance (1862: 225) and *Bothriospermum secundum* Maximowicz (1859: 202); 1 BPP, Fig. 1).

The nuclear tree also shows three independent clades congruent with those of the plastid phylogeny (Fig. 2): the *O. scorpioides* clade is sister to the *Mertensia* clade (1 BPP, Fig. 2), *Omphalodes* s.s. is shown to be an independent clade and the Japanese clade is related to nine genera of Cynoglosseae s.s. (Fig. 2). In contrast to the plastid topology, *Omphalodes* s.s. has a Mediterranean annual subclade (1 BPP, Fig. 2) sister to a subclade of the remaining species of *Omphalodes* s.s. (Mediterranean peripherals, America, western Asia) plus *Myosotidium hortensis* (New Zealand) (0.99 BPP, Fig. 2).

**Fruit morphology**

Descriptions of nutlets of the 15 species of *Omphalodes* sampled, as well as those of the five closest relative genera are shown based on the three *Omphalodes* s.l. clades obtained in the phylogenetic analyses (see above).

Nutlets in Boraginaceae s.s. shows two markedly differentiated adaxial and abaxial surfaces. The nutlet is inserted into the gynobase producing an attachment scar. Its shape and position along the length of the adaxial face, remains constant among most of the species of the genus except for the Japanese *Omphalodes* and *O. scorpioides* (see below). The abaxial side of the fruits of all *Omphalodes* displays a discontinuity of the exocarp resulting in a rounded aperture of varying sizes and a more or less curved aperture margin (Fig. 3A–AW). However, the North American *O. aliena* and *O. cardiophylla* A. Gray ex Hemsl (1882: 377), and Eurasian *O. brassicifolia* Sweet (1826: 293) and *O. luciliae* Boissier (1844: 41) lack such a discontinuity and thus the mesocarp is completely covered by the exocarp. This carpological characteristic was used to define and group species of *Omphalodes*. In the present study, we refer to the base of abaxial aperture as abaxial surface. All the structures mentioned can appear heterogeneously ornamented with different types and densities of trichomes, papillae or spines. In addition to traits already reported in previous studies, for the first time, an air chamber has been observed within the margin of the nutlets in four species of *Omphalodes* (*O. linifolia*, *O. chiangii* Higgins (1976: 412), *O. scorpioides* and *O. japonica* Maximowicz (1872: 452)). *Omphalodes linifolia* forms a small chamber (Fig. 3U) by the ripping of the mesocarp from the exocarp at the top of the margin. In *Omphalodes scorpioides* and *Ophalodes japonica* the air chamber completely occupies the margin, which resembles a floater (Fig. 3AV, 3AS). In contrast, in *Omphalodes chiangii* the margin is actually solid, but it is strongly incurved delimiting the air chamber (Fig. 3H). The air chamber is delimited by a mesocarpic ‘wrapping tissue’-like surface, except in *O. chiangii* that seems to be entirely exocarpic. The significant SEM traits commented above, were also observed on the herbarium specimens studied under the stereomicroscope, with special attention paid to three additional species from Japan. The characteristics of the three clades of *Omphalodes* s.l. are discussed below. A summary table with all the six traits described from the 15 species is provided (Table 2).

**Omphalodes s.s. clade**

The nutlets of the *Omphalodes* s.s clade are suborbicular (or ovoid-subdeltoid), 2–3 (4) × 1–3 (4) mm (Fig. 3A–3AW). Annual European taxa have bigger nutlets (c. 3 mm in diameter) than those of European peripherals (2 mm) (Fig. 3I–X, 3Y–AP). The nutlets of North American species are ovoid to orbicular, 2 (3) × 1–3 mm. The New Zealand genus (*Myosotidium hortensis*) embodied in this clade is morphologically larger in all its parts, including bigger deltoid nutlets (10 × 14 mm). The adaxial nutlet surface of taxa in the *Omphalodes* s.s. clade is densely to sparsely covered by papillae (e.g. Fig. 3L, 3T, 3AJ), and rigid (Fig. 3Z) to hooked (Fig. 3W–X) trichomes. The papillae are conspicuous and range from long towards the edges to short in the central parts of the nutlet (Fig. 3AJ). Trichomes can be papillose or more or less smooth (Fig. 3L, 3AF, 3AL). The endemic North American *Omphalodes chiangii*, unfortunately not sequenced for this study, displays papillae ridges densely covering both adaxial and abaxial sides (Fig. 3I). The nutlets of *M. hortensis* have smooth to slightly wrinkled surface either in the adaxial or abaxial sides as in its margin (Fig. 3BB–BC).
The scar is subdeltoid and it is placed in the centre of the adaxial side (Fig. 3O, 3R, 3T, 3W, 3Z, 3AJ). *Myosotidium hortensia* has the broadest deltoid scar covering nearly all of the adaxial side.

The abaxial aperture is 1–3 × 1–2 (3) mm in the species ascribed to *Omphalodes*, whereas the largest aperture is found in *Myosotidium* (*M. hortensia*, 9 × 10 mm). Major differences between species are found on the edge, curvature and width of the margin, including ornamentation of the epidermis. The edge of the margin varies from entire (Fig. 3F, 3N, 3Y, 3AG, 3AN) to dentate or lobate (Fig. 3A, 3D, 3J, 3S, 3V, 3AD, 3AI). The margin varies from narrow and slightly curved, barely covering the aperture borders (Fig. 3N, 3Y, 3AG, 3AN) to wider than the nutlet body (Fig. 3U), hardly curved and covering nearly one-third of the aperture (Fig. 3Q). The margin can be incurved and covering the aperture (Fig. 3H, 3J, 3AI) to nearly flat and exposing entirely the abaxial surface as in *Omphalodes aliena* (Fig. 3A). All *Omphalodes* taxa have a solid margin except *O. linifolia*, in which the upper part of the margin displays a small air chamber (Fig. 3U). The epidermis on the external surface is usually sparsely to densely covered by papillae and/or straight (Fig. 3AC) to hooked (Fig. 3V) trichomes. These trichomes vary from smooth (Fig. 3AE) to papillose. Sometimes dentate margins become crowded of trichomes (Fig. 3B) towards the edge. The internal surface of the margin is usually glabrous and smooth, especially in those species that have their margins curved inwards (Fig. 3U, 3AB, 3AK, 3AP). The ornamentation of the margin surfaces in the North American studied taxa and the Palearctic *O. brassicifolia* and *O. luciliae* is similar in both internal and external surface (Fig. 3A, 3D, 3H, 3J, 3K). The margin of *M. hortensia* is irregular in width, and is either flat or curved outwards.

The abaxial surface is generally flattish, except for *Omphalodes linifolia* and *Omphalodes brassicifolia* that display a scarcely prominent central rib (Fig. 3K, 3U). The ornamentation of the epidermis at the base of the aperture on the abaxial side usually consists of short papillae, sometimes with sparse trichomes (Fig. 3C, 3AA, 3AM, 3AO).

Omphalodes scorpioides clade and sister group

The nutlets of Omphalodes scorpioides (Fig. 3AU–AW) are orbicular and 2.8 × 2.5 mm. The adaxial side has sparse short papillae and short smooth trichomes (Fig. 3AW). The scar is deltoid in shape and it is in an apical position, which is distinct from the remaining European taxa of the Omphalodes s.s. clade. The margin of O. scorpioides nutlets is deeply inwardly curved, partially covering the abaxial aperture (Fig. 3AU, 3AV). Smooth trichomes occur sparsely on both the outer and inner sides of the margin (Fig. 3AV). Ornamentation of the abaxial aperture displays short papillae and smooth trichomes (Fig. 3AV). This species is also characterized by an air chamber that fills the entire margin (Figs. 3AU–AV).
Mertensia and Asperugo constitute the sister group of O. scorpioides. The nutlets of these taxa are laterally compressed and thus the adaxial and abaxial sides are substituted by ventral and dorsal sides respectively. The studied sample Asperugo procumbens Linnaeus (1753: 138) displays an ovoid, 3.5 × 2 mm nutlet. Both lateral sides are densely covered by fine papillae grouped together and forming prominences and wrinkles (Fig. 3BH). The rounded scar is subapical and ventrally positioned. The flat narrow margin is densely covered by short papillae on the dorsal side (Fig. 3BG). The nutlets of Mertensia are tetrahedral, 3–5 mm in length (Popov 1953, Nazaire & Hufford 2012). As indicated by these authors, both lateral sides are rugose or grummose. The rounded scar is basal and ventrally positioned. Rarely, a narrow wing with prickles is observed.

Japanese clade and its closest relatives

Significant morphological differences have been observed between the species of the Japanese clade and the species of the other two clades of Omphalodes. Despite the single specimen of this clade included in the SEM study, the diagnostic traits detected were confirmed in all the other Japanese samples in the stereomicroscope study.
The nutlets of *O. japonica* (Fig. 3AQ–AT) are orbicular and 2.4 × 2.1 mm. The adaxial side is homogeneously and densely covered by short papillose trichomes (Fig. 3AR). The scar is deltoid and located in an apical position (Fig. 3AR). The margin is strongly inwardly curved and covers most of the abaxial aperture (0.7 × 0.6 mm) (Fig. 3AQ, 3AS). A unique trait is the differentiation of two distinct parts of the margin: (1) the part that is closer to the body of the nutlet is characterized by a dark stony surface with dense short papillose trichomes and dispersed long spines on its external half; and (2) the part of the margin more distant from the body of the nutlet is whitish, papiraceous wrinkled
The sister group of the Japanese clade (*Bothriospermum* and *Thyrocarpus*), displays some similar traits. The fruits of *Bothriospermum zeylanicum* Druce (1917: 610) are reniform and 1 × 1 mm (Fig. 3AX). The nutlet of this species is adaxially orientated contrary to the rest of the species. The scar is apical but completely displaced to the top of the nutlet, which together with the turning of the nutlet has been erroneously interpreted as a basal position (Ge-ling et al. 1995). The abaxial side is densely covered by short and long grouped papillae (Fig. 3BA). The margin is narrow, curved inwards with the entire edge densely covered by short papillae (Fig. 3AY, 3BA). As in *O. japonica*, the nutlet has a wrinkled layer that surrounds the aperture, with some short, sparse and smooth trichomes (Fig. 3AZ). This structure
of Bothriospenum is similar to the inner side of the margin observed in O. japonica (see above), but in this genus the layer is free. The ornamentation of the adaxial side is glabrous, flatish and also densely covered by short papillae. The fruit of Thysanocarpus glochidiatus is from ovoid to reniform and 2 x 1.4 mm (Fig. 3BD). The ornamentation of the adaxial side is similar to that of B. zeylanicum (Fig. 3BE). The margin is wide, curved inwards, with a deeply dentate edge (Fig. 3BD) and densely covered by short papillae that are longer at tips (Fig. 3BD). As in Bothriospenum, there is an inner free layer of wrinkled tissue, with sparsely short, smooth trichomes (Fig. 3BF). Interestingly, the abaxial side of the pericarp seems to be lost or extremely reduced, unlike all other samples of the studied genera (Fig. 3BF).

Discussion

Our phylogenetic results agree with most recent molecular phylogenies of Boraginaceae s.s. (Långström & Chase 2002, Långström & Oxelman 2003, Weigend et al. 2010, Nazaire & Hufford 2012, Weigend et al. 2013, Cohen 2014). The tribe Cynoglosseae s.l., as recently conceived (Långström & Chase 2002, Långström & Oxelman 2003, Weigend et al. 2010, Nazaire & Hufford 2012, Weigend et al. 2013, Cohen 2014), is formed by morphologically heterogeneous groups that were consistently recovered in the plastid (Fig 1), but not in the nuclear trees (Fig 2). The accessions of Omphalodes included in our analyses are placed in Cynoglosseae s.l. in the plastid tree, but not all of them are resolved in this group in the nuclear tree.

Three independent lineages of Omphalodes

Omphalodes is split into three independent lineages in agreement with Weigend et al. (2013). Previous studies already noted the heterogeneity of the genus. Indeed, De Candolle (1846) and Brand (1921) already segregated O. scorpioides from the remaining European taxa at sectional level. Later, Popov (1953) indicated that the Japanese taxa were morphologically distinct to the remaining species of the genus, and O. scorpioides was dissimilar to the remaining European Omphalodes in morphological and embryological features (see below). Pereira Coutinho et al. (2012) also noted the distinctiveness of the pollen of the Japanese species which have a ring-like equatorial aperture, absent in the other taxa. They also found differences in the pollen morphology between the Old World species of Omphalodes s.s. (margins granulate) and the New World taxa (margins smooth). However, they did not observe significant differences between the pollen of O. scorpioides and that of the other European species except that the pollen of O. scorpioides is more compact and globose.

A minor incongruence between plastid and ITS reconstructions was detected within Omphalodes s.s. Concerning the different placement of two European perennial species (O. verna and O. nitida). Only the clade containing the annual taxon of Omphalodes is recovered in both phylogenetic reconstructions (Figs. 1–2). However, no unique characters can be readily found to define any internal grouping within the Omphalodes s.s. clade (see Fig. 3A–AP). In contrast, the central deltoid attachment scar and the solid margin are found in all the species (Table 2). The low number of American species herein included (one (molecular) and three (carpological) species studied out of the total six American species)—prevented us from proposing a more solid phylogenetic hypothesis. Pereira Coutinho et al. (2012) reported common traits in the pollen for all the American species. In contrast, more variability was found in the nutlets of American species, in which O. chiangii has the most dissimilar ornamentation while O. cardiphylla and O. aliena are very similar (Fig 3A–E). The unique carpological traits displayed by O. chiangii (Fig. 3F–I), and the distinctive morphology of O. erecta from northeastern Mexico (erect habit, greater nutlet size (8 mm in width), three of four nutlets aborted and a distinct slightly dentate spreading wing; Johnston 1935), indicate that further taxonomic studies of North American species are necessary as previously suggested by Nesom (2013). The phylogenetic placement of the Oceanic megaherb Myosotidium hortensia within the Omphalodes s.s. clade is in agreement with previous molecular results (Heenan et al. 2011, Nazaire & Hufford 2012, Cohen 2014, Mozaffar et al. 2013, Weigend et al. 2013). There is a single trait shared between Myosotidium and the Omphalodes s.s. clade, namely the marginal wing of the nutlet. The findings of Weigend et al. (2013) and Nesom (2013), in which South American members of Cynoglossum (with no marginal wing) are nested within the Omphalodes s.s. clade, prevented us from proposing the transference of Myosotidium to Omphalodes s.s. One more argument against proposing a new nomenclatural combination is the poor molecular sample of North American species (one of eight). This is an important issue since a great morphological heterogeneity has been found within North American species (Nesom 2013). An extended sample in terms of species and DNA regions will help elucidate the systematics of this group.

The biennial European O. scorpioides is distantly related to Omphalodes s.s. (Fig. 2) because it forms an independent
lineage sister to the Mertensia clade in the ITS tree (Fig. 2). Dense low papillae on the nutlet epidermis is the single character shared by O. scorpioides and the other two genera of its sister group (Asperugo and Mertensia). Indeed, the nutlets of O. scorpioides are fairly distinct from those of Asperugo and Mertensia (Fig. 3AV) and the nearly smooth trichomes (Fig. 3AU-AW) readily distinguish this taxon from the species of the Omphalodes s.s. clade. Some morphological characters, such as the flower disposition along the stems in contrast to terminal inflorescences, and the curved rather than erect embryo (Popov 1953), are additional characters that support the independence of O. scorpioides from Omphalodes s.s. The distinction of O. scorpioides from the remaining taxa of the genus was already indicated by previous authors on the basis of morphological (De Candolle 1846, Brand 1921, Popov 1953) and molecular studies (Cohen 2014; Weigend et al. 2013). Our results help to resolve the sister group and morphological support for the lineage Asperugo-Mertensia-O. scorpioides. O. scorpioides is readily distinguished from Asperugo and Mertensia by characters such as nutlet shape, ornamentation and presence of aperture.

The third clade comprises the Japanese species of Omphalodes. The sample of the plastid phylogeny, which is more complete, reveals its monophyly (Fig. 1). The plastid tree reveals a sister-group relationship of the Japanese Omphalodes with a lineage of two Asian genera (Bothriospermum secundum and Thyrocarpus sampsonii) (Fig. 1), all three of which are placed within Cynoglosseeae s.s., in agreement with Weigend et al. (2013). In addition, they displayed a unique character within Omphalodes s.l.: the two distinct parts of the nutlet margin (see above, Fig. 3AQ). This character was already emphasized in the taxonomic treatment for the Flora of Japan (Yamakazi 1993). Such smooth prolongation seems to be analogous to that found in B. secundum and T. sampsonii as a free inner layer. Other traits that differentiate the fruit of the Japanese Omphalodes from the taxa included in Omphalodes s.s. are the hollow margin completely filled by an air chamber, and the upper position of the attachment scar. Similarly, Thyrocarpus and Bothriospermum have an apical scar. Remarkably, the attachment scar of Bothriospermum has been interpreted to be in a basal position (Ge-ling et al. 1995). However, a close observation shows that the nutlet is completely turned inward, leaving the aperture in an adaxial position, and thus apparently attached by its base.

Taxonomic proposal splitting Omphalodes s.l.

This study is based on a representative sample of Omphalodes. Three independent lineages consistently found in the nuclear and plastid phylogenetic reconstructions (Figs. 1–2) are supported by morphological characters, which should be taxonomically acknowledged. The fruit study provided a detailed source of information that support the partition of Omphalodes, as character states are shared by taxa within each of the three lineages. As a result, a reorganization of Omphalodes s.l. is partly feasible at least for O. scorpioides and the Japanese taxa.

Other names used at the generic level to refer to Omphalodes taxa are Picotia Roemer & Schultes (1819: 10) and Omphalium (Wallr.) Roth (1827: 590). Picotia is an illegitimate name (nomen superfluum) since it explicitly cited Omphalodes as synonym and included Omphalodes verna, which is the type species of the genus Omphalodes (van Ooststroom et al. 1961). Omphalium has Cynoglossum sect. Omphalium Wallr. as basinym, which is a legitimate name. However, this name is also taxonomically superfluous at the genus level because the protologue of its basiosym also cited Omphalodes as a synonym and included O. verna. Omphalodes scorpioides has been included in the monotypic section Maschalanthus (De Candolle 1846, Brand 1921, Popov 1953); however at the genus level Maschalanthus would be illegitimate, as it would be a later homonym of the moss genus Maschalanthus Sprengel ex Schultz (1806: 356). As far as we know, the Japanese species do not have any previous taxon recognition at the genus level, as most of the species are of recent description.

As a result, we propose the creation of two new genera to accommodate O. scorpioides from Europe (Memoremea) and the Japanese species of Omphalodes (Nihon).

Nomenclature


= Picotia Roemer & Schultes, (1819: 10), nom. superfl. Lectotype here designated:—Picotia verna (Moench) Roemer & Schultes (= Omphalodes verna Moench)

= Cynoglossum sect. Omphalium Wallroth (1822: 77). Lectotype here designated:—Cynoglossum omphalodes L. (= Omphalodes verna Moench)

= Omphalium (Wallr.) Roth (1827: 590)
TABLE 3. Key characters defining the three independent lineages of *Omphalodes* s.l.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Habit</th>
<th>Inflorescences</th>
<th>Pollen</th>
<th>Nutlet scar position</th>
<th>Nutlet margin</th>
<th>Embryo position</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Omphalodes</em> s.s.</td>
<td>Annual/Perennial</td>
<td>Terminal</td>
<td>Oblong, no ring-like aperture</td>
<td>Central</td>
<td>Solid, differently ornamented</td>
<td>Erect</td>
</tr>
<tr>
<td><em>Omphalodes scorpioides</em></td>
<td>Biennal</td>
<td>Axilar</td>
<td>Globose, no ring-like aperture</td>
<td>Apical</td>
<td>Hollow, homogeneously ornamented (smooth trichomes)</td>
<td>Curved</td>
</tr>
<tr>
<td><em>(Memoremea scorpioides)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese species (Nihon)</td>
<td>Perennial</td>
<td>Terminal</td>
<td>Oblong, ring-like aperture</td>
<td>Apical</td>
<td>Hollow, abrupt ornamentation change towards the edge (hairy-spiny to wrinkled and nearly smooth)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

**Memoremea** Otero, Jim.-Mejías, Valcárcel & P. Vargas gen. nov. Type:—*Memoremea scorpioides* (Haenke) Otero, Jim.-Mejías, Valcárcel & P. Vargas = *Omphalodes* sect. *Maschalanthus* De Candolle (1846:161)

**Description.** This new genus resembles *Omphalodes* in nutlet shape and size, from which it can be distinguished by the apical attachment scar, the strongly incurved entire, wide and hollow margin of the nutlet with an air chamber, and the presence of smooth trichomes on both adaxial and abaxial sides of the nutlet. In addition, the more compact and globose pollen grains, and axillary inflorescence distinguish *Memoremea* from *Omphalodes* s.s.

**Etymology.** The Latin phrase “Memore me” which means “Remember me”, which would be complementary to “Forget-me-not”, the common name that refers to some species of the tribe Cynoglosseae in many languages, especially species of the genus *Myosotis*.

**Memoremea scorpioides** (Haenke) Otero, Jim.-Mejías, Valcárcel & P. Vargas, **comb. nov.**

≡ *Cynoglossum* scorpioides Haenke (1788: 3) (basionym)
≡ *Omphalodes* scorpioides (Haenke) Schrank (1812: 222)

**Nihon** Otero, Jim.-Mejías, Valcárcel & P. Vargas gen. nov. Type:—*Nihon japonicum* (Maxim.) Otero, Jim.-Mejías, Valcárcel & P. Vargas

**Description.** This new genus resembles *Omphalodes* in nutlet shape and size, from which it can be distinguished by the ornamentation of the margin of the nutlet, which abruptly changes towards the aperture from hairy-spiny to wrinkled and nearly smooth resulting in two distinctive parts. In addition, the nutlet scar is found apically and the margin is completely hollow by an extensive air chamber. An additional key character to distinguish this new genus is the presence of a ring-like equatorial aperture on the pollen grains, that is absent in the rest of species of *Omphalodes*.

**Etymology.** Nihon is the name of Japan in Japanese as written in Latin alphabet.

**Nihon japonicum** (Thunb.) Otero, Jim.-Mejías, Valcárcel & P. Vargas **comb. nov.**

≡ *Cynoglossum* japonicum Thunberg (1784: 187) (basionym)
≡ *Omphalodes* japonica (Thunb.) Maximowicz (1872: 452)

**Nihon akiensis** (Kadota) Otero, Jim.-Mejías, Valcárcel & P. Vargas **comb. nov.**


**Nihon krameri** (Franch. & Sav.) Otero, Jim.-Mejías, Valcárcel & P. Vargas **comb. nov.**

≡ *Omphalodes* krameri Franchet & Savatier (1879: 452) (basionym).

**Nihon laevispermum** (Nakai) Otero, Jim.-Mejías, Valcárcel & P. Vargas **comb. nov.**

≡ *Omphalodes* laevisperma Nakai (1949: 17) (basionym).

**Nihon proliferum** (Ohwi) Otero, Jim.-Mejías, Valcárcel & P. Vargas **comb. nov.**

≡ *Omphalodes* prolifera Ohwi (1956: 98) (basionym).
Conclusions

The phylogenetic reconstructions of our study, coupled with morphological characters of the nutlet, help us to propose a more natural classification of *Omphalodes* species. The inclusion of 14 of the 29 recognized species of *Omphalodes* in our phylogenetic study, clearly supports the polyphylly of the genus. Indeed, we found three independent lineages, which were consistent with results from recent publications. The morphological nutlet differences herein found provided further support for the three lineages of *Omphalodes*. In addition, vegetative and reproductive (inflorescence, pollen ornamentation) characters used in previous taxonomic treatments give solid grounds to recognise three genera, two of them newly proposed: *Memoremea* and *Nihon*. Despite the considerable sampling effort made for this study, additional investigations are needed to infer phylogenetic relationships of all the species of *Omphalodes* from North America and within *Nihon*. Our study has also provided an extended phylogenetic reconstruction of Boraginaceae s.s., especially tribe Cynoglosseae s.l., which also needs further sample of species and DNA sequencing regions.

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http://dx.doi.org/10.5962/bhl.title.304

Popov, M.G. (1953) *Boraginaceae*. *Omphalodes laevisperma*.

http://dx.doi.org/10.1093/molbev/mns083


http://dx.doi.org/10.1093/bioinformatics/btg180
Appendix S1

List of material used for the molecular study. Asterisks indicate new Genbank accession numbers (this study).

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<th>Species</th>
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<th>Voucher</th>
<th>ITS</th>
</tr>
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<td>Actinocarya tibetica Benth.</td>
<td>China (Xizang)</td>
<td>G. &amp; S. Miehe 03-003-08 (MSB)</td>
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<td>Amsinckia spectabilis Fisch. &amp; C. A. Mey. var. microcarpa (Greene) Jeps. &amp; Hoover</td>
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<td>Anchusa crispa Viv.</td>
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<td>Arnebia decumbens (Vent.) Cosson &amp; Kral.</td>
<td>Tunesia</td>
<td>D. Podlech 32857 (M)</td>
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### Appendix S1. (Continued)

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### Appendix S1. (Continued)

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<td>Ogaenema paulliium (Coss. &amp; Durieu ex Bonnet &amp; Barrat) Brunn</td>
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<td>Ophalodes scorpoides Schrank</td>
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<td>H. Merxmüller &amp; O. Angerer 33286 (M)</td>
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<td>Paracarumus laxiflorum Trautv.</td>
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*continued on the next page*
### Appendix S1. (Continued)

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<td>Pseudomerentia sericophylla</td>
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<td>J. Léonard 4493 (MA)</td>
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<td>C.H. Chen 06239 MO /Y.-Y. Huang</td>
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<td>C.Scholeder &amp; M.Jacobs 1809 (MSB)</td>
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### Appendix S2
List of voucher specimens included in the fruit study.

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<th>Collector(s)</th>
<th>Accession number</th>
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<td>J.A. Lázaro Bello s.n. (MA)</td>
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<td>Myosotidium hortensia (Decne.) Baill.</td>
<td>New Zealand (Chatham Islands)</td>
<td>PB Heenan s.n. (CHR)</td>
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<td>Omphalodes aliena A. Gray ex Hemsl.</td>
<td>USA (Texas)</td>
<td>T.R. Van Devender et al. 85-86 (MO)</td>
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<tr>
<td>Omphalodes brassicifolia Sweet</td>
<td>Spain (Salamanca, Aldearcipreste)</td>
<td>J. Fdez Diez 58 (MA)</td>
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<td>Omphalodes cappadocica DC.</td>
<td>Georgia (Adjara)</td>
<td>A. Gröger et al. 1518. (M)</td>
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<td>Omphalodes cardiophylla Gray ex Hemsl.</td>
<td>Mexico (Ciudad Victoria, Tamaulipas)</td>
<td>Clausen &amp; Edwards. 7376 (A)</td>
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<td>Omphalodes caucasica Brand</td>
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<td>V. Vasak &amp; A. Vzda sn.(M)</td>
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<td>Mexico (Galeana, Cerro El Gallo)</td>
<td>Hinton et al. 21036 (A)</td>
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<tr>
<td>Omphalodes commutata G. López</td>
<td>Spain (Cádiz, Grazalema)</td>
<td>A. Aparicio, García &amp; Silvestre s.n. (MA)</td>
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<td>Omphalodes japonica (Thunb.) Maxim.</td>
<td>Japan (Kochi, Mt. Kojio-yama)</td>
<td>Kuroiwa et al. FOK-076224 (MBK)</td>
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<td>Omphalodes kazinskyanae Willk.</td>
<td>Portugal (Estremadura Cascais)</td>
<td>E. Valdés Bermejo (MA)</td>
<td></td>
</tr>
<tr>
<td>Omphalodes littoralis Lehm subsp. gailaecia Lainz</td>
<td>Spain (Madrid, San Martín de la Vega)</td>
<td>J.C. Zamora s.n.</td>
<td></td>
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<tr>
<td>Omphalodes luciliae Boiss.</td>
<td>Turkey (Nigde, Ulukisla)</td>
<td>P.H. Davis 16537 (M)</td>
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<tr>
<td>Omphalodes nitida (Hoffmanns. &amp; Link ex Willd.) Hoffmanns &amp; Link</td>
<td>Spain (Galicia, Lugo)</td>
<td>C. García-Echave s.n.</td>
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<tr>
<td>Omphalodes scorpioides Schrank</td>
<td>Austria (Niederösterreich)</td>
<td>H. Merxmüller &amp; O. Angerer 33286 (M)</td>
<td></td>
</tr>
<tr>
<td>Omphalodes verna Moench</td>
<td>Slovenia (Primorsko)</td>
<td>E. Hörandl &amp; F. Hadacek 5832 (W)</td>
<td></td>
</tr>
<tr>
<td>Thyrocarpus glochidiatus Maxim.</td>
<td>(label in Chinese)</td>
<td>4.Apr.1999 (4707205MO)</td>
<td></td>
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